In vitro somatic embryogenesis of *Musa acuminata* (AAA) cv. Vaibalhla

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

MAISNAM AKBAR SINGH

MZU REGN NO: 1905390

M.PHIL REGN NO: MZU/M.Phil./627 of 12.06.2020

DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF LIFE SCIENCE

MARCH, 2022

In vitro somatic embryogenesis of *Musa acuminata* (AAA) cv. Vaibalhla

BY

MAISNAM AKBAR SINGH

DEPARTMENT OF BIOTECHNOLOGY

Under the Supervision

of

Dr. Thangjam Robert Singh
Professor
Department of Biotechnology
Mizoram University, Aizawl – 796004, INDIA

Submitted

In partial fulfilment of the requirements for the Degree of Master of Philosophy in Biotechnology of Mizoram University, Aizawl.

DECLARATION OF THE CANDIDATE

Mizoram University

December, 2021

I, Maisnam Akbar Singh, hereby declare that the subject matter of this dissertation is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the degree of Master of Philosophy in Biotechnology.

Maisnam Akbar Singh

(Candidate)

Prof. Thangjam Robert Singh,

(Head, Department of Biotechnology)

Prof. Thangjam Robert Singh

(Supervisor)

ACKNOWLEDGEMENT

With respect I wish to express my heartfelt thanks to my supervisor, **Prof. Thangjam Robert Singh**, Professor, Department of Biotechnology, Mizoram University for his active guidance, valuable advice, effective suggestions, unending inspirations and affectionate touch during the course of this study. Without his intellectual inputs and support I might not have achieved this destination. I will always be grateful to him for his enthusiastic encouragement, sympathetic attitude and constructive criticism time to time**. "Thank you, Sir".**

I would like to thank my family for their love, encouragement, moral support and endurance at the time of trouble while doing my research. My sincere gratitude to all the faculty members of Department of Biotechnology namely Dr. N Senthil Kumar, Dr. J Bhattacharya, Dr. Bhim Pratap Singh, Dr. H. Vanlalhruaia and Miss Esther, Mizoram University for their noble knowledge, assistance and encouragement during the course of this work. My sincere appreciation to my seniors and collegues – Thangjam Premabati, Tongbram Punshi Singh, Thingnam Surendrakumar Singh, Soraisam Roshida, Boris Aheibam, Lourembam Dinamani Singh for their cheerful company, immense help in suggesting, co-operation and support during the study.

Heartfelt thanks to all the non-teaching staffs from the Department of Biotechnology, Mizoram University for their valuable assistance and cooperation during the course of my work.

All praises to our **"Almighty God"** whose omnipotence is the success of this study. My period of work has been filled with fun, success, failures, happiness, sickness and through it all, He has helped me to move forward, and I am humbled by His love and compassion.

Date: (MAISNAM AKBAR SINGH)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

NAME OF CANDIDATE : MAISNAM AKBAR SINGH

DEGREE : MASTER OF PHILOSOPHY

DEPARTMENT : BIOTECHNOLOGY

TITLE OF DISSERTATION : *In vitro* somatic embryogenesis of *Musa acuminata* (AAA) cv. Vaibalhla

DATE OF PAYMENTS OF ADMISSION :
06.08.2019

COMMENCENENT OF SECOND SEM/DISSERTATION :

(From conclusion of end semester exam)

APPROVAL OF RESEARCH PROPOSAL

BOS : 23.06.2020

SCHOOL BOARD : 12.06.2020

REGISTRATION NO. & DATE : MZU/M.Phil./627 of 12.06.2020

DATE OF SUBMISSION :

EXTENSION (IF ANY) :

Head

Department of Biotechnology

BIO-DATA

Name : **Maisnam Akbar Singh**

Father's name : **Maisnam Mangol Singh**

Permanent Address : **Ngaikhong Khullen Awang Leikai, Bishnupur, Manipur-795126**

Phone : **9108367655**

Email : **masingh2018@gmail.com**

EDUCATIONAL QUALIFICATION

Sl. No.	Examination passed	Year	Board/University	Percentage
1	HSLC	2011	CBSE	76%
2	HSSLC	2013	CBSE	77.5%
3	B. Sc	2016	Bangalore University	68.12%
4	M. Sc (Biotechnology)	2018	Bangalore University	69.76%

Papers presentation/participation in conference/symposiums:

1. Presented a paper title "In vitro shoot induction of *Musa acuminata* (AAA) cv. Vaibalhla for mutation breeding and flow cytometric analysis of different stages of cell cycle in different developmental stage" in National conference on Microbes in health, agriculture & Environment. Organized by Department of Biotechnology, School of Life Science, Mizoram University, Aizawl – 796004 during 20th & 21st June, 2019
2. Presented a paper title "Determination of LD50 in gamma irradiated aseptically raised *Musa acuminata* (AAA) cv. Vaibalhla inflorescence explants" in International Seminar on Recent Advanced in Science and Technology (ISRAST). Organized by North East (India) Academy of Science and Technology (NEAST), Mizoram University, Aizawl -796004, Mizoram (India) during 16th – 18th November.

National Conference on
Microbes in Health, Agriculture & Environment
Organized by:
Department of Biotechnology
School of Life Sciences
Mizoram University, Aizawl – 796004, Mizoram, INDIA

Certificate

Certified that Mr./Ms./Dr./Prof. Maisnam Akbar Singh *of* Department of Biotechnology (MZU, Aizawl) *participated and presented a paper entitled* In vitro multiple shoot induction of Musa acuminata (AAA) Cv. Vaibalhla for mutation breeding and flow cytometric analysis of different ploidy of cell cycle in different developmental stage *in the National Conference on "Microbes in Health, Agriculture & Environment " organized by Department of Biotechnology, School of Life Sciences, Mizoram University, Aizawl – 796004 during 20th & 21st June, 2019.*

Dr. Thangjam Robert Singh
Professor
Department of Biotechnology
Mizoram University
Aizawl - 796004, India

(Dr. Thangjam Robert Singh)
Department of Biotechnology
Convenor

(Prof. S.K. Mehta)
Dean, School of Life Sciences
Mizoram University

(Prof. K.R.S. Sambasiva Rao)
Vice Chancellor
Mizoram University

2nd Annual Convention of
North East (India) Academy of Science and Technology (NEAST)
&
International Seminar on Recent Advances in Science and Technology (ISRAST)
(16th -18th November 2020)
(Virtual)

Certificate

11215150238

This is to certify that

Mr./Ms./Dr./Prof. Maisnam Akbar Singh
Department of Biotechnology, School of Life Sciences, Mizoram University, Aizawl–796004, India

has attended and presented an Oral Presentation entitled, "Determination of LD50 in Gamma Irradiated Aseptically Raised Musa acuminata (AAA) cv. Vaibalhla Inflorescence Explants" in the 2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST) & International Seminar on Recent Advances in Science and Technology (IRSRAST) during 16th-18th November 2020 (Virtual) organized by NEAST, Mizoram University, Aizawl-796004, Mizoram (India).

Dr. Thangjam Robert Singh
Professor
Department of Biotechnology
Mizoram University
Aizawl - 796004, India

Prof. Diwakar Tiwari
General Secretary, NEAST

Prof. R. Lalthantluanga
President, NEAST

North East (India) Academy of Science & Technology (NEAST), Mizoram University, Aizawl-796004 (India)
Reg. No.: MSR 903 under Mizoram Societies Act-2018

In vitro somatic embryogenesis of *Musa acuminata* (AAA) cv. Vaibalhla

BY

MAISNAM AKBAR SINGH

DEPARTMENT OF BIOTECHNOLOGY

Under the Supervision

of

Dr. Thangjam Robert Singh
Professor
Department of Biotechnology
Mizoram University, Aizawl – 796004, INDIA

Submitted

In partial fulfilment of the requirements for the Degree of Master of Philosophy in Biotechnology of Mizoram University, Aizawl.

DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF LIFE SCIENCES
MIZORAM UNIVERSITY
(A Central University)
P. B. No. 190
TANHRIL, AIZAWL – 796004: MIZORAM, INDIA

Thangjam Robert Singh, Ph.D
Professor & Head

Phone : +91 9436352386(M)
E-mail : robth@mzu.edu.in
robertthangjam@gmail.com

MZU/BTCH/TRS/2007-M.Phil-03 Date:

Certificate

This is to certify that the present dissertation entitled "***In vitro* somatic embryogenesis of *Musa acuminata* (AAA) cv. Vaibalhla"** submitted by **Maisnam Akbar Singh**, M.Phil. Registration No: MZU/M.Phil/627 of dt. 12.06.2020 in partial fulfillment for the award of the Degree of Master of Philosophy in Biotechnology of Mizoram University has been carried out during 2020-21 under my supervision. The dissertation embodies original research and has not been submitted for any degree elsewhere.

The candidate has fulfilled all the requirements under M. Phil ordinance of the Mizoram University.

(Prof. Thangjam Robert Singh)
Supervisor

Dr. Thangjam Robert Singh
Professor
Department of Biotechnology
Mizoram University,
Aizawl - 796004, India

DECLARATION OF THE CANDIDATE

Mizoram University

December, 2021

I, Maisnam Akbar Singh, hereby declare that the subject matter of this dissertation is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the degree of Master of Philosophy in Biotechnology.

Maisnam Akbar Singh

Maisnam Akbar Singh

(Candidate)

Prof. Thangjam Robert Singh,

(Head, Department of Biotechnology)

Head
Deptt. of Biotechnology
MIZORAM UNIVERSITY
AIZAWL - 796004

Prof. Thangjam Robert Singh

(Supervisor)

Dr. Thangjam Robert Singh
Professor
Department of Biotechnology
Mizoram University,
Aizawl - 796004, India

ACKNOWLEDGEMENT

With respect I wish to express my heartfelt thanks to my supervisor, **Prof Dr. Thangjam Robert Singh**, Professor, Department of Biotechnology, Mizoram University for his active guidance, valuable advice, effective suggestions, unending inspirations and affectionate touch during the course of this study. Without his intellectual inputs and support I might not have achieved this destination. I will always be grateful to him for his enthusiastic encouragement, sympathetic attitude and constructive criticism time to time**. "Thank you, Sir".**

I would like to thank my family for their love, encouragement, moral support and endurance at the time of trouble while doing my research. My sincere gratitude to all the faculty members of Department of Biotechnology namely Dr. N Senthil Kumar, Dr. J Bhattacharya, Dr. Bhim Pratap Singh, Dr. H. Vanlalhruaia and Miss Esther, Mizoram University for their noble knowledge, assistance and encouragement during the course of this work. My sincere appreciation to my seniors and collegues – Thangjam Premabati, Tongbram Punshi Singh, Thingnam Surendrakumar Singh, Soraisam Roshida, Boris Aheibam, Lourembam Dinamani Singh for their cheerful company, immense help in suggesting, co-operation and support during the study.

Heartfelt thanks to all the non-teaching staffs from the Department of Biotechnology, Mizoram University for their valuable assistance and cooperation during the course of my work.

All praises to our **"Almighty God"** whose omnipotence is the success of this study. My period of work has been filled with fun, success, failures, happiness, sickness and through it all, He has helped me to move forward, and I am humbled by His love and compassion.

Date: (MAISNAM AKBAR SINGH)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1
Introduction

CHAPTER 1

INTRODUCTION

1.1. General introduction:

Banana (*Musa* spp.), including plantains and dessert varieties., are large perennial monocotyledonous herbaceous plants which belongs to the order *Zingiberales* in the family *Musaceae* (D'Hont et al., 2012). They are mostly cultivated in the humid tropical and subtropical regions. The earliest reference to banana dates back to about 500 BC. The name '*Musa*' proposed by Linnaeus for the genus is taken from the Arabic term for the plant 'Mouz' which itself is derived from 'Moka' a Sanskrit term (Nayar, 2010). Southeast Asia is considered as the region where banana originated from and many of its wild relatives are native (Uma et al., 2002). India is considered to be one of the major centers of origin for *Musa* and origin of wild bananas stretches from India upto Pacific Islands including Papua New Guinea, Micronesia, Melanesia, Samoa etc. (De Langhe et al., 2005). In India more than 970 indigenous banana from around the country were collected and preserved in NRCB (National Research Centre on Banana), Trichy, Tamil Nadu. Out of the total, 109 varieties were collected from northeast states of the country, namely Assam, Arunachal Pradesh, Manipur, Meghalaya, Mizoram, Nagaland and Tripura (Uma and Sathiamoorthy, 2002). Aside from India, the humid lowlands of West and Central Africa are also the center of plantain diversification, whereas the highlands of East Africa are center of cooking and beer banana diversification (Swennen and Vuylsteke, 1991). The northeastern part of India is believed to be the source of the 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). From the region, a total of 39 different banana accessions have been identified and characterized (Uma et al., 2006). From the state Mizoram, 14 different accessions have been identified and characterized (Lalrinfela and Thangjam, 2012). Various banana and plantains are believed to have spread from Asia throughout the humid tropics (Swennen and Ortiz, 1997; Valmayor et al., 2000) solely by humans through suckers.

1.2. Taxonomy

Banana is a monocotyledonous herb that comes under the kingdom plantae belongs to class *Liliopsida* and division *Magnoliophyta* order *Zingiberales* belonging to the family *Musaceae* and genus *Musa* (D'Hont et al., 2012). The family *Musaceae* comprises of two genera *Musa* L. (Linnaeus, 1753) and *Ensete* (Horaninow, 1862), where *Musa* differs from *Ensete* in producing suckers and small seeds (Li et al., (2010). The *Musa* genus consists of approximately 50 species while the genus *Ensete* has 9 species (Simmonds & Shepherd, 1955). The genus *Musa*, on the basis of basic chromosomal number and phenotypic characteristic, has been divided into four different sections namely *Rhodochlamys*, *Eumusa*, *Callimusa* and *Australimusa* (Simmonds & Shepherd, 1955). *Eumusa* constitute the largest, most widely distributed, highly diversified and the most important section containing all the edible bananas (Uma et al., 2006). Mainly cultivars in the *Eumusa* section are mainly derived from two species namely *Musa acuminata* having A genome and *M. balbisiana* having B genome (D' Hont et al., 2000). The species, *Musa acuminata* is the most widespread of the *Eumusa* section (Daniells, 2001). India has 70% of the total diversity of *Rhodochlamys,* which is distributed throughout the southern and northern states (Uma et al., 2005). Both the species, *M. acuminata* and *M. balbisiana* serves as progenitor for majority of the cultivated bananas, which may be diploid (2n=22), triploid (2n=33) or tetraploid (2n=44). Most cultivated *Musa* are triploids (2n=33) with characteristic genome constitutions (Lebot et al., 1993), while diploids and tetraploids are lesser in number. Simmonds and Shepherd (1955), proposed the following genomic classes for edible *Musa* AA, AB, AAB, AAA, ABB, AAAA and ABBB which are derived from two wild progenitor species *M, acuminata* (AA) and *M. balbisiana* (BB). These genomic groupings are almost exclusively used for the classification of edible *Musa* (Turner et al., 2007). Crosses between diploid and triploid types of *M. acuminata* with *M. balbisiana* result in the generation of heterogenomic triploid hybrids which are mostly plantains (AAB) and other cooking bananas (ABB). The generation of heterogenomic triploid (2n=3x) hybrids with the 'AAA' genotype occurred within *M. acuminata* leads to the formation of cultivars that comprises the sweet bananas (Daniells, 2001).

1.3. Uses and economic importance

Banana, including plantains, is among the oldest and major tropical fruit crops known and after rice, maize, and wheat, it is the fourth most crucial food crop (Moffat, 1999; Tripathi et al., 2007). Bananas are multifunctional plants since most of their components, depending on the species, can be used in a number of ways. It is nutritionally significant as it provides a well-balanced diet with a calorific value of 67 to 137 calories per 100 gm. The nutritive value of banana when compared to an apple, one banana contains 4 times the proteins, twice the carbohydrates, 3 times the phosphorous, 5 times vitamin A and iron including twice the other minerals and vitamins an apple contains (Nakirya, 2007). The high sugar content in a ripe banana supplies almost double the energy of an apple and nearly three times that of citrus fruits. The pulp of a ripe banana contains about 70 % water with 1.03 % protein, 0.48 % fat, 23.43 % carbohydrate, 0.6 % calcium, 0.03 % Iron, 39.6 % potassium, 0.1 % sodium and seven types of vitamins including vitamin B6, vitamin A, vitamin C, Folacin, Thiamin, Riboflavin and Niacin. (Aurore et al., 2009). The unripe fruits are the good sources of starch and amylase (Van den Houwe et al., 2000). Male floral buds can be consumed as vegetables whereas pseudostems can be used as a source of fiber and used in rope manufacturing, paper and textiles (Khan et al., 2014). Banana leaves can also be used for roof thatching, fabric and cordage manufacture, and animal feed (Smith et al., 2005). *Musa* species including *M. veluntina* and *M. ornata* are renowned banana ornamental plants (Heslop-Harrison and Schwarzacher, 2007). Essentially, the high intake of fibre, sugar, minerals, and vitamins, as well as the low consumption of fat, highlight the nutritional benefits of bananas (Forster et al., 2002; FAOSTAT, 2013). Several *Musa* and *Ensete* spp. are also commonly marketed in international markets as ornamental plants. Banana and plantain plants are grown in over 130 nations across the tropical and subtropical areas, covering nearly 10 million hectares of harvested land (FAOSTAT, 2013). The yearly global production is estimated to be over 145 million tonnes (39 million tons for plantain and 106 million tons for banana), with India as the world's largest banana grower (29 million tonnes) (FAOSTAT, 2013). However, being the top producers of banana, India does not include among the top exporters due to its high domestic consumption. Ecuador, Costa Rica, Philippines, Colombia are countries include in the large exporters list producing about 80 % of the world's banana export, while the United States, Canada and the European countries are

the major importers of banana that accounts for over 75 % of world imports (Worobetz, 2000).

1.4. Problems and need for research

Banana production all over the world has been severely affected by several diseases and pests (Jones, 2007). Banana production is lowered by several biotic and abiotic stresses. Abiotic stresses like continuous rainfall, soil moisture deficit, salinity, extreme temperatures and strong winds are few of the bottle necks for successful banana production. Most of the problems were associated with plantation of poor-quality clones in the field (Novak, 1992). Pests and diseases constraints to banana production are: Fusarium wilt or Panama disease is caused by the *Fusarium oxysporum* which is a fungus, Sigatoka leaf spot diseases both black and yellow caused by the *Mycosphaerella fijiensis* and *M. musicola*. Cucumber Mosaic Virus causes chlorosis, mosaic and heart rot. Bunchy top of banana caused by BBTV (Banana Bunchy Top Virus) is a very destructive disease. The major vector of BBTV is an aphid namely *Pentalonia nigronervosa*. Banana Streak Virus (BSV) being another important virus affect both banana and plantain cultivation. Large number of root knot nematodes such as *Radopholous similis, Pratylenchus coffeae, Meloidogyne incognita, Helicotylenchus multicinctus,* etc. are also found infecting banana by burrowing roots, affecting the vigour and yield (Cronauer and Krikorian, 1986). In Northeast India, banana diseases like BBTV, Black Sigatoka and Yellow Sigatoka are prevalent (Uma et al., 2005). The conventional method of propagation through suckers cannot fulfill the large requirement of planting materials as very few suckers are produced from the mother plant and also not free from diseases. The development of desired varieties of banana through conventional breeding methods remains challenging because of the sterility and polyploidy of most of the edible cultivars. Furthermore, suckers commonly transmit soil nematodes and disease-causing organisms including leaf spot, BBTV, and others, that leads to the reduction of crop productivity and ultimately destroying the entire plantation. Genetic improvement by cross breeding is a difficult task in this plant because most of the edible bananas are sterile polyploids. The combination of both *in vitro* culture and mutation breeding has been suggested as an alternative method for banana improvement program (Novak et al., 1990). Plant tissue culture techniques involve the culturing of cells, tissues or organs of the plants under an aseptic and controlled environment (George, 1993). This is based on the totipotency

principles of plant cells, the phenomenon first proposed by (Haberlandt, 1902). In vitro propagation has several excellent advantages over traditional propagation method including physiological uniformity, higher multiplication rate, year-round availability of disease-free plant material, rapid dissemination of new plant materials around the world, shoots uniformity, faster growth in the early growing stages when compared to conventional materials and a shorter harvest interval when compared to conventional plants (Vuylsteke, 1989). It has unique advantage of rapid multiplication, uniformity of planting materials, availability of a greater number of plants in short time, disease-free and also possibility of non-seasonal production of plants over conventional propagation methods. Thereby, tissue culture approaches can offer faster and efficient means of producing huge number of genetically uniform planting material within shorter period of time. In addition, there are several reports which proves that in vitro regenerated banana plants are superior to the conventional method due to their maximum growth (Daniells, 1988), precocity and higher yields (Drew and Smith, 1990). Moreover, in vitro system forms the basis for the successful programme of plant genetic engineering. Plant genetic engineering requires the mastery of a regeneration process by means of organogenesis or somatic embryogenesis, preferably of unicellular origin to avoid the problem of chimeras (Grapin et al., 1996).

1.5. Somatic embryogenesis

Somatic embryogenesis which results in embryo development and regeneration of plant offers advantages for efficient mass propagation of improved cultivars and can also provide a useful platform for genetic manipulation studies. Somatic embryogenesis is the developments of an embryo from a cell other than a gamete or the product of gametic fusion (Merkle et al., 1995). Somatic embryogenesis technique used in the genus *Musa* is aimed at two main objectives-the development of high performance micropropagation and regeneration system useful for genetic improvement. Embryogenesis is regarded as a model for testing the totipotency of crop tissues. Analysis of the impacts of different medium, growth conditions, and modifying factors, particularly quantities and types of plant growth regulators, is important for the improving of high frequency tissue culture responses. As basic material for genetic manipulation, research groups emphasized on the utilization of protoplasts or embryogenic cells as well as meristems (May et al., 1995) of banana in the early stages of genetic transformation of banana. Generation of chimeric

transformants hamper the wide application of the techniques used. On the other hand, majority of the genetic transformation studies, even using Agrobacterium-mediated transformation (Moy et al., 1999; Perez et al., 1998) are currently relies on embryogenic cells/tissues. For numbers of species, successful generation of somatic embryos and subsequent recovery of viable plants is neither standard nor efficient. Understanding the mechanisms underlying the expression and stimulation of somatic embryogenesis in various species will improve the genotype potentiality. Somatic embryogenesis in banana has been demonstrated using different explants like leaf bases and fragments of rhizome (Novak et al., 1989, Ganapathi et al., 1999), proliferating meristem (Dhed'a et al., 1991; Schoofs, 1997; Mayer et al., 1998), female flower and immature male flowers (Escalant et al., 1994; Grapin et al., 1998). Ganapathi et al. (1999) reported complete regeneration of plants through somatic embryogenesis from young male flowers of Indian banana cultivars belonging to AAA and AAB genomic groups. Even though embryogenic cell suspensions were obtained and plants were regenerated (Dhed'a et al., 1991; Schoofs et al., 1999; Grapin et al., 1998; Cote et al., 1996), there are still problems associated with the generation of embryogenic cell suspensions from meristem and premature flowers would be free from problems and standard.

1.6. Somaclonal variation in *Musa*

In order to regenerate and proliferate the existing clone, numerous micropropagation techniques have been established, and a significant number of plantlets have been generated, (Venkatachalam et al., 2007). Regeneration of plantlets in tissue culture mostly depends on the presence or absence of different plant growth regulators in the culture medium (Dahot, 2007). In the presence of high concentrations of cytokinins, the probability of abnormalities and non-true-to-type plants increases (Aremu et al., 2013). Moreover, during the in vitro phases, growth conditions and material management can cause chromosomal translocations, loss or duplication of DNA fragments, small mutations, and DNA methylation (Sahijram et al., 2003), which can result in the phenotypic changes known as somaclonal variation (Abdellatif et al., 2012). somaclonal variation, in a broad sense encompasses inheritable phenotypic and genotypic variability (Israeli et al., 1991; Jain, 2001) that affects up to 88 percent of banana plants regenerated in vitro (Sahijram et al., 2003; Bairu et al., 2006). It is also called tissue or culture-induced variation (Soniya et al. 2001). Explant source, culture

period, subculture time, plant growth regulators, number of subcultures, genotype, medium composition, ploidy level, and genetic mosaicism are all factors that might cause in vitro variability (Silvarolla, 1992).

1.6.1. Identification of genetic variation

The detection of genetic variations within regenerated plants and their stability in subsequent generations is a key challenge to somaclonal variability. Crop improvement programmes require genetic stability and the preservation of variant germplasm. Several approaches can be utilized to determine tissue culture-induced variations at the cytological, morphological, biochemical, and molecular levels. Despite the fact that morphological features can be utilized to consistently distinguish off types, specific clones and mutations. Molecular markers that can be employed to create DNA profiles have been found to be a useful technique for determining the genetic stability of regenerated plants. Environmental factors have no effect on these markers, which produce consistent and reproducible results. At the genetic level, molecular markers are commonly utilised to detect and describe somaclonal variation (Sabir et al., 1992; Barrett et al.,1997). various such markers have successfully been utilised in *Musa*. They are: Randomly Amplified Polymorphic DNA (RAPD) markers, isozyme Analysis, Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeats (SSR), Short Tandem Repeats (STRs), Inter Simple Sequence Repeats (ISSR), Allele specific Amplified Fragment Length Polymorphism (AS-AFLP).

1.7. Objectives

Mizoram lies between 21˚58' & 24˚35' N latitude and 92˚15' & 93˚29'E longitude spread over 21,081 sq.kms area (Kar et., al 2013). Wild and edible banana varieties are distributed throughout the state. Considering the aforementioned biotechnological strategies for banana improvement of local varieties of the state and understanding the level of their genetic diversity will provide useful information for the status, breeding purposes, and producing uniform superior planting for large scale plantation. Despite the availability of numerous reports on somatic embryogenesis in banana, many of which are complicated, standardisation of specific protocols for a specific cultivar is critical. As a result, the current study was designed to fill scientific gaps in the identification of Mizoram's local banana cultivars, as well as to standardise the mass

propagation of quality planting materials through the use of biotechnological approaches. The present study was therefore under initiated following objectives:

- To establish aseptic culture of *Musa acuminata* cv. Vaibalhla using male flowers
- To induce callus and somatic embryogenesis
- To study the proliferation and *in vitro* regeneration of the somatic embryos.

Chapter 2
Review of Literature

CHAPTER - 2

REVIEW OF LITERATURE

2.1. General Description

Banana (*Musa* spp.), being one among the most widely cultivated fruit crop in the world, is grown 0ver 120 countries with almost covering 10 million hectares under cultivation and an annual yield of 95 million tonnes. After rice, maize and wheat it is the fourth most significant food crop (Tripathi et al., 2007). Bananas is an important tropical fruit, with yearly production exceeding 100 million tonnes. Banana is also the world's fourth most significant food crop, with over 600 million people globally relying on it as a staple diet. It is also India's most important fruit crop, with significant socioeconomic implications. (Moffat 1999; Tripathi et al. 2007). Schleiden (1838) presented the basis of cellular theory that the cell is capable of autonomy and is even totipotent, which leads to the starting of plant tissue culture. They didn't have any proof, however, that somatic cells are capable of this process. The earliest report of banana tissue culture came from Taiwan, where shoot-tips were cultivated that regenerate into in vitro adventitious buds (Ma and Shii, 1972; 1974). Berg and Bustamante (1974) employed meristem culture in combined with thermotherapy to generate virus-free banana plants. Since then, several procedures for increasing banana yield and improving banana tissue culture have been developed. In early days, tissue culture studies were initiated from 'Cavendish' banana (Berg and Bustamante, 1974) and from some dessert bananas (De Guzman, 1975; De Guzman et al., 1976). Tissue culture experiments on the 'Cavendish' banana (Berg and Bustamante, 1974) and certain dessert bananas days (De Guzman 1975; De Guzman et al., 1976) were started in the early. Plant growth regulators in the medium and also growth hormones secreted by the cultured cell, tissue, or organ are the main regulators of In vitro growth and development of the plants. In vivo and in vitro, Plant growth regulators like auxins, ethylene, gibberellins, cytokinins and abscisic acid activate a range of growth and development processes (George and Sherrington, 1984). To induce varied morphogenic responses on inoculated plant cells, tissues, and organs, growth regulators are employed in varying quantities. Because these compounds influence in apical dominance, they aid in the generation of new shoots (Madhulatha, et al., 2004). Auxins and cytokinins are utilised as growth regulators in *Musa* spp. in micro

propagation of *Musa* spp. Cytokinins are primarily involved in cell division and apical dominance modification. They're used in tissue culture for axillary shoot development and shoot differentiation. Adenine-based cytokinins like benzylaminopurine (BAP), isopentyladenin (2-ip), and kinetin (KN) are the most successful in tissue culture. Cytokinins like BAP and KN have shown to decrease apical dominance in banana meristematic explants by encouraging the development of both adventitious and auxiliary shoots (Jafari et al., 2011).

In banana shoot tip culture, Wong (1986) looked at the effect of cytokinins on roots formation and shoot proliferation in shoot tip culture of banana. Arinaitwe et al. (2000) studied the influence of 24 cytokinins (BAP, TDZ, 2ip, and KN) on the efficiency of shoot proliferation in three banana cultivars: 'Kibuzi', 'Ndiziwemti' and 'Bwara'. In the cultivar 'Grand Naine,' the influence of endogenous hormone activity on lateral bud development in vitro was examined (Zaffari et al., 2000). Smitha and Nair (2011) investigated the effect of picloram on somatic embryogenesis in 'Njali Poovan' leaf sheath explants. inmaIn 'Grand Naine' (Shankar et al., 2014) and 'Matti,' (Lohidas and Sujin, 2015) benzyl adenine produced more bud from shoot tips than KN in mass proliferation. Jafari et al. (2011) investigated the effects of BA on shoot apex explants in 'Berangan. 'Bhosale et al. (2011) observed that varying BA levels had distinct effects on different cultivars. According to Muhammad et al. (2004), the rate of multiplication differed among shoot tip explants of the similar genotype during various subcultures of 'Basrai'. Thiadiazuron (TDZ), a urea-based cytokinin is commonly utilised in banana in vitro experiments. Gubbuk and Pekmezci (2004) investigated the effects of TDZ and BAP on shoot multiplication in bananas, either alone or in conjunction with IAA, and found that TDZ considerably increased proliferation and elongation of shoots compared to BAP. With the use of TDZ, shoot regeneration from 'Gros Michel' callus cultures was achieved (Srangsam and Kanchanapoom, 2003). The effects of varying TDZ concentrations on shoot proliferation rate in six banana cultivars were studied by Youmbi et al. (2006). Strosse et al. (2008) cultivated shoot tip explants to see how five cytokinins (BA, KN, 2iP, zeatin, and TDZ) affected their ability to initiate multiple shoots. They found that the most explants initiated multiple shoots in m miedium containing TDZ, followed by BA.

2.2. Somatic embryogenesis

The discovery of the development of somatic embryos in cell cultures is one of the

most stunning discoveries in plant tissue culture (Steward et al., 1958, Reinert, 1958). Somatic embryogenic systems were established with two primary purposes: bulk micropropagation and development of cellular mechanisms for genetic improvement through genetic transformation. Double fertilization occurs when a haploid sperm fertilizes a haploid egg cell that produce a diploid zygote in flowering plants. The zygote then goes through a series of morphological, biochemical and molecular changes to become an embryo which is termed as embryogenesis (Goldberg et al., 1994). Somatic embryogenesis is the production of somatic embryos in vitro by dedifferentiation of somatic cell (Verdeil et al., 2007). Somatic embryogenesis has also been documented in a variety of plant species (Tautorus et al., 1991; Jain et al., 2013). In terms of regulatory and developmental mechanisms, the somatic embryogenesis stages are identical to those of zygotic embryogenesis (Dodeman et al., 1997). As a result, somatic embryogenesis can be employed to examine the zygotic embryo's initial developmental stages of higher plants life cycle (Zimmerman, 1993). In various banana cultivars like 'Grand Naine'- AAA (Cote et al., 1996; Novak et al., 1989; Becker et al., 2000), 'Bluggoe'- ABB (Novak et al., 1989; Dhed'a et al., 1991), 'Mysore', 'Rasthali' and 'Rajeli'- AAB (Escalant et al., 1994; Ganapathi et al., 2001; Kulkarni et al., 2006), somatic embryogenesis has been done successfully. Various banana explants have been employed in somatic embryogenesis, including leaf sheath and rhizome (Novak et al., 1989), proliferating meristem (Cronauer and Krikorian, 1983; Escalant and Teisson, 1989; Marroquin et al., 1993; Asif et al., 2001), female flowers parts (Grapin et al., 2000) and young male flowers (Ma, 1991; Shii et al., 1992; Grapin et al., 1996; Cote et al., 1996; Ganapathi et al., 1999; Kosky et al., 2002; Khalil et al., 2002; Jalil et al., 2003; Khalil and Elbanna, 2003; Sidha et al., 2007). Escalant et al., 1994) observed somatic embryogenesis using different immature male flowers of triploid banana varieties. In a brief immersion technique, explants were able to multiply and maintain embryogenic cultures. Plantains have also been shown to have somatic embryogenesis, according to Grapin et al. (1996). Regeneration of plants from embryogenic cultures of diploid and triploid 'Cavendish' banana from immature zygotic as well as male flower bud primordial was described by Navarro et al. (1997). Lee et al. (1997) described the initiation and organogenesis of somatic embryos in *Musa* sp. rhizome explants, however the large number of the somatic embryos showed typical root development and a poorly formed shoot apex. In tissue culture, experimental somatic embryogenesis has been described in more than 30 plant

families. Yang and Zhang, (2010) described the induction of somatic embryogenesis in two different ways - indirect somatic embryogenesis and direct somatic embryogenesis. Indirect somatic embryogenesis, however, occurs in most species via an intermediary callus stage (Cuenca et al., 1999; Gaj, 2004; Montalbán et al., 2012; Corredoira et al., 2013, 2015). As both direct and indirect somatic embryogenesis are found to occur simultaneously, differentiating them in the same tissue culture environment is found to be challenging (Turgut et al., 1998). Indirect somatic embryogenesis on the other hand is a multi-step regeneration approach that starts with the formation of proembryogenic mass (PEM) and ends with the development, maturation, and somatic embryo conversion (Arnold et al., 2002). The development of PEM, which consists of embryogenic cells that are proliferating in a state halfway between callus and the somatic embryo, and a relatively disorganised structure, is a crucial step in indirect somatic embryogenesis (Halperin, 1966). Auxin is essential for PEM proliferation, but it prevents PEMs from somatic embryos development (Yang and Zhang, 2010). To promote somatic embryogenesis, tissues inoculated in vitro must normally be exposed to plant growth regulators (PGRs) and other stress factors. PGRs are critical media components for in vitro regulation of explant growth and development. The pattern of development in vitro is frequently determined by their concentration and ratio in the media. Banana tissue culture uses cytokinins and auxins as growth regulators. 6 benzylaminopurine (BAP), -Naphthalene acetic acid (NAA), Kinetin (kin), and thidiazuron (TDZ) are the most often used PGRs (Harirah and Khalid, 2006; Darvari et al., 2010; Mahadev et al., 2011). Wiggan (1954) and Steward et al. (1958), pioneers of in vitro somatic embryogenesis, described bud development and proembryo-like structures in Daucus. They were the first to establish totipotency in somatic plant cells, although they were unable to clearly detect SE. Instead, they observed organogenesis and filamentous proembryos. In the early 1960s, Steward reports the formation of bipolar embryos and regeneration of plant there from cultures derived from mature parts of the carrot plant (Steward et al., 1958) or from cultured zygotic embryos (Norstog, 1961). Proembryo genic masses (PEMs) were coined by Halperin (1966) to characterize the embryogenic cell masses from which somatic embryos can be generated. PEMs go through cycles of growth and fragmentation until auxin is withdrawn from the culture media or the level of auxin in old cultures falls below a certain threshold.

2.2.1. Factors controlling somatic embryogenesis

Plant tissues have the ability to develop callus in vitro, according to Litz and Jarrett (1991), however only a few explants have the ability to produce callus having embryogenic structures. It might be possible to delete or reduce the correct gene expression for the development of somatic embryogenesis by exposing the explant culture to unusual conditions (stress). Heat, increasing the hypochlorite ion concentration, anaerobiosis, low temperature (4°C), high osmotic pressures, and exposure to auxin have all been utilized to trigger somatic embryogenesis based on this (Merkle et al., 1995). For the stable development of somatic embryos, monitoring these potentials of culture would be beneficial (Ibaraki et al., 1998). When suitable explants at defined developmental stages are excised from plants under optimal conditions, and when the appropriate growth regulators and medium are used, highly recalcitrant plants or genotypes could be induced to undergo morphogenesis in culture, according to Vasil (1987) and Close and Gallagher-Ludeman (1989). A lowered auxin-cytokinin balance in the culture medium is essential for inducing callus towards embryogenesis (Michaux Ferriere and Schwendiman, 1992). However, auxin is regarded as the most essential hormone in the regulation of somatic embryogenesis (Cooke et al., 1993). During the expression and induction phases of somatic embryogenesis, both endogenous contents and the administration of exogenous auxins are determining components. The auxin employed during induction has a major impact on the frequency and development of somatic embryos (Levi and Sink, 1991, Rodriguez and Wetzstein, 1994). Out of the 65 dicot species as reviewed by Raemakers et al., (1995), 17 dicot species were found to induced Somatic embryogenesis on hormone-free medium, 29 species on medium containing auxin, and 25 species on medium supplemented cytokinin were also found to induced somatic embryogenesis. The auxin 2,4-D was the most commonly utilized (49 percent), which was followed by auxin naphthalene acetic acid (27 %), indole-3-butyric acid (IBA) (6%), indole-3-acetic acid (IAA) (6%), Picloram (5%), and Dicamba (5%). N6 benzyl aminopurine was the most commonly utilized cytokinin (57%), which was followed by kinetin (37%), then zeatin (Z) (3%) and thidiazuron (3%). One of the most potent features of somatic embryogenesis is that it can be used for gene transfer and mass regeneration (Merkle et al., 1995). Secondary embryogenesis, recurrent or repetitive embryogenesis are all terms used to describe these processes (Gomez et al., 2001).

Without the presence of exogenous auxin, repetitive embryogenesis can occur. This process is referred to as autoembryogenesis, which is also known as mass or propagation proliferation (Merkle et al., 1995). Cytokinin is also important in banana ECS in the development embryos (Novak et al., 1989; Ma, 1991; Dhed'a et al., 1991; Cote et al., 1996). Arinaitwe (2000) also found that increasing TDZ concentration from 0.045 to 6.81 mM enhanced the rate of bud proliferation. Nahamya (2000) reported high multiple bud proliferation and early scalp formation in EAHBs with TDZ than BAP when these two cytokinins were supplemented at concentrations of 10 mM and 100 mM, respectively. Similar observation was reported by Sales et al. (2001), in which effects of different benzyladenine concentration on scalp initiation was observed to produce embryogenic callus from them.

2.2.2. Unicellular and multicellular origin of somatic embryos

The coordinated behavior of surrounding cells as a morphogenetic group is closely associated to the question of whether a single-cell or multiple-cell origin for somatic embryoids. Haccius (1971) characterized a nonzygotic embryo as a new individual developing from single cell with no vascular link to maternal tissues, in keeping with Street (1974). Raghavan (1976) was more cautious, recognising that in several cases where apparently normal bipolar embryoids were produced cell from aggregates, a single cell origin had not been conclusively confirmed. Embryoids can form from single cells (unicellular origin) or groups of cells (multicellular origin) in both processes, depending on whether neighbouring cells have the same interval development status and/or can interact and behave as a group rather than as individual cells (Williams and Maheswaran, 1986). In multicellular explants, the cell maturity or epigenetic distance from the embryogenic state determines whether somatic embryos are unicellular or multicellular. This was demonstrated by Maheswaran and Williams (1985) for the Somatic embryo induction from epidermal cells. They believe that Embryogenesis from matured tissue is only achievable through callus induction. Direct embryogenesis was observed in Cichorium from matured cortical cells in the root and mesophyll cells in the leaf (Dubois et al., 1990; Verdus et al., 1993). Direct embryogenesis has also been observed in Orchard grass mesophyll cells (Conger et al., 1983) and Dactylis glomerata mesophyll cells (Trigiano et al., 1987).

2.2.3. Application of somatic embryogenesis

One of the most prominent applications of somatic embryogenesis is to study the earliest stages of zygotic embryogenesis in higher plants. The most commercially appealing use of somatic embryogenesis is mass propagation of plantlets by the proliferation of embryogenic propagules (Merkle et al., 1995). Other applications include:

a) The utilization of the somatic embryos in synthetic seed technologies has become possible because of somatic embryogenesis (Gray, 1987).

b) Another application of somatic embryogenesis is regeneration of plants with varied ploidy levels, such as generating haploid embryos by culturing anthers and raising triploids from endosperm, which has been suggested and exploited to a limited extent (Terzi and Lo Schiavo, 1990).

c) To make use of totipotency, embryogenic callus, cell suspension, and somatic embryos can all be exploited as a source of protoplasts (Merkle et al., 1995).

d) Application in invitro production of embryo metabolites remains a huge potential as secondary embryogenesis has been found in at least 80 species as reported by Raemakers et al., 1995.

2.3. Somaclonal variation

Tissue culture techniques were believed to be capable of producing identical replicas of the parent plant (Denton et al., 1977; Wright, 1983). Somaclonal variation, on the other hand, has been discovered in a large number of plant species and can be found in both sexually and asexually propagated plants, as well as at all ploidy levels (Al-Zahim, 1996). One of the key challenges to modern micropropagation and plant regeneration by somatic embryos is somaclonal variation (De Klerk, 1990). It also makes it difficult to use biotechnological breeding techniques like somatic hybridization to recombine the genomes of sexually incompatible species, recombinant DNA techniques to modify plants, and anther cultures to accelerate homozygosity (Al-Zahim, 1996). Although there have been reports on the selection of desirable genetic variations caused by tissue culture (Smith and Drew, 1990), most somaclones show reduced vigor, yield, and other characteristics (Johnson et al., 1984; Jackson and Dale, 1989). Somaclonal variation is believed to occur only in plants

propagated from adventitious or newly produced apical meristems (Hussey, 1983), whereas epigenetic (non-genetic) modifications can be seen in plants originating from pre-existing axillary/apical meristems (De Klerk, 1990). Stress application in flax was observed to cause somaclonal variation (Cullis and Cleary, 1986). Stress pretreatment of Vicia faba roots, on the other hand, was found to make these meristems less responsive to mutagenesis by Heindorff et al., (1987). The effect of plant growth regulators particularly 2,4-D in somaclonal diversity is a matter of discussion. Without being directly mutagenic, 2,4-D is thought to induce aberrations by encouraging rapid, disorganized development (Bayliss, 1980). Bayliss (1980) discovered that NAA and 2,4-D induce chromosomal abnormalities only at extremely high concentrations (50 mg/l), not at doses commonly employed in tissue culture (less than 10 mg/l). At low doses, 2,4-D was also found to cause cell cycle alterations and increase the frequency of sister chromatid exchange (Dolezel and Novak, 1986). Auxins are also thought to impact the ploidy level in callus cells (Ghosh and Gadgil, 1979), increasing the frequency of aneuploidy and polyploidy (Ramulu et al., 1983). Somaclonal variation is indeed a regular phenomenon in *Musa* plants cultivated in vitro and in vivo. In vitro-regenerated bananas and plantains have more somolcones (off-types) than conventionally grown *Musa* species (Drew and Smith, 1990; Vuylsteke et al., 1988). Variations in stature, leaf, colour, and the morphology of pseudostem and reproductive organs are the most often reported in banana and plantain (Israeli et al., 1995). Sub-group Cavendish's prevalent variant is dwarfism, which accounts for 75-90 percent of all variants (Stover, 1987). It's not unusual for the same plant to have two separate varieties (Israeli et al., 1991). Hwang and Ko (1987) observed somoclonal variations in the cultivar 'Giant Cavendish' that exhibited putative field resistance to Fusarium wilt, but they all had unsatisfactory horticultural characteristics, such as low yield and poor fruit quality. The somaclones were mainly inferior to the 'Cavendish' replica from which they were generated, in that their bunches and fingers were smaller and of little commercial value (Hwang and Ko, 1987; Smith and Drew, 1990). However, further studies of these in vitro or sucker-propagated clones of these particular variants revealed plants with improved attributes when compared to the original variety, but somewhat lower yielding than the true-to-type cultivar (Hwang and Ko, 1987). The first authors to report on somaclonal differences in banana plants regenerated from embryogenic cell suspensions were Dhed'a (1992) and Grapin (1995). Dhed'a et al. (1991) found that 5-10% of abnormal somatic embryos obtained from a 'Bluggoe's

suspension can grow into healthy plants despite their abnormalities. Only one off-type (0.7 percent) with deformed leaves and retarded growth was observed out of 140 plants studied in the field. Plants regenerated from a 'French Sombre' culture had 16-22 percent somaclonal variations, according to Grapin (1995), but all plants grown via in vitro propagation were found normal. Mosaic variations (partial discoloration of the leaf) were observed, along with dropping leaves that revealed stunted growth. Variegated leaves and deformed laminae tend to revert to the source plant-type in the second or even first cycle (Israeli et al., 1991 and 1995; Vuylsteke et al., 1991), hence banana plants must be studied for at least three cycles before definitive claims about off-type frequency can be made. According to Stover (1987), less than 5% of off-types are commercially acceptable.

2.3.1. Use of molecular markers to detect variation

Several approaches can be used to study tissue culture-induced alterations at the morphological, cytological, molecular and biochemical levels. Molecular markers that can be utilized to generate DNA profiles have been found to be a useful technique for determining the genetic stability of regenerated plants. These indicators are not affected by environmental parameters and produce consistent, repeatable results. Amplified fragment length polymorphism (AFLP), Restriction fragment length polymorphism (RFLP), and random amplified polymorphic DNA (RAPD) are the most often used DNA-based markers.

RAPD is one of the approaches that is becoming increasingly popular for detecting genetic variation because it is technically simple, faster to perform, and only takes a low amount of DNA (Williams et al., 1990). Amplified Fragment Length Polymorphism (AFLP) and Random RAPD analysis were used to determine the genetic fidelity of in vitro regenerated plants of Arachis, and no polymorphism was found in the genomic regions studied (Gagliardi et al., 2003). The use of arbitrary oligonucleotide primers in RAPD analysis allows the detection of alterations in the plant genome at the DNA level. This approach has been used to check the somaclonal variations in plants generated from cultured cells and tissues (Vidal and de Garcia, 2000). Polymorphism is caused by the presence or absence of an amplification product from a single locus, as reported by Tingey and del Tufo (1993). In addition to the marker DNA band, variations in intensity of the band between the variant and normal clones have been observed. ISSR (Inter simple sequence repeats) (Zietjiewicz et al.,

1994) and RAPD (Williams et al., 1990) markers have shown to be effective in the determining of genetic variations. In micro propagated samples from various plants, both ISSR and RAPD markers have been successfully employed to determine genetic similarities and dissimilarities (Martin et al., 2006).

ISSR is a PCR-based approach that requires amplification of DNA segments that are located at amplifiable distance between two comparable microsatellite repeat regions that are located in opposite directions. Microsatellites, generally 16-25 base pair long primers in a single PCR reaction targeting various genomic loci, are used in this technique to amplify primarily inter-SSR sequences of various sizes. According to Vendrame et al. (1999), genetic variation in a culture line is influenced more by genotype than by culture period. This genetic instability could be a danger related with using in vitro culture methodology in germplasm management and preservation. Somaclonal variation, on the other hand, may provide source of unique and valuable variability (Vuylsteke, 1998) that can be employed in banana genetic improvement (Sahijram et al., 2003). In most of the primers tested, banding pattern of the PCR amplified product from regenerated plantlets was found to be monomorphic. When compared to the mother plant, the majority of the primers showed identical DNA patterns. Only a few plants displayed genetic variance, but they were all physically identical.

Chapter 3
Materials and Methods

CHAPTER - 3

METHODOLOGY

The current study was conducted at Mizoram University, Department of Biotechnology. *Musa*. *acuminata* cv. Vaibalhla (AAA) was chosen for the current study because of its high preference and popularity in Mizoram.

3.1. Sample Collection:

For the current study, 5 different *M. acuminata* cv. Vaibalhla (AAA) individual plant were selected from the field gene bank maintained at the Department of Biotechnology, Mizoram University, Aizawl.

Sample ID	Sample name
TRS-VB1	*M. acuminata* cv. Vaibalhla
TRS-VB2	*M. acuminata* cv. Vaibalhla
TRS-VB3	*M. acuminata* cv. Vaibalhla
TRS-VB4	*M. acuminata* cv. Vaibalhla
TRS-VB5	*M. acuminata* cv. Vaibalhla

Table 1: Details of *Musa acuminata* cv. Vaibalhla (AAA) samples selected for the study.

Fig.1: *Musa acuminata* cv. Vaibalhla (AAA group) in field gene bank Department of Biotechnology, Mizoram University, Aizawl

3.2. Screening of banana bunchy top virus (BBTV) in the selected *Musa acuminata* (AAA) cv. Vaibalhla.

3.2.1. DNA extraction:

Genomic DNA isolation of the selected *Musa acuminata* cv. Vaibalhla samples were carried out from the cigar leaves following the modified CTAB method (Thangjam et al., 2003) with slight modifications as described below.

1. 100 mg of young leaves from each sample were collected, rinse with tap water and blot dry. 2. Cigar leaves were transferred in the micro-centrifuge tube (2 ml) containing 400 µl of extraction buffer.

3. The leaves were ground in the buffer inside the tube with sterile glass rod.

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

5. Addition 400 µl of 2% CTAB solution and incubation at 65°C for 120 mins.

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

7. Centrifugation at 10,000 rpm in a microcentrifuge at 4°C for 10 mins.

8. The upper aqueous layer was gently transferred to another new tube and extraction process was repeated for 3 times to remove the cloudiness of the upper layer.

9. Addition of 2/3rd of ice-cold isopropanol in volume and gently mixing by repeated inversions followed by incubation at room temperature for 30 mins to precipitate DNA.

10. Centrifugation at 10,000 rpm for about 15 mins at a temperature of 4°C and supernatant was removed.

11. The pellet was washed with ice cold 70% ethanol.

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

13. Resuspension of the pellet in 50µl of Tris-EDTA buffer and removal of RNA by adding 1µl of RNase.

14. Incubate the tube at 37°C for approximately 60 mins.

3.2.2. 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 also at 280 nm using Multiskan GO (Thermo Scientific) according to the manufacturer's instruction. The purity of DNA was determined by calculating the ratio of the 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. Each sample's extracted DNA was separated on 0.8% agarose gel in 1× TAE buffer. For this, aliquot of 2 µl (100 ng/µl) was loaded into the gel and then photographed using gel documentation system (BIO-PRINT, Vilber).

3.2.3. PCR amplification of BBTV DNA-R genome:

In the study, molecular genetic approach was used for screening BBTV using primer specific to BBTV DNA-R genome. DNA-R genome which is a replication initiation encoding protein is a single stranded DNA component with a size of about 1.1 kb. BBTV DNA-R genome was amplified from the genomic DNA of the selected *Musa* samples using the BBTV DNA-R forward primer 5'-GGA AGA AGC CTC TCA TCT GCT TCA GAG AGC-3' and reverse primer 5'-CAG GCG CAC ACC TTG AGA AAC GAA AGG GAA-3' as described by Harding et. al, 2000. The PCR amplification was carried out with 15 µl reaction mixture containing 10× PCR buffer, 50 ng of genomic DNA as template, 0.5 µM each of the primers, 1.5 mM of MgCl2, 0.2 mM of dNTP (Himedia, India), 1 U of Taq Polymerase (Sigma-Aldrich Pvt. Ltd, Bangalore). The PCR amplification was carried out in a Thermal Cycler (Applied Biosystem) following the condition of 94 °C for 4 minutes, 30 cycles of 94 °C for 1 minute, 56 °C for 1 minute and 72°C for 2 minutes for initial extension which was followed by step of final extension at 72°C for 10 minutes. Amplified fragments were resolved on a 2% agarose gel and the gels were stained using ethidium bromide and the visualized using UV light with a 100 bp DNA ladder as a marker and then photograph using gel documentation system (BIO-PRINT, Vilber).

3.3. Establishment of aseptic culture of *Musa acuminata* cv. Vaibalhla using male flowers

3.3.1. Media preparation:

MS basal medium (Murashige & Skoog, 1962) was used for the explant regeneration in the study. MS media were supplemented with different concentrations of plant growth hormones (Table 3) and the pH was adjusted to 5.5 - 5.8 using 0.1N NaOH and 0.1N HCl. The MS media was solidified by addition of 0.8% (w/v) agar which was followed by sterilization at 121°C (15 lb psi pressure) for 15 minutes.

Sl. No.	Plant Growth Regulators (mg/L)			
	NAA	BAP	TDZ	IBA
1	0.0	0.0	0.0	0.0
2	0.0	2.0	1.0	0.0
3	0.1	0.0	2.0	0.0
4	0.1	1.0	1.0	0.0
5	0.5	0.5	0.1	0.0
6	0.5	0.5	0.2	0.0
7	0.5	1.0	0.3	0.0
8	0.5	1.0	0.5	0.0
9	0.5	0.5	0.5	0.0
10	0.5	0.5	1.0	0.0
11	1.0	0.5	2.0	0.0
12	1.0	0.0	2.0	0.0
13	0.0	0.0	0.0	1.0

Table 2: Details of Plant Growth Regulators (PGRs) use in the study.

3.3.2. Preparation of explants:

In the current study, mature male flower bud of *Musa acuminata* cv. Vaibalhla were collected from the field gene bank and used as explants. The collected male flower buds were then sterilized using 70% (v/v) ethanol for 5 minutes and rinsed well using sterile distilled water to remove the adhered ethanol. The male buds were then reduced to 4-6 cm in length by removing the upper layer of enclosing bracts. The male buds were then dissected to isolate the young immature male flowers by using sterile forceps and scalpel. The resulting young young immature male flowers that emerged were employed as explants for the study. The sterilized male buds were inoculated on Petri plates containing ~20 ml of MS solid basal medium which was supplemented with different concentrations of PGRs.

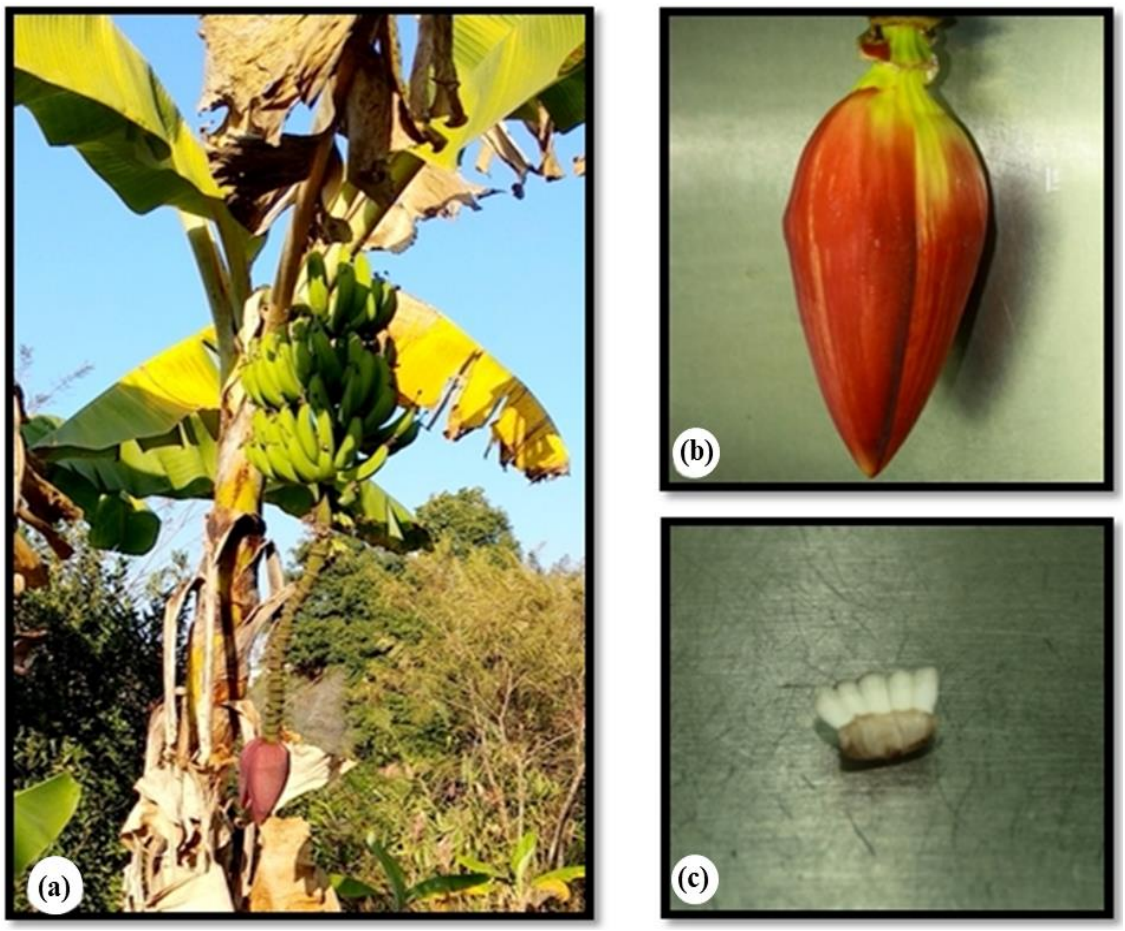

Fig. 2. *Musa acuminata* cv. Vaibalhla (AAA group), (b) Male flower bud and (c) Male flower explants of *Musa acuminata* cv. Vaibalhla

3.3.3. Culture conditions for regeneration:

The cultures were incubated and kept in culture room at 25±1°C, and kept 8:16 hours light-dark photoperiod with 55 µmol $m^{-2}s^{-1}$ light intensity by using white fluorescent light (Bajaj Ltd., India) and maintaining a relative humidity of around 50-60 %.

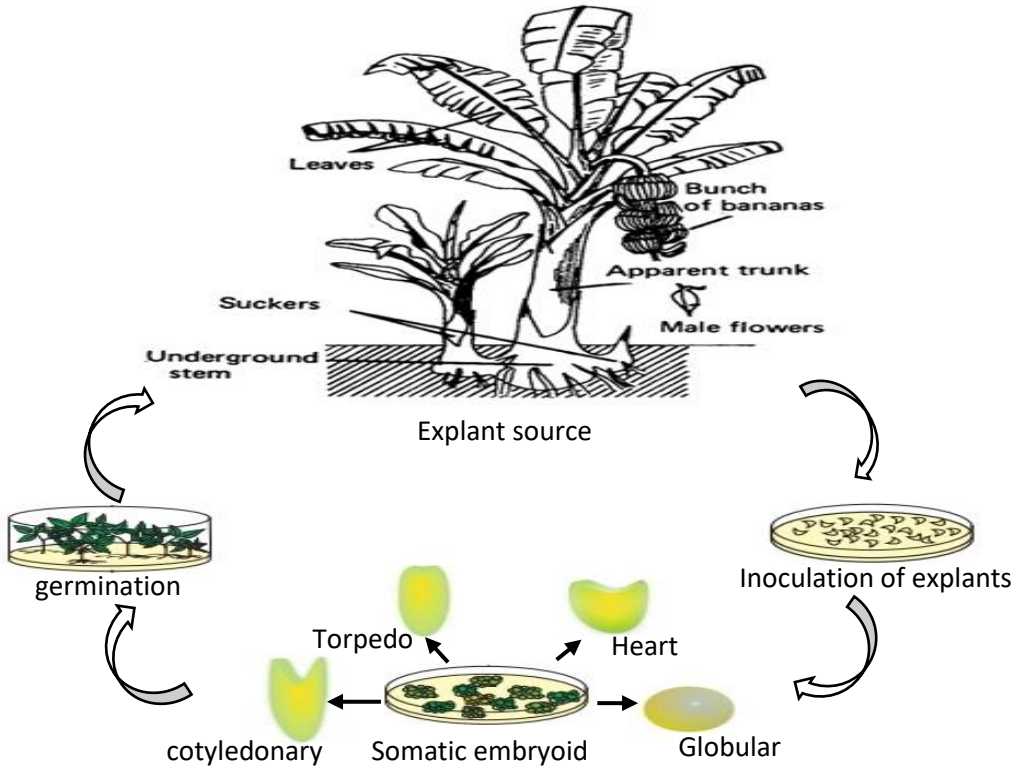

Fig. 3. Flow diagrammatic representation of regeneration of plantlets through Somatic embryogenesis

The explants were first inoculated on MS medium which was supplemented with 0, 0.1, 0.5 mg/L of NAA (α – naphthalene acetic acid), 0, 0.5, 1 mg/L of 6-Benzylaminopurine (BAP) and 0, 0.1, 0.2, 0.3, 1, 2 mg/L of Thidiazuron (TDZ) PGRs in combination. The explants were subculture every 2 weeks and the morphological changes such as bulging and formation of whitish bud-like structures (WBLs) were observed and recorded depending upon the different PGRs used. The same MS medium with same concentration and also in same combination of PGRs was used for induction of somatic embryos. Differences in time taken and also number of embryos which was formed in each petri plate according to different PGRs were recorded. The embryos formed were subculture into freshly prepared MS basal medium which was supplemented with the same PGRs as above but in different combination to induced formation of shoots and leaves.

For regeneration of the somatic embryo into plantlets, the embryos were transferred into freshly prepared MS medium that was supplemented with NAA (0, 0.5, 1 mg/L), BAP (0, 0.5, 1, 2 mg/L) and TDZ (0, 0.5, 1, 2 mg/L). All the changes of embryos into

shoots and further induction of leaves were observed and recorded. Once shoots and leaves were formed, the plantlets were transferred to root inducing medium which was MS medium that was supplemented with 1 mg/L of IBA PGR. The number of shoots, leaves and roots per explants and also length of each of the developmental stages were observed carefully and data was recorded for further analysis.

3.3.5. Hardening:

The effect of various plant growth regulators on potential of in vitro regeneration of the selected *Musa. acuminata* cv. Vaibalhla (AAA) was evaluated. The regenerated plants with fully developed leaves and roots were transferred for primary hardening. The plantlets were removed and also the roots were then thoroughly washed to remove agar and transferred in pots containing soil, fine sands and farm yard manure mixture in the ratio of 2:1:1 respectively. Hardened plantlets were maintained in the greenhouse at a temperature of 27°C - 28°C and a relative humidity of 60% to 80%.

3.3.6. Statistical Analysis:

All the experimental work were done with three replicates each for every treatment and a total of 20 explants were cultured for each replica. The cluster were sub cultured in every two weeks. Data were transformed to arcsine for the analysis of significance by using analysis of variance (ANOVA; $P<0.05$) and is represented as percentage data in tabular format. The mean differences were contrasted by using Duncan's new multiple range test of ANOVA. All the statistical analysis were done by using IBM SPSS software version 26.0.

3.4. Evaluation of genetic fidelity of the regenerated plantlets:

Genomic DNA from young leaves (100 mg) of 10 randomly selected banana plantlets that was regenerated as well as from the mother plant which was maintained in the field gene bank was isolated by using modified CTAB method (Thangjam et al, 2003). DNA was quantified by measuring absorbance at 260 nm and also at 280 nm using Multiskan GO (Thermo Scientific) according to the manufacturer's instruction. The purity of DNA was determined by calculating the ratio of 260 nm to that of 280 nm absorbance. ISSR-PCR amplification was carried out using 10 UBC primers with 15 µl reaction mixture containing 10× PCR buffer, 50 ng of genomic DNA as template, 0.5 µM each of the primers, 0.2 mM of dNTP (Himedia, India), 1.5 mM of MgCl2,

and 1 U of Taq Polymerase (Sigma-Aldrich Pvt. Ltd, Bangalore) performed in Thermal Cycler (Applied Biosystem) maintaining the condition as follows: 94 °C for 4 minutes, 35 cycles of 94 °C for 1 minute, annealing at 45-50°C for 1 minute, initial extension for 30 seconds at 72°C which was followed by final extension of 72°C for 5 minutes. The amplified fragments were resolved on a 2% agarose gel and the gels were treated with ethidium bromide and then visualized using UV light with a 100 bp DNA ladder as a marker and then photograph using gel documentation system (BIO-PRINT, Vilber). All the PCR reactions were performed at least twice in order to check the reproducibility and only the distinct and well-resolved fragments were scored.

3.5. Screening of banana bunchy top virus (BBTV) in the regenerated plantlets of *Musa acuminata* cv. Vaibalhla *(*AAA):

Young cigar leaves (100 mg) of 10 randomly selected regenerated plantlets, one among the 5 selected *Musa* samples and one from a symptomatic plant were collected. DNA isolation was carried out following the modified CTAB method (Thangjam et al., 2003) with slight modification. BBTV DNA-R genome amplification was amplified from the genomic DNA of the selected regenerated plantlets using the BBTV DNA-R forward primer 5'-GGA AGA AGC CTC TCA TCT GCT TCA GAG AGC-3' and reverse primer 5'-CAG GCG CAC ACC TTG AGA AAC GAA AGG GAA-3' as described by Harding et. al, 2000.

The PCR amplification was carried out with 15 µl reaction mixture containing 10× PCR buffer, 50 ng of genomic DNA as template, 0.5 µM each of the primers, 1.5 mM of MgCl2, 0.2 mM of dNTP (Himedia, India), 1 U of Taq Polymerase (Sigma-Aldrich Pvt. Ltd, Bangalore). The PCR amplification was performed in a Thermal Cycler (Applied Biosystem) with the following condition of 94 °C for 4 mins, 30 cycles of 94 °C for 1 min, 56 °C for 1 min and 72°C for 2 min; followed by a final extension at 72°C for 10 min. The amplified fragments were resolved on a 2% agarose gel and the gels were stained using ethidium bromide which were then visualized under UV light with a 100 bp DNA ladder as a marker and then photograph using gel documentation system (BIO-PRINT, Vilber).

Chapter 4
Results

CHAPTER - 4

RESULTS

In the current study, *in vitro* morphogenetic responses of *Musa acuminata* (AAA) cv. Vaibalhla such as bulging of the explants, formation of the white body like structure (WBL) formation, formation of somatic embryos, shoot regeneration, leaf formation and formation of roots was successfully achieved thereby establishing a suitable technique for mass production of the particular explants through somatic embryogenesis. The effect of plant growth regulators (PGRs) on each of the developmental stages were analysed.

4.1. Screening of banana bunchy top virus (BBTV) in the selected *Musa acuminata* cv. Vaibalhla (AAA).

4.1.1. Quality of the extracted genomic DNA:

The quality of the extracted genomic DNA from all the selected samples were evaluated by loading 2 µl (100 ng/µl) of the DNA and electrophorized on 0.8 % agarose gel and then photographed using a gel documentation system (BIO-PRINT, Vilber). The electropherogram showed a distinct sharp band of high molecular weight DNA.

4.1.2. PCR amplification of BBTV DNA-R genome:

PCR amplification of BBTV DNA-R genome of the selected sample showed no band while a band with a size range of 1100bp was observed in the BBTV symptomatic plant sample. This confirmed that the selected *Musa* samples were successfully screened and found free from Banana Bunchy Top Virus. The donor plants for tissue culture must be virus-free in order to produce virus-free plantlets, hence the sample selected were screened successfully and found free from BBTV and was used for the *in vitro* somatic embryogenesis of *Musa acuminata* (AAA) cv. Vaibalhla.

Sample	Tm (°C)	Observed band	Size range (bp) of observed band
Symptomatic plant	56°C	Observed	1100 bp
TRS-VB1	56°C	No band observed	0
TRS-VB2	56°C	No band observed	0
TRS-VB3	56°C	No band observed	0
TRS-VB4	56°C	No band observed	0
TRS-VB5	56°C	No band observed	0

Table 3. Observations recorded using the BBTV DNA-R primer in the selected *M. acuminata* samples.

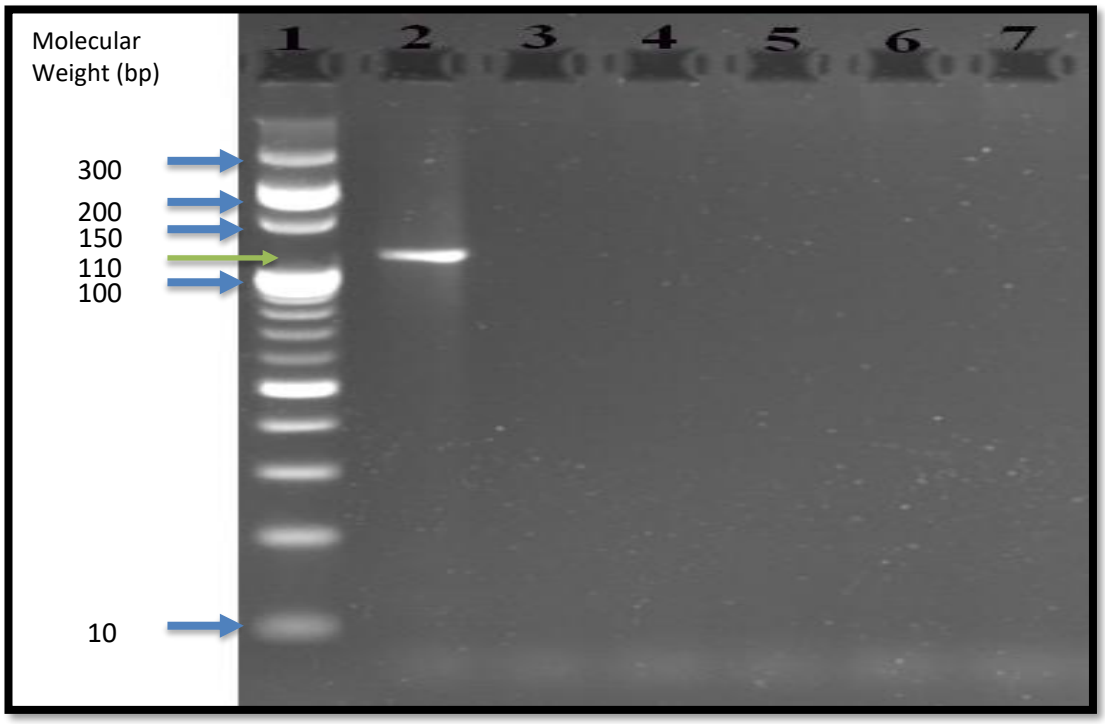

Fig. 4: Documented gel picture of PCR assay for screening of Banana Bunchy Top Virus in the selected donor *M. acuminata* Vaibalhla (AAA), 1. 100 bp DNA ladder, 2. symptomatic plant, 3-7: TRS-VB1 to TRS-VB5 respectively.

4.2. Establishment of aseptic culture of *Musa acuminata* cv. Vaibalhla using male flowers:

4.2.1. Sterilization of the explants:

Male flower buds were surface sterilized using 70% ethanol and the immature male flowers were successfully isolated in sterile environment inside the laminar air flow. The isolated male flowers were used as explant and showed no contamination on

inoculation into the culture medium. Hence, sterilization with 70% ethanol was found effective for the regeneration process.

4.2.2. Somatic embryogenesis and plantlet regeneration:

In vitro somatic embryogenesis and regeneration of plantlets of *Musa acuminata* (AAA) cv. Vaibalhla was successfully established. Plant growth regulators (PGRs) at various concentrations and combinations elicited varied morphogenic responses and differed in the length of each developmental stage. Morphological changes were observed on the explants after the second week of inoculation (Fig 5). Explants cultured on MS medium (Media set 4) supplemented with 0.1 mg/L of NAA, 1 mg/L of BAP and 1 mg/L of TDZ PGRs showed bulging on the basal part with the maximum percentage of bulging (70.11) (Fig 9). Explants cultured on the same MS medium (Media set 4) was also found to be superior in terms of responses and time duration for the development (Table 4).

Media Set	PGRs (mg/L)			Time duration for bulging (weeks)	Percentage of cluster giving bulging (mean ± SE)
	NAA	BAP	TDZ		
1*	0.0	0.0	0.0	2-3	00.00 ± 0.00a
2	1.0	0.5	2.0	4-5	51.74 ± 0.99b
3	0.5	1.0	0.3	2-3	61.14 ± 1.14c
4	0.1	1.0	1.0	2-3	70.11 ± 1.45d
5	0.1	0.0	2.0	4-5	27.7 ± 1.14e
6	0.5	0.5	0.1	3-4	40.2 ± 0.96f
7	0.5	0.5	0.2	3-4	37.26 ± 0.98f

Table 4: Effect of plant growth regulators on immature male flower *Musa acuminata* (AAA) cv. Vaibalhla explants on bulging
Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column were not significantly different at $P<0.05$ using Duncan's new multiple range test. * Control without any PGR

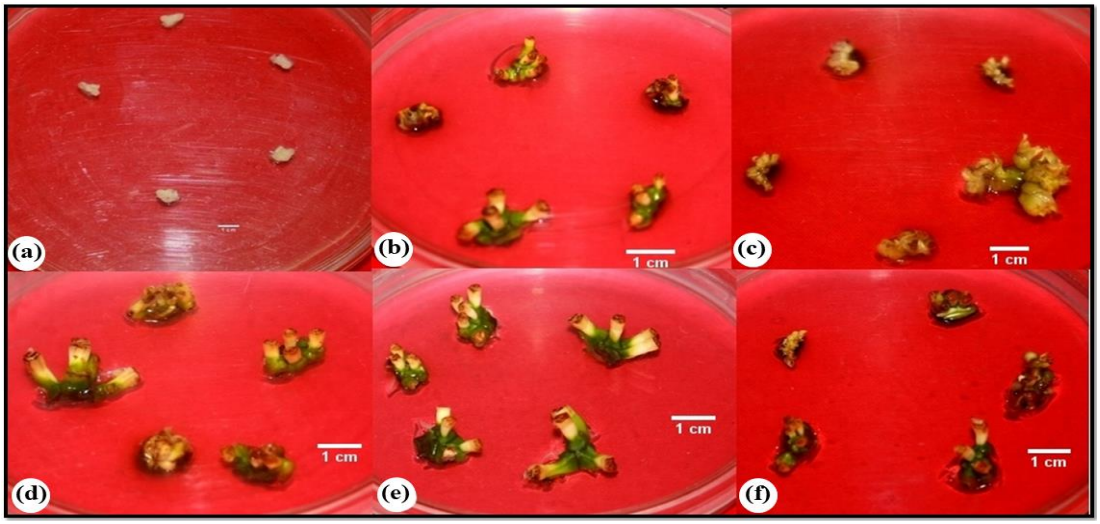

Fig 5: Bulging observed on basal part of immature male flower cluster of *M. acuminata* (AAA) cv. Vaibalhla cultured on media set 4 after 2-3 weeks, a) inoculated explants (b) explants after 1st week, (c) explants after 2nd weeks, (d) explants after 3rd weeks, (e) explants after 4th weeks, and (f) explants starting to form WBL after 4th weeks.

Explants were subculture every two weeks and formation of white bud like structure was observed after 4th week of inoculation (Fig 6). Inoculated clusters of immature male flower, sub cultured on MS medium supplemented with 0.1 mg/L of NAA, 1 mg/L of BAP and 1 mg/L of TDZ (media set 4) showed maximum WBLs percentage (70.32) (Fig 9) and the minimum was observed on medium supplemented with 0.1 mg/L of NAA and 2 mg/L TDZ (39.78). The same media set was found superior in producing WBLs per explants (fig 10). The cluster cultured on control medium without any PGRs showed no development, started browning and was found dead after 3-4th week of the culture.

Media set	PGRs (mg/L)			Time taken to form WBLS (weeks)	Percentage of cluster showing WBLS induction (mean ± SE)	Number of WBLS per cluster (mean ± SE)
	NAA	BAP	TDZ			
1*	0.0	0.0	0.0	3-4	00.00 ± 0.00a	00.00 ± 0.00a
2	1.0	0.5	2.0	5-6	46.47 ± 0.73b	8.14 ± 0.18b
3	0.5	1.0	0.3	5-6	59.27 ± 0.36c	8.88 ± 0.17c
4	0.1	1.0	1.0	4-5	70.32 ± 0.19d	11.64 ± 0.05d
5	0.1	0.0	2.0	7-8	39.78 ± 0.55e	7.19 ± 0.5e
6	0.5	0.5	0.1	6-7	53.07 ± 0.83f	7.99 ± 0.14b
7	0.5	0.5	0.2	6-7	50.15 ± 1.04g	9.3 ± 0.13c

Table 5: Effect of different plant growth regulators on immature male flower explants of *M. acuminata* (AAA) cv. Vaibalhla on white Bud like structure (WBLS) formation Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column were not significantly different at P<0.05 using Duncan's new multiple range test.
* Control without ay PGRs

Formation of proembryonal globular protuberances was observed from the WBLs on the 10-12 weeks of inoculation. These proembryonal globular protuberance on further subculture on the same PGRs concentration finally give rise to embryos through the formation of heart, torpedo and cotyledonary stage without the formation of callus (Fig:7).

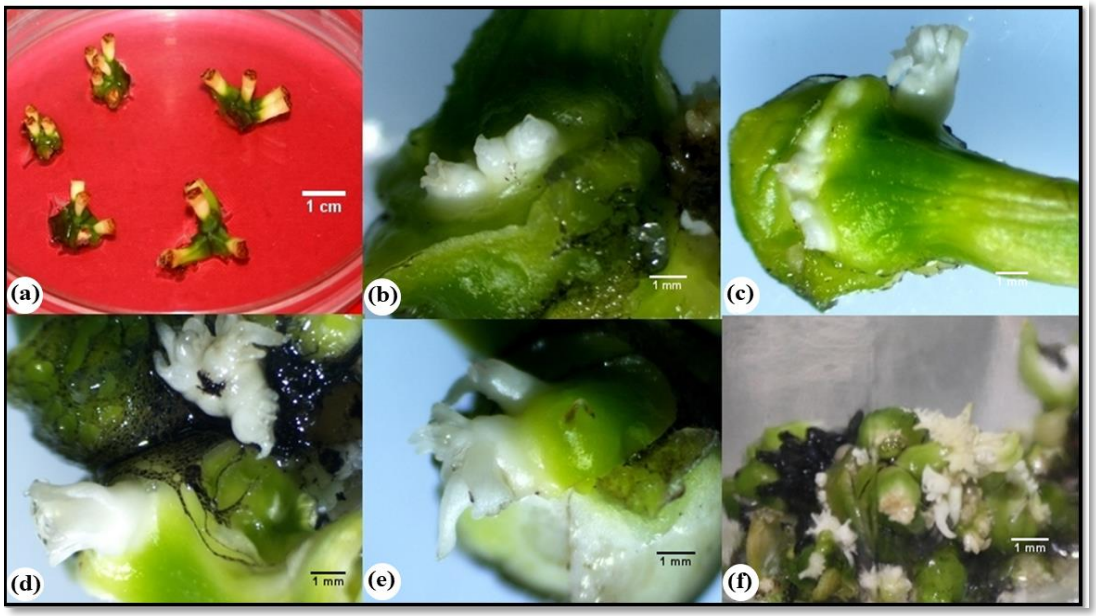

Fig 6: Formation of WBL observed from 4th week on immature male flower cluster of *M. acuminata* (AAA) cv. Vaibalhla cultured on media set 4 supplemented with different PGRs, (a) bulged cluster, (b) WBL observed after 4th weeks, (c) WBLs observed after 5th weeks, (d) WBLS after 6th weeks, (e) WBLs after 7th weeks, and (f) WBLs observed after 8th weeks.

The maximum number of embryo induction per explant was observed on MS medium supplemented with 0.1 mg/L of NAA, 1 mg/L of BAP and 1 mg/L of TDZ PGRs with 33.81 per explant (Fig 10) and maximum percentage of the total explants were found to generate embryos (48.84) (Fig 9) in the same media set.

Media set	PGRs (mg/L)			Time duration for embryos induction (weeks)	Percentage of clusters giving embryos (mean ± SE)	Number of embryos per cluster (mean ± SE)
	NAA	BAP	TDZ			
1*	0.0	0.0	0.0	00	00 ± 0.00a	00 ± 0.00a
2	1.0	0.5	2.0	13-15	21.33 ± 1.45b	12.2 ± 0.12b
3	0.5	1.0	0.3	11-13	40.2 ± 0.96c	26.11 ± 0.22c
4	0.1	1.0	1.0	10-12	48.84 ± 0.96d	33.81 ± 0.04d
5	0.1	0.0	2.0	13-15	19.88 ± 1.45b	10.42 ± 0.23e
6	0.5	0.5	0.1	12-14	34.23 ± 1.02e	21.21 ± 0.04f
7	0.5	0.5	0.2	12-14	28.85 ± 1.14f	18.57 ± 0.07g

Table 6: Effect of different plant growth regulators on Embryo formation
Medium: MS + PGRs.
Mean (±) followed by the same letter(s) in each column were not significantly different at P<0.05 using Duncan's new multiple range test.
* Control without ay PGRs

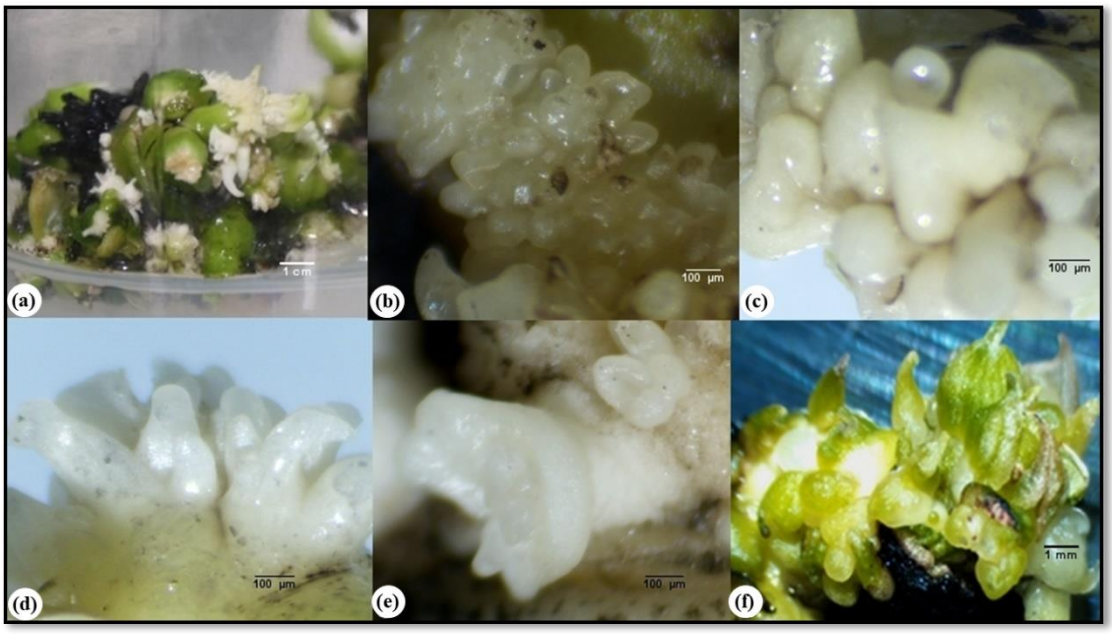

Fig. 7: Formation of embryos from WBLs observed on clusters cultured on media set 4 (a) male flower cluster with WBLs, (b) proembryonal globular stage, (c) heart stage, (d) torpedo stage, (e) cotyledonary stage, and (f) shoots formation.

The embryos so formed after subculturing into freshly prepared shoot medium in addition with different PGRs concentrations showed shoot formation after 20-22 weeks of inoculation (Fig 8). It was observed that 44.04% of the total explants showed shoots formation (Fig 9) with the maximum number of 25.81 per explant (Fig 10) and the longest shoot length (13.36 cm) (Fig 12) was observed on MS medium supplemented with 2 mg/L BAP and 1 mg/L TDZ.

Media set	PGRs (mg/L)			Time taken to form shoots (weeks)	percentage of embryos showing shoot induction (mean ± SE)	Number of shoots per cluster (mean ± SE)	shoot length (mean ± SE)
	NAA	BAP	TDZ				
1*	0.0	0.0	0.0	0	00.00 ± 0.00a	00.00 ± 0.00a	00.00 ± 0.00a
2	1.0	0.5	2.0	24-26	21.33 ± 1.45b	7.33 ± 0.15b	6.36 ± 0.17b
3	0.5	1.0	0.5	21-23	37.26 ± 0.98c	15.39 ± 0.05c	11.18 ± 0.21c
4	0.0	2.0	1.0	20-22	44.04 ± 0.95d	25.81 ± 0.05d	13.63 ± 0.14d
5	1.0	0.0	2.0	24-26	19.88 ± 1.45b	7.55 ± 0.31b	6.18 ± 0.23b
6	0.5	0.5	0.5	23-25	31.07 ± 1.07e	12.75 ± 0.08e	9.74 ± 0.15e
7	0.5	0.5	1.0	23-25	27.71 ± 1.14f	11.08 ± 0.07f	8.39 ± 0.17f

Table 7: Effect of plant growth regulators on shoot formation from the induced embryos after 20 weeks
Medium: MS + PGRs.
Mean (±) followed by the same letter(s) in each column were not significantly different at $P<0.05$ using Duncan's new multiple range test.
* Control without ay PGRs

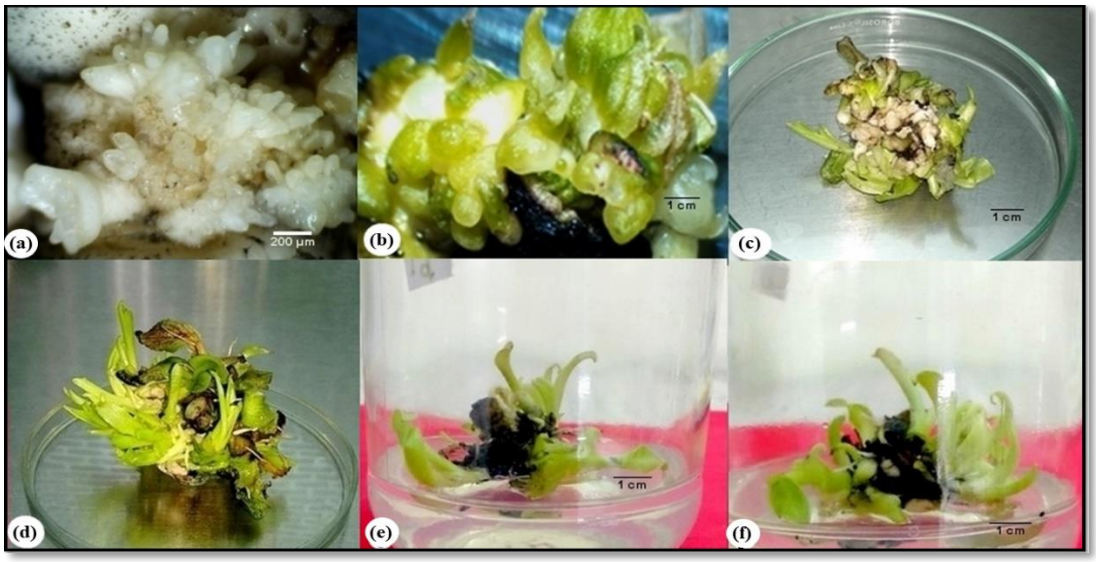

Fig 8: Shoots formation from embryos of immature male flower clusters of *M. acuminata* (AAA) cv. Vaibalhla observed on media set 4 (a) clusters with matured somatic embryos, (b) shoots formation after 20th weeks, (c) shoots after 22nd weeks, (d) shoots after 23rd weeks, (e) shoots after 25 weeks, and (f) shoots after 27th weeks.

Leaves formation from the regenerated shoots were observed within 23-25 weeks of the inoculation (Fig 11) with the highest percentage of shoot showing leaf induction (38.24%) (Fig 9) with maximum number of leaves per shoot (11.32) (Fig 10) and longest leaf length (11.87 cm) (Fig 12) on MS medium supplemented with 2 mg/L BAP and 1 mg/L TDZ.

Media set	PGRs (mg/L)			Time taken to form leaves (weeks)	Percentage of shoots showing leaf induction (mean ± SE)	Number of leaves per shoot (mean ± SE)	Leaf length (mean ± SE)
	NAA	BAP	TDZ				
1*	0.0	0.0	0.0	0	00.00 ± 0.00a	00.00 ± 0.00a	00.00 ± 0.00a
2	1.0	0.5	2.0	27-28	16.59 ± 1.83b	7.76 ± 0.36b	7.48 ± 0.12b
3	0.5	1.0	0.5	24-26	34.23 ± 1.02c	9.71 ± 0.14c	10.41 ± 0.14c
4	0.0	2.0	1.0	23-25	38.24 ± 0.98d	11.32 ± 0.12d	11.87 ± 0.12d
5	1.0	0.0	2.0	27-28	14.75 ± 1.83b	6.16 ± 0.43e	6.94 ± 0.21e
6	0.5	0.5	0.5	26-27	27.71 ± 1.14e	9.11 ± 0.29cf	9.63 ± 0.1f
7	0.5	0.5	1.0	26-27	25.3 ± 1.26e	8.51 ± 0.91bf	8.91 ± 0.11g

Table 8: Effect of plant growth regulators on leaves formation from the germinated shoots derived after 23 weeks formation. Mean (±) followed by the same letter(s) in each column were not significantly different at P<0.05 using Duncan's new multiple range test. * Control without ay PGRs

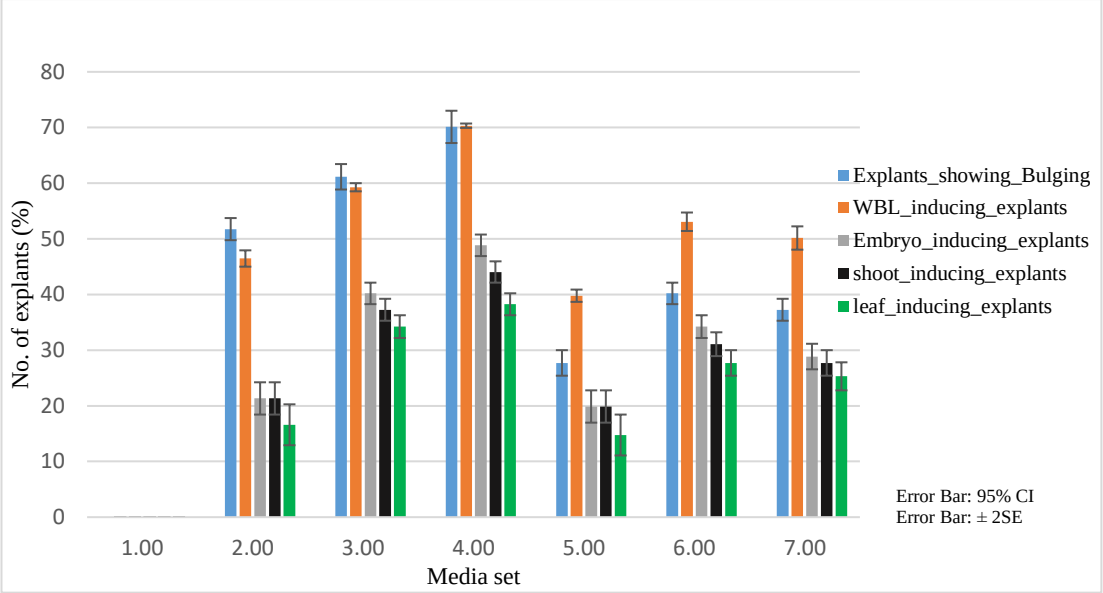

Fig 9: Percentage of explants showing bulging, WBLs formation, Embryo formation, shoot induction and leaf formation

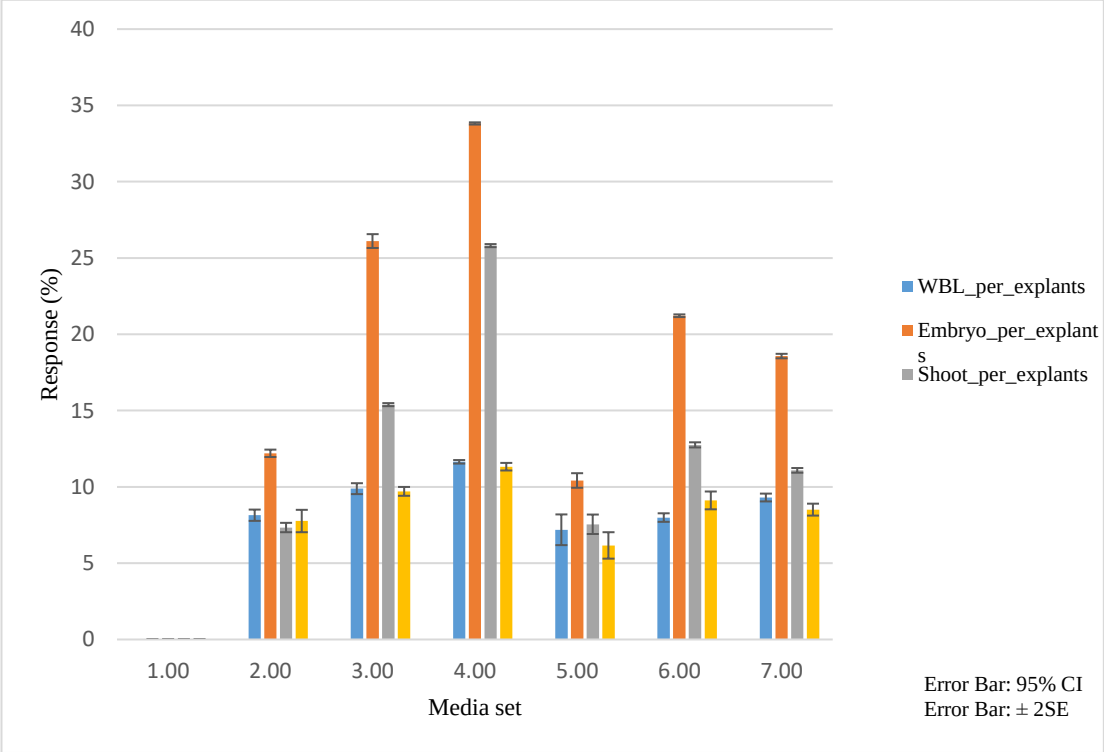

Fig 10: Effect of different media set on induction of WBLs, Embryo formation, shoot induction and leaf formation per explant

Fig 11: Formation of leaves and roots from the germinated shoots of *M. acuminata* (AAA) cv. Vaibalhla (a) leaves formations observed after 23rd weeks on media set 4, (b) leaves after 25th weeks on media set 4, (c) root formation observed on met set 9, (d) growth of roots and leaves observed after 29th weeks, (e) leaves and roots after 35th weeks, and (f) fully developed plantlets.

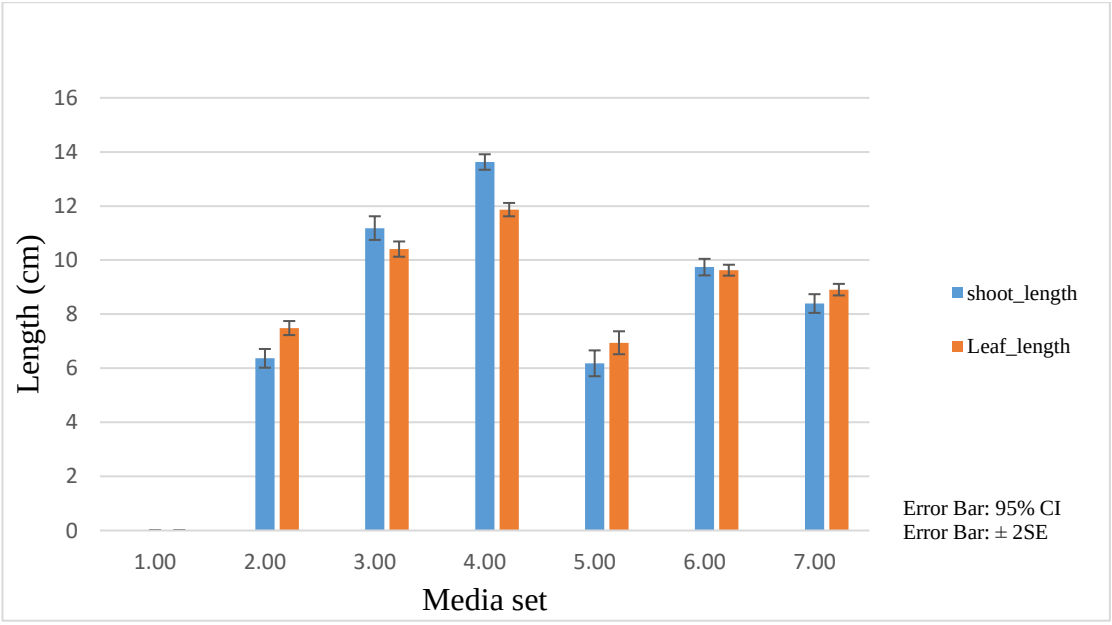

Fig 12: Effect of different media set on shoot and leaf length

Fully developed young plantlets after transferring to root inducing medium containing Indole-3-butyric acid (IBA) and control medium without any PGR showed formation of roots (Fig 11). Highest percentage of root per explants (13.93 ± 0.14) (Fig 13) and maximum root length (16.44 ± 0.06 cm) (Fig 14) was observed in MS medium supplemented with 1 mg/L of IBA as shown in table 9.

Media set	IBA (mg/L)	No. of plantlets	Root formation		
			Time taken in week (range)	Percentage of root per explants (mean ± SE)	Root Length (mean ± SE)
8*	0.0	10	2-4	7.49 ± 0.12	11.09 ± 0.08
9	1.0	10	2-4	13.93 ± 0.14	16.44 ± 0.06

Table 9: Effect of roots formation of the germinated plantlets on medium with and without IBA PGR.
Medium: MS + PGRs. Mean (±) SE
* Control without any PGRs

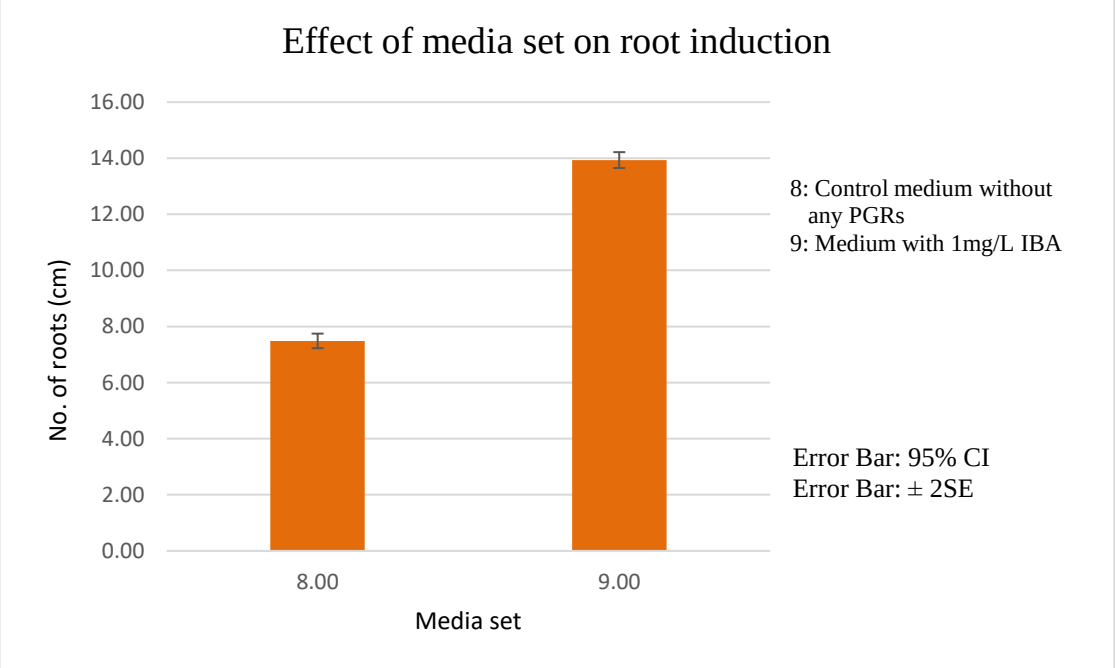

Fig 13: Effect of media set on root induction

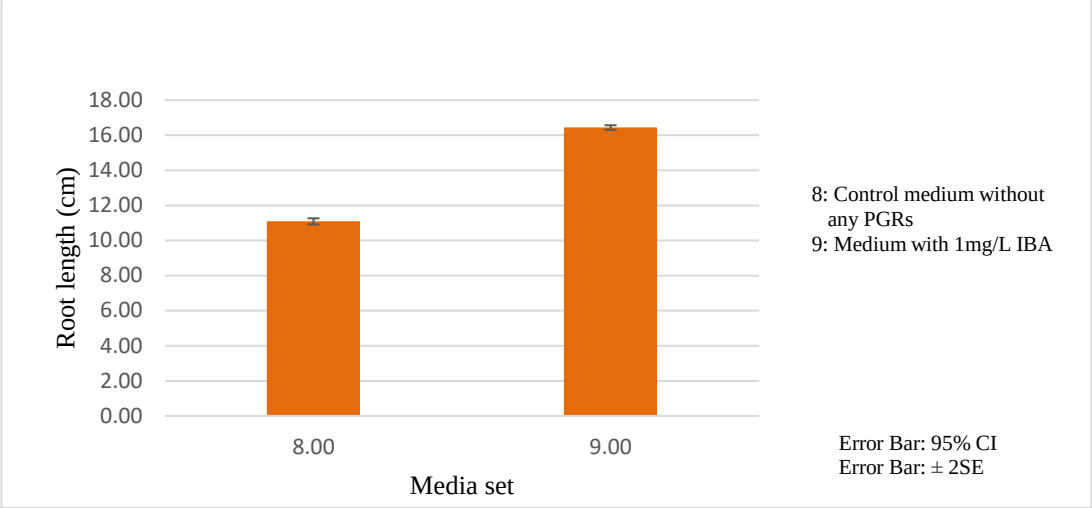

Fig 14: Effect of media set on root length

The regenerated plantlets having fully developed leaves and roots were transplanted for primary hardening in the laboratory. The plantlets were removed and also the roots were then thoroughly washed to remove agar and transferred in pots containing soil, fine sands and farm yard manure mixture in the ratio of 2:1:1 respectively which is shown in figure 15. Hardened plantlets were maintained in the greenhouse at a temperature of 27°C - 28°C and a relative humidity of 60% to 80%.

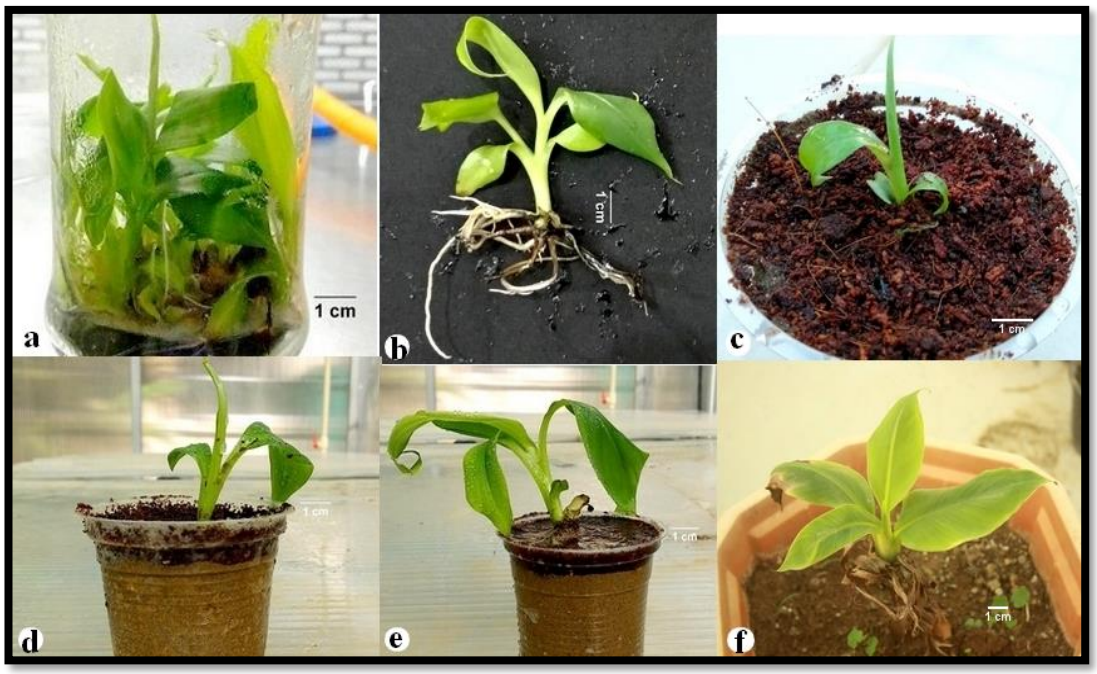

Fig 15: Preparation of fully developed young plantlets for hardening (a) Fully developed clusters of young plantlets of *M. acuminata* (AAA) cv. Vaibalhla, (b) separated plantlet after washing with sterile distilled water, (c) hardened plantlets, (d) plantlet after 2nd weeks of hardening, (e) plantlet after 4th weeks of hardening, and (f) well grown plantlet.

4.3. Evaluation of genetic fidelity of the regenerated plantlets

The ISSR banding patterns of the 9 randomly selected regenerated plants of *M. acuminata* cv. Vaibalhla (AAA) in comparison with the donor mother plant verified their genetic stability. Out of the total 15 ISSR primers used for the screening, 10 primers showed clear, sharp reproducible amplicons with a size range of 100 to 1200 base pairs in the regenerated plantlets as well as in the donor plant and none of the primers showed polymorphism as shown in table 10. The optimum annealing temperature of the selected primers varied between 45.0 and 47°C. The number of scoreable bands were found different for each primer with the minimum band of 2 (UBC-899) and a maximum band of 6 (UBC-886) with an average band of 4 per primer. Representative ISSR profiles of the control donor plant and 9 *in vitro* regenerated plantlets with ISSR primer (UBC-899) is shown in figure 16.

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-866	(CTC)6	47°C	4	0	4	300-1000
UBC-879	(CTTCA)3	46°C	4	0	4	300-2000
UBC-880	(GGAGA)3	47°C	4	0	4	200-1000
UBC-886	VDV(CT)7	47°C	6	0	6	100-900
UBC-887	DVD(TC)7	47°C	5	0	5	100-900
UBC-889	DBD(AC)7	46°C	4	0	4	200-1000
UBC-890	VHV(GT)7	46°C	5	0	5	100-1000
UBC-891	HVH(TG)7	45°C	3	0	3	300-900
UBC-895	AGAGTTGGTAGCTCTTGATC	46°C	3	0	3	400-1000
UBC-899	CATGGTGTTGGTCATTGTTCCA	46°C	2	0	2	600-1000
Total			40	0	40	

Table 10: Details of primer used and result observed
Where V represents A or C or G, D represents A or G or T, B represents C or G or T
H represents A or C or T.

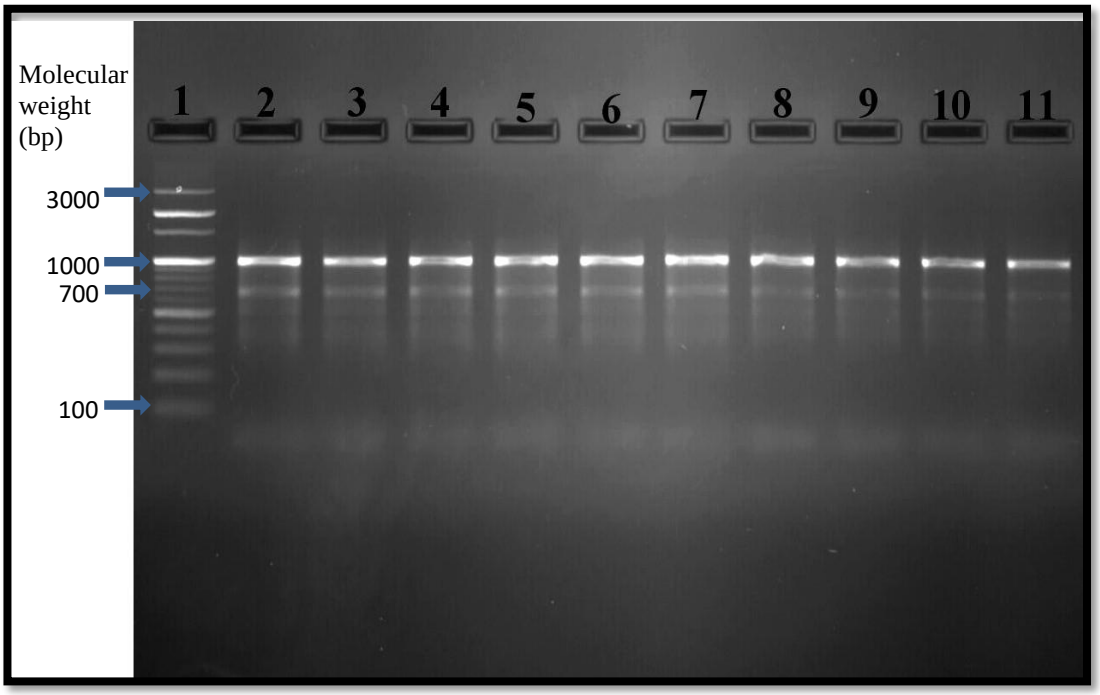

Fig 16: ISSR profile of the *in vitro* regenerated plantlets derived from male floral explants of *M. acuminata* Vaibalhla (AAA), Amplification with UBC ISSR Primer #899 Where Lane 1: 100 bp DNA lader, 2: donor plant, 3-11 regenerated plantlets.

4.4. Screening of banana bunchy top virus (BBTV) in the regenerated plantlets of *Musa acuminata* cv. Vaibalhla *(*AAA):

The screening of banana bunchy top virus using DNA-R primer of the 10 randomly selected regenerated plantlets of *M. acuminata* cv. Vaibalhla (AAA) in comparison with the donor mother plant and a symptomatic control plant showed the absence of BBTV in the regenerated plantlets. A sharp amplicon was observed with a size 1100 bp in the symptomatic control plant whereas no band were observed on the remaining regenerated plantlets as well as in the donor plant as shown in table 11. The documented gel picture of PCR assay for screening of Banana Bunchy Top Virus in the regenerated plantlets of *M. acuminata* Vaibalhla (AAA) is shown in figure 17.

Sample	BBTV (DNA-R) Primer Sequence (5'-3')	Tm (°C)	Band observed	Size range of the observed band (bp)
Selected plants donor plant	Forward – GGA AGA AGC CTC TCA TCT GCT TCA GAG AGC Reverse – CAG GCG CAC ACC TTG AGA AAC GAA AGG GAA	56°C	Not observed	0
Symptomatic plant			Observed	1100
RP1			Not observed	0
RP2			Not observed	0
RP3			Not observed	0
RP4			Not observed	0
RP5			Not observed	0
RP6			Not observed	0
RP7			Not observed	0
RP8			Not observed	0
RP9			Not observed	0
RP10			Not observed	0

Table 11: Details of primer used and result observed
Where R represent regenerated plantlets

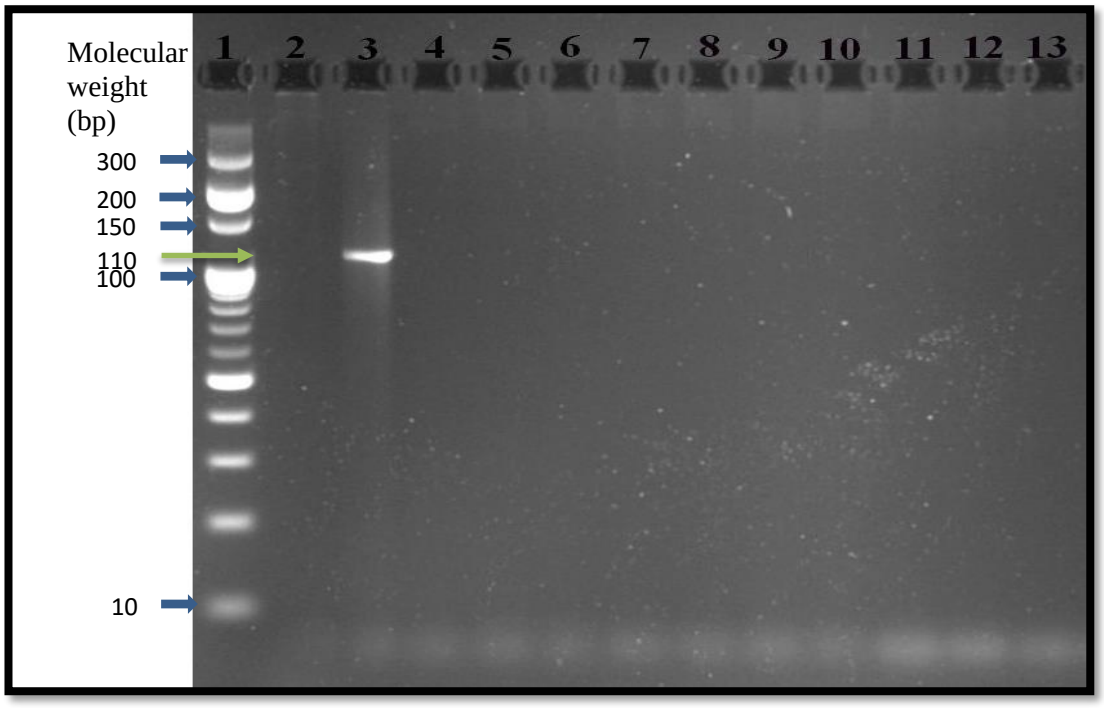

Fig 17: Documented gel picture of PCR assay for screening of Banana Bunchy Top Virus in the regenerated plantlets of *M. acuminata* Vaibalhla (AAA) where Lane 1: 100 bp DNA ladder, lane 2: asymptomatic donor plant, lane 3: symptomatic plant, lane 4-13 regenerated plantlets.

Chapter 5
Discussion

CHAPTER 4

DISCUSSION:

The current study tried to establish a means to generate a significant number of plantlets of the triploid cultivar *Musa acuminata* (AAA) cv. Vaibalhla, which is one of the most commonly grown and economically important banana in Mizoram and has a sweet flavour and texture, employing somatic embryogenesis. Using immature male flowers as explants, young plantlets were successfully produced through somatic embryogenesis. Even though in vitro plant regeneration is preferred more than the conventional breeding method, it has various limitations such as the need for a high number of manual processes and low multiplication rates, that leads to the increment of the cost of production (Savangikar, 2004). Somatic embryogenesis allows for greater multiplication in a shorter time and at a lower expense, making it potentially more efficient than organogenesis-based regeneration (Ibaraki, 2001).

The selection of explant determines the success or failure of most of the plant regeneration approaches. Somatic embryogenesis in commercial *Musa* spp. cultivars, on the other hand, is often performed employing two explants: premature male flower buds (Escalante and Teisson, 1994) and propagating meristems (Schoofs, 1997), scalps which consist of 3–4-mm-thick epidermal layer from the leafless meristematic clumps (Schoofs et al., 1997; Sholi et al., 2009; Sadik et al., 2007). The current study was performed by using immature male flower of *Musa accumiata* Vailalhla as explants. The use of proliferating meristems as a source of explants requires a greater number of subcultures of 5 to 14 months when the explants was inoculated in MS medium with supplementation of high concentration of 6-BAP (6-benzylaminopurine) 22.5 mg/l (Schoofs et al., 1999). Using of scalp as explants has several limitations as it is difficult to generate the ideal meristematic structure for the excision of scalp and requires the addition of high concentration of Thidiazuron (TDZ) and 6-benzylaminopurine (BAP) (100–400 μM). This particular explants also requires several cycles of multiplications (Strosse et al., 2006; Sadik et al., 2007). Even though scalps have already been commonly used as explant material, the percentage of responsive scalps was typically low (< 10%), most possibly due to shortage of homogeneity of scalps. Moreover, the duration for initiation of embryo ffrom scalps generally required longer that ranged between 3 to 8 months. The main disadvantage of the scalp procedure is the use

of lengthy material preparation step (Schoofs et al., 1999; Strosse et al., 2006). Immature male flowers (IMF) are demonstrated to be one of the most effective starting materials for embryogenic cell suspension initiating and regeneration of plantlets among these explants (Nandhakumar et al., 2018). However, supplementation of elevated concentration of PGR cytokinin increases the genetic variability up to 4-10 times over immature male flower bud and axillary buds (Reuveni et al., 1993). Therefore, current study was performed by utilization of immature male flowers as explant that promotes plant development with lower levels of somaclonal variation. Using this particular explants of *Musa acuminata* Vaibalhla, somatic embryos were generated within 10- 12 weeks of inoculation of the explants without the formation of callus when the medium was supplemented with different concentration of 1-Naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) and Thidiazuron (TDZ). More over the explants were easily available inside the campus and material preparation phase requires a short period of time.

In this study, PCR as a molecular tool was used for detection of BBTV in randomly selected *Musa acuminata* cv. Vailbalhla (AAA) by using a symptomatic banana sample as control. Two oligonucleotides belonging to the BBTV- DNA-R component were used as primers (Harding et al., 1993). The screening of banana bunchy top virus using DNA-R primer of the selected 5 sample showed the absence of BBTV whereas a sharp amplicon was observed with a size 1100 bp in the symptomatic control plant which was observed in leaf samples of naturally infected banana collected resulted in *1.1 kb amplifications with the same primer sequence (Vishoi *et al*. 2009). This result is consistent with the findings of Harding et al. (2000) and Abdel-Hamid et al., (2003), who emphasised the importance of detecting BBTV as an early step in virus control, particularly in plants generated via tissue culture as well as symptomless plants. Hence, the sample selected were screened successfully and found free from BBTV and were used for the *In vitro* somatic embryogenesis of *Musa acuminata* (AAA) cv. Vaibalhla.

In previous study, Male flower bud of *Musa accuminata* Vailalhla were sterilized by using 70 % (v/v) ethanol which was followed by 1 % sodium hypochlorite and rinsing with sterile distilled water (Hrahsel et al., 2014). The sterilisation process requires multiple steps and treatment with sodium hypochlorite affects the explants and leads to dead of low percentage of the explant. Banana inflorescence is comprised of densely overlapping bracts with floral hands organised in a semi helical pattern. As a result of

this natural protection Flower buds were neither harmed or dehydrated during transportation to the lab. Hence, in the current study, sterilization of the immature male flower buds was performed in laminar air flow by removing upper layer of bracts and using 70 % (v/v) ethanol. Immature male flowers were excised and used as explants. Explants after inoculation showed no contamination and 100% survival was observed. Previous studies have shown that the antibiotics used in bananas, that can be strain specific (Habiba et al., 2002), are phytotoxic to plant tissues (Van den houwe, 1998). HgCl2 (mercuric chloride) is employed for sterilization, sometimes along with sodium hypochlorite (Banerjee and Sharma, 1989) however heavy metals are not typically suggested for sterilization. According to the findings, no particular antibiotic or sterilant is necessary for controlling contamination when immature male flowers are utilised as explants, which are naturally protected.

In the present study, bulging of the explant was observed at the basal part within 2nd week of the culture. MS medium supplemented with 0.1 mg/L, 1.0 mg/L BAP and 1.0 mg/L TDZ was found to be superior among the different PGRs tested. Similar bulging of the explants at the basal part was observed on *Musa acuminata* cv. Vaibalhla (AAA) (Hrahsel et al., 2014). It was found that increasing the concentration of NAA and TDZ result in reduction of the bulging percentage. Explants inoculated in MS medium without any PGRs doesn't give any response and was found browning that leads to the dead of the explants.

The formation of white bud-like structures in the present study was found similar to the white buds which were observed in different banana cultivars like *Musa acuminata* cv. Vaibalhla (AAA) (Hrahsel et al., 2014), Sannachenkadali (AA), Red banana (AAA) (Resmi and Nair, 2007) and Virupakshi (AAB) and Sirumalai (AAB) (Mahadev et al., 2011). WBLs mainly appeared after 2 months in previous reports when inoculated in medium high BAP concentrations (8 mg/L, 5 mg/L) (Mahadev et al., 2011), on *Musa acuminata* cv. Vaibalhla (AAA) WBLs formation was observed within 5-7 weeks when inoculated in MS medium supplemented with BAP (2 mg/L) and NAA (0.5 mg/L) in combination. Whereas in the present study, WBLS formation was observed after 4-5 weeks with maximum number of explants forming (WLs 70.32 ± 0.19) when inoculated on MS medium supplemented with 0.1 mg/L NAA, 1.0 mg/L BAP and 1.0 mg/L TDZ in combination.it was found that increasing the concentration of NAA, TDZ and decreasing the concentration of BAP reduced the percentage of WBL formation thereby increasing the during (7-8 weeks) which was less preferable.

Similar observations were reported on cultivars like Berangan (AAA), Rastali (AAB), Nangka (AAB) and Abu (ABB) (Darvari *et al*., 2010).

The development of the embryo (zygotic and non-zygotic) follows a sequence of organized events, which comprise active cell division that contribute to the formation of an undifferentiated globular shape embryo followed by cellular differentiation and enlargement of the embryo. Maturation, the final stage of embryo development is distinguished by overall cell expansion and accumulation of reserve substances of the embryo. In several edible banana genotypes, the basis for the low quality and quantity of the somatic embryos, and the poor regeneration and conversion to vigorous plants is frequently the least studied aspects in banana (Krikorian and Scott, 1995). Proembryonal protuberances formation was observed with the maximum percentage when the explants were transferred to MS medium supplemented with different concentration of NAA, BAP and TDZ without the formation of callus. Similar pattern of development was observed when basal leaf sheaths was used as explants which was cultured on medium containing Dicamba with TDZ (Novak et al.,1989). In previous reports, Supplementation of TDZ on the medium with (0.45 µM) TDZ produced off white friable calli while (3.6 µM) and (9.00 µM) TDZ initiated yellow compact calli (Smitha et al., 2020). Embryogenic calluses were initiated from MS solid medium containing 2.0 mg/l TDZ whereas application of BAP and NAA on medium leads to the formation of leaf-like structure was and developed further until plantlets regenerate (Srangsam and Kanchanapoom , 2003). Novak et al., (1989) reported that combination of 30 µM Dicamba with 5 µM TDZ improved callus formation and overcame the problem of browning. The highest germination rate of somatic embryos was observed in Rosa hybrida 'Samantha' on MS medium containing 1.0 mg/L TDZ and 0.5 mg/L BA (Bao et al., 2012). Many plant species require a significant cytokinin-supplemented medium for embryo formation, according to studies like Coffea canephora (Hatanaka et al., 1991), Medicago truncatula (Nolan et al., 1989) and Eleusina caracana (Eapen and George, 1989). Novak et al., 1989; Dhed'a et al., 1991; Grapin et al., 1996; Cote et al., 1996) observed that cytokinin was required for the formation of banana ECS embryos.

Thus, based on the above findings combination of BAP, NAA and TDZ in different concentrations were used in the present study which result in the formation of the embryos without the callus stages. The maximum number of embryo induction per

explant was observed on MS medium supplemented with 0.1 mg/L of NAA, 1 mg/L of BAP and 1 mg/L of TDZ PGRs with 33.81 per explant and also maximum percentage of explants generate embryos (48.84) in the same media set. It was observed that application low concentrations of TDZ induced multiple shoots, but high concentrations reduced the frequency of shoot regeneration (Jafari et al., 2015). Compare to BAP and KN, TDZ is more effective at low concentrations (Parveen et al., 2010; Devi et al., 2011). BAP has been believed to be one of the most effective cytokinin for the generation of shoot in plant tissue culture (Tan et al., 2011; Hrahsel et al., 2014). In the present study, Embryos were transferred to medium containing BAP, NAA and TDZ for regeneration of shoots. It was observed that 44.04% of the total explants showed shoots formation with the maximum number of 25.81 per explant and the longest shoot length (13.36 cm) was observed on MS medium supplemented with 2 mg/L BAP and 1 mg/L TDZ after 20-22nd week after inoculation. Similarly, highest frequency of germination (56.2%) of somatic embryos was observed on medium supplemented with 1.0 mg/L TDZ and 0.5 mg/L BA in Rosa hybrida 'Samantha' (Bao et al., 2012). The germinated shoots were transferred to root inducing medium containing IBA and 100 % root induction was observed as IBA is very effective to increase endogenous auxin contents and show higher stability against catabolism and in activation by conjugation with growth inhibitors (George et al., 2008; Hasan et al., 2010).

True-to-type clonal fidelity is among the most crucial requirements for any crop species' micropropagation. A key concern with in vitro cultures is the appearance of somaclonal variation across subclones of one parental line, which occurs as a direct result of in vitro culture of plant cells, tissues, or organs. Though the process for banana micropropagation is known to produce clonal material, there have been instances of somaclonal variation, particularly in cv. Williams (Damasco et al., 1996). A good examination of genetic stability of the regenerated plantlets may be done by combining two types of markers that amplify different areas of the genome (Lattoo et al., 2006; Ray et al., 2006; Faisal et al., 2012; Phulwaria et al., 2013). In the current work, PCR-based approaches, ISSR, was used to assess genetic variability of the regenerated banana plantlets. While genetic variants exist, most organised cultures, particularly shoot tips, exhibit strict genotypic and phenotypic stability under tissue culture conditions (Bennici et al., 2004). This is also relevant for cv. Vaibalhla (AAA), because no variability was noticed when the set of primers examined using the

randomly chosen regenerated plantlets. It was discovered that high levels of plant growth regulators generated variants such as shoot length suppression, resulting in morphological deformities and genetic modifications in other banana genotypes (Shenoy and Vasil, 1992; Martin et al., 2006). As a result, minimal amounts of growth regulators were used in the current investigation. A significant number of publications documented the development of PCR primer pairs and highly sensitive assays for almost all viral pathogens (Hadidi et al., 1993; Harding et al., 2000). Mullis and Faloona (1987) demonstrated that polymerase chain reaction (PCR) is one of the most effective molecular approaches for pathogen identification currently available. A succession of hot, cold, and warm cycles is used in the PCR process. Hot cycles separate DNA strands into single strands. Cold cycles enable the primers to adhere. The enzyme duplicates each piece of primed DNA during warm cycles. If a virus is present, PCR will generate a huge amount of a specific fragment of DNA. Electrophoresis was employed, which separates fragments of DNA by size, to determine whether or not that piece of DNA is present. Several studies found that new molecular approaches have become accessible for analyzing plant viruses (Levy and Hadidi, 1991; Smith et al., 1992; Hu et al., 1993; Sadik et al., 1997; Harding et al., 2000; Abdel-Hamid et al., 2003).

In this study, PCR as a molecular tool was used for detection of BBTV in randomly selected regenerated banana plantlets as well as in symptomatic banana samples. Two oligonucleotides belonging to the BBTV- DA-R component were used as primers (Harding et al., 1993). The screening of banana bunchy top virus using DNA-R primer of the 10 randomly selected regenerated plantlets of *M. acuminata* cv. Vaibalhla (AAA) in comparison with the donor mother plant and a symptomatic control plant showed the absence of BBTV in the regenerated plantlets. A sharp amplicon was observed with a size 1100 bp in the symptomatic control plant whereas no band were observed on the remaining regenerated plantlets as well as in the donor plant. The same amplicon was observed in leaf samples of naturally infected banana collected resulted in *1.1 kb amplifications with the same primer sequence (Vishnoi et al., 2009).

This result is consistent with the findings of Harding et al., (2000) and Abdel-Hamid et al., (2003), who emphasised the importance of detecting BBTV as an early step in virus control, particularly in plants generated via tissue culture as well as symptomless plants. Thus, the absence of BBTV in the regenerated plantlets was confirmed by the screening results.

Chapter 6

SUMMARY

CHAPTER 6

SUMMARY

Screening of Banana Bunchy Top Virus (BBTV):

Five *Musa acuminata* cv. Vaibalhla (AAA) samples collected for the study were successfully screened for BBTV using the DNA-R primer. PCR amplification of the selected samples showed no band while a band with a size range of 1100bp was observed in the symptomatic sample. The absence of band confirms that the selected samples were free from BBTV and can be used for further study of *in vitro* somatic embryogenesis.

Establishment of aseptic culture of *Musa acuminata* cv. Vaibalhla using male flowers:

Male flower buds were surface sterilized with 70% ethanol and was found effective in successful regeneration of plantlets through somatic embryogenesis. In vitro somatic embryogenesis and plantlet regeneration of the explants were successfully achieved with varied morphogenic responses. Media supplemented with 0.1 mg/L of NAA, 1 mg/L of BAP and 1mg/L TDZ showed the maximum percentage of bulging (70.11). Maximum white bud like structure (WBL) was observed in media supplemented with 0.1 mg/L of NAA, 1 mg/L of BAP and 1 mg/L TDZ while minimum was observed in media supplemented with 0.1 mg/L of NAA and 2 mg/L TDZ.

Somatic embryogenesis was observed from the proembryonal globular protuberances which were developed from the WBLs after 10 week of inoculation giving rise to embryos through formation of heart, torpedo and cotyledonary stages. Maximum embryo induction per explant (33.81) and maximum explants generating embryos (48.84) was observed on media supplemented with 0.1 mg/L of NAA, 1 mg/L of BAP and 1 mg/L TDZ.

Shoot formation of the cultured embryos was observed on the 22nd week after inoculation. Media supplemented with 2 mg/L BAP and 1 mg/L of TDZ showed superior response of 44.04% of the total explants showing shoot formation with highest number of 25.81 per explant and longest shoot length of 13.36 cm.

Leaf formation from the regenerated shoots was observed on the 25th week after inoculation. Media supplemented with 2 mg/L BAP and 1 mg/L of TDZ was found most effective with the highest percentage of shoot showing leaf induction (38.24%) with maximum number of leaves per shoot (11.32) and longest leaf length (11.87 cm).

Regenerated young plantlets showed high percentage of root induction with 13.93±0.14 per explant and maximum root length of 16.44±0.06 when transferred in media supplemented with 1 mg/L of IBA.

In the current study, successful regeneration of plantlets through somatic embryogenesis was achieved and the regenerated plantlets were transplanted in pots containing soil, fine sand and farm yard manure mixture in the ratio of 2:1:1 respectively and maintained in the greenhouse.

Evaluation of genetic fidelity of the regenerated plantlets:
ISSR primers was successful in evaluation of genetic fidelity in the regenerated *M. acuminata* cv. Vaibalhla samples. A total of 15 ISSR primers were used for the screening out of which 10 primers showed clear, sharp and reproducible bands. Only monomorphic bands with a maximum of 6 (UBC-886) and a minimum of 2 (UBC-899) was observed in the primers tested and no polymorphic band was found among the samples tested.

Screening of banana bunchy top virus (BBTV) in the regenerated plantlets of *Musa acuminata* cv. Vaibalhla (AAA):
Fully regenerated plantlets through somatic embryogenesis were screened for BBTV using the DNA-R primer. PCR amplification of the regenerated plantlet samples showed no band which indicates the absence of BBTV, thereby confirming the successful regeneration of disease-free plantlets through somatic embryogenesis.

In the present study, an efficient standardized protocol for regeneration of plantlets through somatic embryogenesis was successfully established. A single male inflorescence was used in the study which gave around 300 male flowers leading to WBL formation, SE induction and finally successful regeneration of plantlets of around 1500 disease free, genetically stable clones of the mother plant. This approach is cost-effective, where the male flower buds of banana were made beneficial in young plantlets regeneration, which fulfils the demand for large scale cultivations. Banana genetic improvement through traditional hybridization is complicated and challenging because of its characteristics like polyploid and vegetatively propagation crop. Establishing embryogenic culture systems having consistent regeneration efficiency from significant banana cultivars is a necessity for achieving the potential of crop

improvement. The current methodology can also be applicable for future conservation and genetic transformation investigations on this commercially significant banana.

Chapter 7

LIST OF ABBREVIATIONS

LIST OF ABBREVIATIONS

NRCB	: National Research Centre on Banana
BSV	: Banana Streak Virus
CMV	: Cucumber Mosaic Virus
BBTV	: Banana Bunchy Top Virus
DNA	: Deoxyribonucleic acid
RAPD	: Random Amplified Polymorphic DNA
RFLP	: Restriction Fragment Length Polymorphism
SSR	: Simple Sequence Repeats
STRs	: Short Tandem Repeats
ISSR	: Inter Simple Sequence Repeats
NAA	: Naphthalene acetic acid
BAP	: 6-Benzylaminopurine
TDZ	: Thidiazuron
2ip	: isopentyladenin
KN	: Kinetin
PEM	: Proembryogenic Mass
PGR	: Plant growth regulators
IAA	: Indole-3-acetic acid
IBA	: Indole-3-butyric acid

PEDCs	: Pre-embryogenic determined cells
IEDCs	: Induced embryogenic determined cells
2, 4-D	: 2, 4-Dichlorophenoxyacetic acid
PCR	: Polymerase Chain Reaction
CTAB	: Cetyltrimethyl Ammonium Bromide
$MgCl_2$	: Magnesium Chloride
EDTA	: Ethylene Diaminetetra Acetic Acid
RNA	: Ribonucleic Acid
dNTP	: De-oxyribonucleotide Triphosphate
UV	: Ultra Violet
MS	: Murashige & Skoog
WBLs	: white bud-like structures
UBC	: University of British Columbia
SE	: Standard error
IMF	: Immature male flowers
$HgCl_2$	: Mercuric Chloride
ECS	: Embryogenic cell suspensions

Chapter 8
BIBLIOGRAPHY

REFERENCES

Abdel-Hamid, I. A., Wagih, E. E., Mahfouz, H. T., & Sadik, A. S. (2003). Non-radioactive detection of banana bunchy top nanovirus. Arab Journal of Biotechnology, 6(2), 327-336.

Abdellatif, K. F., Hegazy, A. E., Aboshama, H. M., Emara, H. A., & El-Shahed, A. A. (2012). Morphological and molecular characterization of somaclonal variations in tissue culture-derived banana plants. Journal of Genetic Engineering and Biotechnology, 10(1), 47-53.

Al-zahim, M. A. (1996). Induction of somatic embryogenesis and the use of RAPD to classify and to detect somaclonal variation in garlic (Allium sativum L.) (Doctoral dissertation, University of Birmingham).

Aremu, A. O., Bairu, M. W., Szüčová, L., Doležal, K., Finnie, J. F., & Van Staden, J. (2013). Genetic fidelity in tissue-cultured 'Williams' bananas–The effect of high concentration of topolins and benzyladenine. Scientia Horticulturae, 161, 324-327.

Arinaitwe, G., Rubaihayo, P. R., & Magambo, M. J. S. (2000). Proliferation rate effects of cytokinins on banana (*Musa* spp.) cultivars. Scientia Horticulturae, 86(1), 13-21.

Arnold, S. V. Izabela. S, Peter, B, Julia. D, Lada. F. 2002. Developmental pathways of somatic embryogenesis. Plant Cell, Tissue and Organ Culture, 69, 233-249.

Asif, M. J., Mak, C., & Othman, R. Y. (2001). In vitro zygotic embryo culture of wild *Musa acuminata* ssp. malaccensis and factors affecting germination and seedling growth. Plant cell, Tissue and Organ culture, 67(3), 267-270.

Aurore, G., Parfait, B., & Fahrasmane, L. (2009). Bananas, raw materials for making processed food products. Trends in Food Science & Technology, 20(2), 78-91.

Bairu, M. W., Fennell, C. W., & van Staden, J. (2006). The effect of plant growth regulators on somaclonal variation in Cavendish banana (Musa AAA cv.'Zelig'). Scientia Horticulturae, 108(4), 347-351.

Banerjee, N., & Sharma, A. K. (1989). Chromosome constitution and alkaloid content in Rauwolfia L. (Apocynaceae). Cytologia, 54(4), 723-728.

Bao, Y., Liu, G., Shi, X., Xing, W., Ning, G., Liu, J., & Bao, M. (2012). Primary and repetitive secondary somatic embryogenesis in Rosa hybrida 'Samantha'. Plant Cell, Tissue and Organ Culture (PCTOC), 109(3), 411-418.

Barrett, C., Lefort, F., & Douglas, G. C. (1997). Genetic characterisation of oak seedlings, epicormic, crown and micropropagated shoots from mature trees by RAPD and microsatellite PCR. Scientia horticulturae, 70(4), 319-330.

Bayliss, M. W. (1980). Chromosomal variation in plant tissues in culture. 5. International Review of Cytology.

Becker, D. K., Dugdale, B., Smith, M. K., Harding, R. M., & Dale, J. L. (2000). Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv'Grand Nain'via microprojectile bombardment. Plant Cell Reports, 19(3), 229-234.

Bennici, A., Anzidei, M., & Vendramin, G. G. (2004). Genetic stability and uniformity of Foeniculum vulgare Mill. regenerated plants through organogenesis and somatic embryogenesis. Plant science, 166(1), 221-227.

Berg, L. A., & Bustamante, M. (1974). Heat treatment and meristem culture for the production of virus-free bananas. Phytopathology, 64: 320-322.

Bhosale, U. P., Dubhashi, S. V., Mali, N. S., & Rathod, H. P. (2011). In vitro shoot multiplication in different species of Banana, Asian Journal of Plant Science and Research, 1(3), 23-27.

Close, K. R., & Gallagher-Ludeman, L. A. (1989). Structure-activity relationships of auxin-like plant growth regulators and genetic influences on the culture induction response in maize (Zea mays L.). Plant Science, 61(2), 245-252.

Conger, B. V., Hanning, G. E., Gray, D. J., & McDaniel, J. K. (1983). Direct embryogenesis from mesophyll cells of orchardgrass. Science, 221(4613), 850-851.

Cooke, T. J., Racusen, R. H., & Cohen, J. D. (1993). The role of auxin in plant embryogenesis. The Plant Cell, 5(11), 1494.

Corredoira, E., Ballester, A., Ibarra, M., & Vieitez, A. M. (2015). Induction of somatic embryogenesis in explants of shoot cultures established from adult Eucalyptus globulus and E. saligna× E. maidenii trees. Tree physiology, 35(6), 678-690.

Corredoira, E., Valladares, S., Martínez, M. T., Vieitez, A. M., & San José, M. C. (2013). Somatic embryogenesis in Alnus glutinosa (L.) Gaertn. Trees, 27(6), 1597-1608.

Cote, F. X., Domergue, R., Monmarson, S., Schwendiman, J., Teisson, C., & Escalant, J. V. (1996). Embryogenic cell suspensions from the male flower of *Musa* AAA cv. Grand nain. Physiologia Plantarum, 97(2), 285-290.

Cronauer, S. S., & Krikorian, A. D. (1983). Somatic embryos from cultured tissues of triploid plantains (*Musa* 'ABB'). Plant cell reports, 2(6), 289-291.

Cronauer, S. S., & Krikorian, A. D. (1986). 'Banana (*Musa* Spp.)', YPS Bajaj. Trees I. Biotechnology in Agriculture and Forestry. Springer Verlag Berlin Heidelberg.

Cuenca, B., San-José, M. C., Martínez, M. T., Ballester, A., & Vieitez, A. M. (1999). Somatic embryogenesis from stem and leaf explants of Quercus robur L. Plant Cell Reports, 18(7), 538-543.

Cullis, C. A., & Cleary, W. (1986). DNA variation in flax tissue culture. Canadian journal of genetics and cytology, 28(2), 247-251.

D'Hont, A., Denoeud, F., Aury, J. M., Baurens, F. C., Carreel, F., Garsmeur, O., ... & Wincker, P. (2012). The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. *Nature*, *488*(7410), 213-217.

D'Hont, A., Paget-Goy, A., Escoute, J., & Carreel, F. (2000). The interspecific genome structure of cultivated banana, *Musa* spp. revealed by genomic DNA in situ hybridization. Theoretical and Applied Genetics, 100(2), 177-183.

Dahot, M. U. (2007). Micro-propagation efficiency in banana (Musa sp.) under different immersion systems. Pakistan journal of biological sciences: PJBS, 10(5), 726-733.

Damasco, O. P., Graham, G. C., Henry, R. J., Adkins, S. W., Smiths, M. K., & Godwin, I. D. (1996). Random amplified polymorphic DNA (RAPD) detection of dwarf

off-types in micropropagated Cavendish (*Musa* spp. AAA) bananas. Plant Cell Reports, 16(1), 118-123.

Daniells, J. (2001). *Musalogue*: a catalogue of *Musa* germplasm: diversity in the genus *Musa*. Bioversity International.

Daniells, J. W. (1988). Comparison of growth and yield of bananas derived from tissue culture and conventional planting material, Banana Newsletter, 11(2).

Darvari, F. M., Sariah, M., Puad, M. P., & Maziah, M. (2010). Micropropagation of some Malaysian banana and plantain (*Musa* sp.) cultivars using male flowers. African Journal of Biotechnology, 9(16), 2360-2366.

Darvari, F. M., Sariah, M., Puad, M. P., & Maziah, M. (2010). Micropropagation of some Malaysian banana and plantain (*Musa* sp.) cultivars using male flowers. African Journal of Biotechnology, 9(16), 2360-2366.

De Guzman, E. V. (1975). Project on production of mutants by irradiation of in vitro cultured tissues of coconut and banana and their mass propagation by the tissue culture technique (No. IAEA--173).

De Guzman, E. V., Ubalde, E. M., & Del Rosario, A. G. (1976). Banana and coconut in-vitro cultures for induced mutations studies (No. IAEA--194).

De Klerk, G. J. (1990). How to measure somaclonal variation. Acta Botanica Neerlandica, 39(2), 129-144.

De Langhe, E., Pillay, M., Tenkouano, A., & Swennen, R. (2005). Integrating morphological and molecular taxonomy in *Musa*: the African plantains (*Musa* spp. AAB group). Plant Systematics and Evolution, 255(3), 225-236.

Denton, I. R., Westcott, R. J., & Ford-Lloyd, B. V. (1977). Phenotypic variation of Solanum tuberosum L. cv. Dr McIntosh regenerated directly from shoot-tip culture. Potato Research, 20(2), 131-136.

Devi, P. S., Arundathi, A., & Rao, T. R. (2011). Multiple shoot induction and regeneration of whole plants from cotyledonary node and nodal explants of Sterculia urens Roxb., a gum yielding tree. Journal of plant biochemistry and biotechnology, 20(2), 161-165.

Dhed'a, D. (1992). Culture de suspensions cellulaires embryogéniques et régénération en plantules par embryogénèse somatique chez le bananier et le bananier plantain (*Musa* spp.). Katholieke Universiteit Leuven, Faculteit der Wetenschappen, Belgium: 168p.

Dhed'a, D. B., Dumortier, F., Panis, B., & Vuylsteke, D. (1991). Plant regeneration in cell suspension cultures of the cooking banana cv. Bluggoes'(*Musa* spp. ABB group). Fruits. 46, 125-135.

Dhed'a, D. B., Dumortier, F., Panis, B., & Vuylsteke, D. (1991). Plant regeneration in cell suspension cultures of the cooking banana cv. Bluggoes' (*Musa* spp. ABB group).

Dodeman, V. L., Ducreux, G., & Kreis, M. (1997). Zygotic embryogenesis versus somatic embryogenesis. Journal of Experimental Botany, 48(8), 1493-1509.

Doležel, J., & Novak, F. J. (1986). Sister chromatid exchanges in garlic (Allium sativum L.) callus cells. Plant cell reports, 5(4), 280-283.

Drew, R. A., & Smith, M. K. (1990). Field evaluation of tissue-cultured bananas in south-eastern Queensland. Australian Journal of Experimental Agriculture, 30(4), 569-574.

Dubois, T., Guedira, M., Dubois, J., & Vasseur, J. (1990). Direct somatic embryogenesis in roots of Cichorium: is callose an early marker?. Annals of Botany, 65(5), 539-545.

Eapen, S., & George, L. (1989). High frequency plant regeneration through somatic embryogenesis in finger millet (Eleusine coracana Gaertn). Plant Science, 61(1), 127-130.

Escalant, J. V., & Teisson, C. (1989). Somatic embryogenesis and plants from immature zygotic embryos of the species *Musa acuminata* and *Musa balbisiana*. Plant Cell Reports, 7(8), 665-668.

Escalant, J. V., Teisson, C., & Cote, F. (1994). Amplified somatic embryogenesis from male flowers of triploid banana and plantain cultivars (*Musa* spp.). In Vitro–Plant, 30(4), 181-186.

Faisal, M., Alatar, A. A., Ahmad, N., Anis, M., & Hegazy, A. K. (2012). An efficient and reproducible method for in vitro clonal multiplication of Rauvolfia tetraphylla L. and evaluation of genetic stability using DNA-based markers. Applied biochemistry and biotechnology, 168(7), 1739-1752.

FAOSTAT (Food and Agriculture Organization of the United Nations), 2013. http://faostat.fao.org/ (accessed 26.11.2021.).

Forster, M. P., Rodríguez Rodríguez, E., & Díaz Romero, C. (2002). Differential characteristics in the chemical composition of bananas from Tenerife (Canary Islands) and Ecuador. Journal of Agricultural and Food Chemistry, 50(26), 7586-7592.

Gagliardi, R. F., Pacheco, G. P., Carneiro, L. A., Valls, J. F. M., Vieira, M. L. C., & Mansur, E. (2003). Cryopreservation of Arachis species by vitrification of in vitro-grown shoot apices and genetic stability of recovered plants. CryoLetters, 24(2), 103-110.

Gaj, M. D. (2004). Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to Arabidopsis thaliana (L.) Heynh. Plant Growth Regulation, 43(1), 27-47.

Ganapathi, T. R., Higgs, N., Van Eck, J., Balint-Kurti, P., & May, G. D. (1999). Transformation and regeneration of the banana cultivar “Rasthali” (AAB). In International symposium on the molecular and cellular biology of banana (pp. 22-25).

Ganapathi, T. R., Srinivas, L., Suprasanna, P., & Bapat, V. A. (2001). Regeneration of plants from alginate-encapsulated somatic embryos of banana cv. Rasthali (*Musa* spp. AAB group). In Vitro Cellular & Developmental Biology-Plant, 37(2), 178-181.

Ganapathi, T. R., Suprasanna, P., Bapat, V. A., Kulkarni, V. M., & Rao, P. S. (1999). Somatic embryogenesis and plant regeneration from male flower buds in banana. Current Science, 1228-1231.

George, E. F., & Sherrington, P. D. (1984). Plant propagation by tissue culture. Plant propagation by tissue culture.

George, E. F., (1993). Plant Propagation by Tissue Culture. Philosophical Transactions of the Royal Society of London, 351, 1291-1298.

George, E. F., Hall, M. A., & De Klerk, G. J. (2008). Plant propagation by tissue culture 3rd Edition. Springer, Dordrecht, The Netherlands, 1, 501.

Ghosh, F., & Gadgil, V. N. (1979). Shift in ploidy level of callus tissue: a function of growth substances. Indian journal of experimental biology.

Gleddie, S., Keller, W., & Setterfield, G. (1983). Somatic embryogenesis and plant regeneration from leaf explants and cell suspensions of Solanum melongena (eggplant). Canadian Journal of Botany, 61(3), 656-666.

Goldberg, R. B., De Paiva, G., & Yadegari, R. (1994). Plant embryogenesis: zygote to seed. Science, 266(5185), 605-614.

Gomez, K. R., Sol, L. D., Reyes, V. M., Seijo, M., Posada, P. L., Herrera, I., & Vincent, E. J. (2001). Somatic embryogenesis in banana and plantain (*Musa* sp.) from male immature flowers. Biotech-Veg, 1, 29-35.

Grapin, A. (1995). Régénération par embryogénèse somatique en milieu liquide et transformation génétique par biolistique de bananiers di-et triploïdes. Regeneration by somatic embryogenesis in a liquid medium and genetic transformation of diploid and triploid bananas by biolistics.) ENSAM, Montpellier (FRA) 93pp.

Grapin, A., Ortiz, J. L., Domergue, R., Babeau, J., Monmarson, S., Escalant, J. V., ... & Côte, F. (1998). Establishment of embryogenic callus and initiation and regeneration of embryogenic cell suspensions from female and male immature flowers of *Musa*.

Grapin, A., Ortiz, J. L., Lescot, T., Ferrière, N., & Côte, F. X. (2000). Recovery and regeneration of embryogenic cultures from female flowers of False Horn Plantain. Plant Cell, Tissue and Organ Culture, 61(3), 237-244.

Grapin, A., Schwendiman, J., & Teisson, C. (1996). Somatic embryogenesis in plantain banana. In Vitro-Plant, 32(2), 66-71.

Gray, D. J. (1987). Quiescence in monocotyledonous and dicotyledonous somatic embryos induced by dehydration. HortScience (USA).

Gubbuk, H., & Pekmezci, M. (2004). In vitro propagation of some new banana types (*Musa* spp.). Turkish Journal of Agriculture and Forestry, 28(5), 355-361.

Haberlandt, G. (1902). Kulturversuche mit isolierten Pflanzenzellen. Sitzungsber. Akad. Wiss. Wien. Math.-Naturwiss. Kl., Abt. J. 111, 69–92.

Habiba, U., Reza, S., Saha, M. L., Khan, M. R., & Hadiuzzaman, S. (2002). Endogenous bacterial contamination during in vitro culture of table banana: Identification and prevention. Plant Tissue Cult, 12(2), 117-124.

Haccius, B. (1971). Zur derzeitigen situation der angiospermen-embryologie. Bot Jahrbucher, 91, 309.

Hadidi, A., Montasser, M. S., Levy, L., Goth, R. W., Converse, R. H., Madkour, M. A., & Skrzeckowski, L. J. (1993). Detection of potato leafroll and strawberry mild yellow-edge luteoviruses by reverse transcription-polymerase chain reaction amplification. Plant Disease, 77(6), 595-601.

Halperin, W. (1966). Alternative morphogenetic events in cell suspensions. American Journal of Botany, 53(5), 443-453.

Harding, R. M., Burns, T. M., Hafner, G., Dietzgen, R. G., & Dale, J. L. (1993). Nucleotide sequence of one component of the banana bunchy top virus genome contains a putative replicase gene. Journal of General Virology, 74(3), 323-328.

Harding, R. M., Sadik, A. S., Bahieldin, A., & Dale, J. L. (2000). A sensitive detection of banana bunchy top nanovirus using molecular genetic approaches. Arab Journal of Biotechnology, 3(1), 103-114.

Harirah, A. A., & Khalid, N. (2006). Direct regeneration and RAPD assessment of male inflorescence derived plants of *Musa acuminata* cv. Berangan. Asia Pacific Journal of Molecular Biology & Biotechnology, 14(1), 11-17.

Hasan, S. Z. U., Ahmad, T., Hafiz, I. A., & Hussain, A. (2010). Direct plant regeneration from leaves of Prunus rootstock GF-677 (Prunus amygdalus× P. persica). Pak. J. Bot, 42(6), 3817-3830.

Hatanaka, T., Arakawa, O., Yasuda, T., Uchida, N., & Yamaguchi, T. (1991). Effect of plant growth regulators on somatic embryogenesis in leaf cultures of Coffea canephora. Plant Cell Reports, 10(4), 179-182.

Heindorff, K., Rieger, R., Schubert, I., Michaelis, A., & Aurich, O. (1987). Clastogenic adaptation of plant cells—reduction of the yield of clastogen-induced chromatid aberrations by various pretreatment procedures. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 181(1), 157-171.

Heslop-Harrison, J. S., & Schwarzacher, T. (2007). Domestication, genomics and the future for banana. Annals of botany, 100(5), 1073-1084.

Horaninow P. F., (1862). Prodromus Monographiae Scitaminarum. Academiae Caesareae Scientiarum, Petropoli (in Latin).

Hrahsel, L., Basu, A., Sahoo, L., & Thangjam, R. (2014). In vitro propagation and assessment of the genetic fidelity of *Musa acuminata* (AAA) cv. Vaibalhla derived from immature male flowers. Applied biochemistry and biotechnology, 172(3), 1530-1539.

Hu, J. S., Xu, M. Q., Wu, Z. C., & Wang, M. (1993). Detection of banana bunchy top virus in Hawaii. Plant Disease, 77: 952-952.

Hussey, G. (1983). In vitro propagation of horticultural and agricultural crops. In Seminar series-Society for Experimental Biology, 18, 111-138.

Hwang, S. C., & Ko, W. H. (1987). Somaclonal variation of bananas and screening for resistance to Fusarium wilt. Banana and plantain breeding strategies, 21, 151-156.

Ibaraki, Y. (2001). Automation in somatic embryo production. Progress in Biotechnology, 18, 365-374.

Ibaraki, Y., Kaneko, Y., & Kurata, K. (1998). Evaluation of embryogenic potential of cell suspension cultures by texture analysis. Transactions of the ASAE (USA). 41: 247- 252.

Israeli, Y., Lahav, E., & Reuveni, O. (1995). In vitro culture of bananas. In Bananas and plantains (pp. 147-178). Springer, Dordrecht.

Israeli, Y., Reuveni, O., & Lahav, E. (1991). Qualitative aspects of somaclonal variations in banana propagated by in vitro techniques. Scientia Horticulturae, 48(1-2), 71-88.

Jackson, J. A., & Dale, P. J. (1989). Somaclonal variation in Lolium multiflorum L. and L. temulentum L. Plant cell reports, 8(3), 161-164.

Jafari, N., Othman, R. Y., & Khalid, N. (2011). Effect of benzylaminopurine (BAP) pulsing on in vitro shoot multiplication of *Musa acuminata* (banana) cv. Berangan. African Journal of Biotechnology, 10(13), 2446-2450.

Jafari, N., Othman, R. Y., Tan, B. C., & Khalid, N. (2015). Morphohistological and molecular profiles during the developmental stages of somatic embryogenesis of *Musa acuminata* cv.'Berangan'(AAA). Acta physiologiae plantarum, 37(3), 45.

Jain, S. M. (2001). Tissue culture-derived variation in crop improvement. Euphytica, 118(2), 153.

Jain, S. M., Gupta, P. K., & Newton, R. J. (Eds.). (2013). Somatic Embryogenesis in Woody Plants: Volume 6 (Vol. 67). Springer Science & Business Media.

Jalil, M., Khalid, N., & Othman, R. Y. (2003). Plant regeneration from embryogenic suspension cultures of *Musa acuminata* cv. Mas (AA). Plant Cell, Tissue and Organ Culture, 75(3), 209-214.

Johnson, L. B., Stuteville, D. L., Schlarbaum, S. E., & Skinner, D. Z. (1984). Variation in Phenotype and Chromosome Number in Alfalfa Protoclones Regenerated from Nonmutagenized Calli 1. Crop science, 24(5), 948-951.

Jones, D. R. (2007). Disease and pest constraints to banana production. In III International Symposium on Banana: ISHS-Pro*Musa* Symposium on Recent Advances in Banana Crop Protection for Sustainable 828 (pp. 21-36).

Kar, A., Bora, D., Borthakur, S. K., Goswami, N. K., & Saharia, D. (2013). Wild edible plant resources used by the Mizos of Mizoram, India. Kathmandu University Journal of Science, Engineering and Technology, 9(1), 106-126.

Khalil, S. M., & Elbanna, A. A. M. (2003). Highly efficient somatic embryogenesis and plant regeneration via suspension cultures of banana (*Musa* spp.). Arab Journal Biotechnology, 7(1), 99-110.

Khalil, S., Cheah, K., Perez, E., Gaskill, D., & Hu, J. (2002). Regeneration of banana (*Musa* spp. AAB cv. Dwarf Brazilian) via secondary somatic embryogenesis. Plant Cell Reports, 20(12), 1128-1134.

Khan, M. Z. H., Sarkar, M. A. R., Al Imam, F. I., Khan, M. Z. H., & Malinen, R. O. (2014). Paper making from banana pseudo-stem: Characterization and comparison. Journal of Natural Fibers, 11(3), 199-211.

Kosky, R. G., de Feria Silva, M., Pérez, L. P., Gilliard, T., Martínez, F. B., Vega, M. R., ... & Mendoza, E. Q. (2002). Somatic embryogenesis of the banana hybrid cultivar FHIA-18 (AAAB) in liquid medium and scaled-up in a bioreactor. Plant Cell, Tissue and Organ Culture, 68(1), 21-26.

Krikorian, A. D., & Scott, M. E. (1995). Somatic embryogenesis in bananas and plantains (*Musa* clones and species). In Somatic embryogenesis and synthetic seed II (pp. 183-195). Springer, Berlin, Heidelberg.

Kulkarni, V. M., Suprasanna, P., & Bapat, V. A. (2006). Plant regeneration through multiple shoot formation and somatic embryogenesis in a commercially important and endangered Indian banana cv. Rajeli. Current Science, 842-846.

Lalrinfela, P. C., & Thangjam, R. (2012). Genome characterization of banana genetic resources of Mizoram, India. Science Vision, 12(1), 32-36.

Lattoo, S. K., Bamotra, S., Dhar, R. S., Khan, S., & Dhar, A. K. (2006). Rapid plant regeneration and analysis of genetic fidelity of in vitro derived plants of Chlorophytum arundinaceum Baker—an endangered medicinal herb. Plant cell reports, 25(6), 499-506.

Lebot, V., Aradhya, K. M., Manshardt, R., & Meilleur, B. (1993). Genetic relationships among cultivated bananas and plantains from Asia and the Pacific. Euphytica, 67(3), 163-175.

Lee, K. S., Zapata-Arias, F. J., Brunner, H., & Afza, R. (1997). Histology of somatic embryo initiation and organogenesis from rhizome explants of *Musa* spp. Plant Cell, Tissue and Organ Culture, 51(1), 1-8.

Levi, A., & Sink, K. C. (1991). Somatic embryogenesis in asparagus: the role of explants and growth regulators. Plant cell reports, 10(2), 71-75.

Levy, L., & Hadidi, A. (1991). Development of a reverse-transcription/polymerase chain reaction assay for the detection of plum pox potyvirus from microgram quantities of total nucleic acids. Phytopathology, 81(21), 154.

Li, L. F., Häkkinen, M., Yuan, Y. M., Hao, G., & Ge, X. J. (2010). Molecular phylogeny and systematics of the banana family (*Musaceae*) inferred from multiple nuclear and chloroplast DNA fragments, with a special reference to the genus *Musa*. Molecular Phylogenetics and Evolution, 57(1), 1-10.

Linnaeus, C. (1753). Species Plantarum, vol. 1. Imprensis Laurentiii Salvii, Stockholm, 560 pp.

Litz, R. E., & Jarret, R. L. (1991). Regeneración de plantas en el cultivo de tejidos: Embriogénesis somática y organogénesis. Roca, William M.; Mroginski, Luis A.(eds.). Cultivo de tejidos en la agricultura: Fundamentos y aplicaciones.

Lohidas, J., & Sujin, D. (2015). Effect of growth hormones in the micro propagation of banana CV. Matti. Plant Archives, 15(1), 307-314.

Ma SS (1991) Somatic embryogenesis and plant regeneration from cell suspension culture of banana. In: Department of Agriculture, National Taiwan University (eds) Proceedings of symposium on tissue culture of horticultural crops. Taibei, Taiwan, 8, 9 March, 1988, pp 181–188

Ma, S. S., & Shii, C. T. (1972). In vitro formation of adventitious buds in banana shoot apex following decapitation. J. Chinese Soc. Hort. Sci, 18, 135-142.

Ma, S. S., & Shii, C. T. (1974). Growing banana plantlets from adventitious buds. Journal of Chinese Society of Horticultural Science, 20, 6-12.

Madhulatha, P., Anbalagan, M., Jayachandran, S., & Sakthivel, N. (2004). Influence of liquid pulse treatment with growth regulators on in vitro propagation of banana (*Musa* spp. AAA). Plant cell, tissue and organ culture, 76(2), 189-192.

Mahadev, S. R., Kathithachalam, A., & Marimuthu, M. (2011). An efficient protocol for large-scale plantlet production from male floral meristems of *Musa* spp. cultivars Virupakshi and Sirumalai. In Vitro Cellular & Developmental Biology-Plant, 47(5), 611-617.

Mahadev, S. R., Kathithachalam, A., & Marimuthu, M. (2011). An efficient protocol for large-scale plantlet production from male floral meristems of *Musa* spp. cultivars Virupakshi and Sirumalai. In Vitro Cellular & Developmental Biology-Plant, 47(5), 611-617.

Maheswaran, G., & Williams, E. G. (1985). Origin and development of somatic embryoids formed directly on immature embryos of Trifolium repens in vitro. Annals of Botany, 56(5), 619-630.

Marroquin, C. G., Paduscheck, C., Escalant, J. V., & Teisson, C. (1993). Somatic embryogenesis and plant regeneration through cell suspensions in *Musa acuminata.* In Vitro Cellular & Developmental Biology-Plant, 29(1), 43-46.

Martin, K. P., Pachathundikandi, S. K., Zhang, C. L., Slater, A., & Madassery, J. (2006). RAPD analysis of a variant of banana (*Musa* sp.) cv. grande naine and its propagation via shoot tip culture. In Vitro Cellular & Developmental Biology-Plant, 42(2), 188-192.

May, G. D., Afza, R., Mason, H. S., Wiecko, A., Novak, F. J., & Arntzen, C. J. (1995). Generation of transgenic banana (*Musa acuminata*) plants via Agrobacterium-mediated transformation. Bio/technology, 13(5), 486-492.

Mayer, K. F., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G., & Laux, T. (1998). Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. Cell, 95(6), 805-815.

Merkle, S. A., Parrott, W. A., & Flinn, B. S. (1995). Morphogenic aspects of somatic embryogenesis. In In vitro embryogenesis in plants (pp. 155-203). Springer, Dordrecht.

Michaux-Ferriere, N., Schwendiman, J., Y (ed.). Dattee, C (ed.). Duman, A. Gallais. (1992). Histology of somatic embryogeneisis. Reprod. boil. and plant breeding., pp.247-259.

Moffat, A. S. (1999). Crop engineering goes south. Science, 285, 370-371.

Molina, A. B., & Kudagamage, C. (2002). The international network for the improvement of banana and plantain (INIBAP): PGR activities in South Asia. In South Asia network on plant genetic resources (SANPGR) meeting held on December (pp. 9-11).

Montalbán, I. A., De Diego, N., & Moncaleán, P. (2012). Enhancing initiation and proliferation in radiata pine (Pinus radiata D. Don) somatic embryogenesis through seed family screening, zygotic embryo staging and media adjustments. Acta physiologiae plantarum, 34(2), 451-460.

Moy, Y., Hanson, B., Engler, D., & Gutterson, N. (1999). Analysis of uidA gene expression from transgenes in field-grown 'Grand Nain'(AAA) banana plants. In The 1st International symposium on the molecular and cellular biology of banana.

Muhammad, A., Hussain, I., Naqvi, S. S., & Rashid, H. (2004). Banana plantlet production through tissue culture. Pakistan Journal of Botany, 36(3), 617-620.

Mullis, K. B., & Faloona, F. A. (1987). [21] Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods in enzymology, 155, 335-350.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia plantarum, 15(3), 473-497.

Nahamya, P. (2000). Development of Embryogenic cell suspensions for East African Highland bananas (Doctoral dissertation, Tesis para optar por el Grado Académico de Master en Ciencias, Makerere University, Kampala, Uganda).

Nakirya M (2007). The Value of the Banana Fruit. https://celac.wordpress.com/2007/05/17/the-value-of-the-banana-fruit/ (accessed 07.11.2021).

Nandhakumar, N., Kumar, K., Sudhakar, D., & Soorianathasundaram, K. (2018). Plant regeneration, developmental pattern and genetic fidelity of somatic embryogenesis derived *Musa* spp. Journal of Genetic Engineering and Biotechnology, 16(2), 587-598.

Navarro, C., Escobedo, R. M., & Mayo, A. (1997). In vitro plant regeneration from embryogenic cultures of a diploid and a triploid, Cavendish banana. Plant Cell, Tissue and Organ Culture, 51(1), 17-25.

Nayar, N. M. (2010). 2 The bananas: Botany, origin, dispersal. Horticultural reviews, 36(3), 117-164.

Nolan, K. E., Rose, R. J., & Gorst, J. R. (1989). Regeneration of Medicago truncatula from tissue culture: increased somatic embryogenesis using explants from regenerated plants. Plant Cell Reports, 8(5), 278-281.

Norstog, K. (1961). The growth and differentiation of cultured barley embryos. American Journal of Botany, 48(10), 876-884.

Novak, F. J. (1992). *Musa* (bananas and plantains). Biotechnology of perennial fruit crops., 449-488.

Novak, F. J., Afza, R., Duren, M. V., & Omar, M. S. (1990). Mutation induction by gamma irradiation of in vitro cultured shoot-tips of banana and plantain (*Musa* cvs). Tropical Agriculture, 67(1), 21-28.

Novak, F. J., Afza, R., Van Duren, M., Perea-Dallos, M., Conger, B. V., & Xiaolang, T. (1989). Somatic embryogenesis and plant regeneration in suspension cultures of dessert (AA and AAA) and cooking (ABB) bananas (*Musa* spp.). Bio/Technology, 7(2), 154-159.

Parveen, S., Shahzad, A., & Saema, S. (2010). In vitro plant regeneration system for Cassia siamea Lam., a leguminous tree of economic importance. Agroforestry systems, 80(1), 109-116.

Perez, E. A., Brunner, H., & Afza, R. (1998). Somatic embryogenesis in banana (*Musa* ssp.) cv. lakatan and latundan. Philippine Journal of Crop Science (Philippines).

Phulwaria, M., Rai, M. K., & Shekhawat, N. S. (2013). An improved micropropagation of Arnebia hispidissima (Lehm.) DC. and assessment of genetic fidelity of micropropagated plants using DNA-based molecular markers. Applied biochemistry and biotechnology, 170(5), 1163-1173.

Raemakers, C. J. J. M., Jacobsen, E. A., & Visser, R. G. F. (1995). Secondary somatic embryogenesis and applications in plant breeding. Euphytica, 81(1), 93-107.

Raghavan, V. (1976). Experimental embryogenesis in vascular plants.

Ramulu, K. S., Dijkhuis, P., & Roest, S. (1983). Phenotypic variation and ploidy level of plants regenerated from protoplasts of tetraploid potato (Solanum tuberosum L. cv.'Bintje'). Theoretical and Applied Genetics, 65(4), 329-338.

Ray, T., Dutta, I., Saha, P., Das, S., & Roy, S. C. (2006). Genetic stability of three economically important micropropagated banana (*Musa* spp.) cultivars of lower Indo-Gangetic plains, as assessed by RAPD and ISSR markers. Plant cell, tissue and organ culture, 85(1), 11-21.

Reinert, J. (1958). Untersuchungen uber die Morphogenese an Gewebekulturen. Ber Dtsch Bot Ges, 71, 15.

Resmi, L., & Nair, A. S. (2007). Plantlet production from the male inflorescence tips of *Musa acuminata* cultivars from South India. Plant cell, tissue and organ culture, 88(3), 333-338.

Reuveni, O., Golubowicz, S., & Israeli, Y. (1993). Factors that influence the incidence of somaclonal variation in micropropagated bananas. Acta Horticulturae , 357 , 364.

Rodriguez, A. P., & Wetzstein, H. Y. (1994). The effect of auxin type and concentration on pecan (Carya illinoinensis) somatic embryo morphology and subsequent conversion into plants. Plant cell reports, 13(11), 607-611.

Sabir, A., Newbury, H. J., Todd, G., Catty, J., & Ford-Lloyd, B. V. (1992). Determination of genetic stability using isozymes and RFLPs in beet plants regenerated in vitro. Theoretical and Applied Genetics, 84(1), 113-117.

Sadik, A. S., Abdel-Gaffar, M. H., Salama, M. I., & Allam, E. K. (1997). Evaluation of three different methods for detection of banana bunchy top nanoviruses. In Proceeding of the 9th Conference of Microbiology, Cairo (pp. 188-202).

Sadik, K., Rubaihayo, P. R., Magambo, M. J. S., & Pillay, M. (2007). Generation of cell suspensions of East African highland bananas through scalps. African Journal of Biotechnology, 6(11).

Sahijram, L., Soneji, J. R., & Bollamma, K. T. (2003). Analyzing somaclonal variation in micropropagated bananas (*Musa* spp.). In Vitro Cellular & Developmental Biology-Plant, 39(6), 551-556.

Sales, E. K., Carlos, L. R., & Espino, R. R. C. (2001). Initiation of meristematic buds by benzyl amino purine on banana (*Musa* spp.) cultivars for somatic embryogenesis.

Savangikar, V. A. (2004). Role of low-cost options in tissue culture. In Low-cost options for tissue culture technology in developing countries. Proceedings of a Technical Meeting organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (pp. 11-15).

Schleiden, M. J. (1838). Beiträge zur phytogenesis, Arch. Anat. Physiol. Wiss Med.

Schoofs, H. (1997). The origin of embryogenic cells in *Musa* (Doctoral dissertation, Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen van de UK Leuven).

Schoofs, H., Panis, B., & Swennen, R. (1997, November). Competence of scalps for somatic embryogenesis in *Musa*. In II International Symposium on Banana: I International Symposium on Banana in the Subtropics 490 (pp. 475-484).

Schoofs, H., Panis, B., Strosse, H., Mayo Mosqueda, A., Lopez Torres, J., Roux, N., ... & Swennen, R. (1999). Bottlenecks in the generation and maintenance of morphogenic banana cell suspensions and plant regeneration via somatic embryogenesis therefrom.

Shankar, C. S., Balaji, P., & Sekar, D. S. (2014). Mass propagation of banana (*Musa* sp.) cv. grand naine through direct organogenesis by benzyl adenine purine and kinetin. Journal of Academia and Industrial Research, 3(2), 92-97.

Shenoy, V. B., & Vasil, I. K. (1992). Biochemical and molecular analysis of plants derived from embryogenic tissue cultures of napier grass (Pennisetum purpureum K. Schum). Theoretical and Applied Genetics, 83(8), 947-955.

Shii, C. T., Ma, S. S., Huang, I. C., & Ching, W. H. (1992). Somatic embryogenesis and plantlet regeneration in suspension cell cultures of triploid bananas (*Musa* AAA) subgroup Cavendish. In International Symposium on Recent Development in Banana Cultivation Technology (pp. 14-18).

Sholi, N. J., Chaurasia, A., Agrawal, A., & Sarin, N. B. (2009). ABA enhances plant regeneration of somatic embryos derived from cell suspension cultures of plantain cv. Spambia (*Musa* sp.). Plant Cell, Tissue and Organ Culture (PCTOC), 99(2), 133-140.

Sidha, M., Suprasanna, P., Bapat, V. A., Kulkarni, U. G., & Shinde, B. N. (2007). Developing somatic embryogenic culture system and plant regeneration in banana. Bhabha At. Res. Centre (BARC) Newslett, 285, 153-161.

Silvarolla, M. B. (1992). Plant genomic alterations due to tissue culture. Ciëncia e Cultura, 44, 329-329.

Simmonds, N. W., & Shepherd, K. (1955). The taxonomy and origins of the cultivated bananas. Botanical Journal of the Linnean Society, 55(359), 302-312.

Smith, G. R., Van de Velde, R., & Dale, J. L. (1992). PCR amplification of a specific double-stranded RNA region of Fiji disease virus from diseased sugarcane. Journal of virological methods, 39(3), 237-246.

Smith, M. K., & Drew, R. A. (1990). Current applications of tissue culture in plant propagation and improvement. Functional Plant Biology, 17(3), 267-289.

Smith, M. K., Hamill, S. D., Becker, D. K., & Dale, J. L. (2005). 13.1 *Musa* spp. Banana and Plantain. General Editor: Gabrielle J. Persley, The Doyle Foundation, Glasgow, Scotland., 366.

Smitha P. D., kr, B., & Nair, A. S. (2020). Enhanced secondary somatic embryogenesis in suspension culture of four diploid banana cultivars from Kerala. International Journal of Fruit Science, 20(sup2), S617-S626.

Smitha, P. D., & Nair, A. S. (2011). Effect of picloram on somatic embryogenesis from leaf-sheath explants in diploid *Musa acuminata* cv. Njalipoovan. Plant Tissue Culture and Biotechnology, 21(1), 83-87.

Soniya, E. V., Banerjee, N. S., & Das, M. R. (2001). Genetic analysis of somaclonal variation among callus-derived plants of tomato. Current science, 1213-1215.

Srangsam, A., & Kanchanapoom, K. (2003). Thidiazuron induced plant regeneration in callus culture of triploid banana (*Musa* sp.) 'Gros Michel', AAA group. Songklanakarin Journal of Science and Technology, 25(6), 689-696.

Steward, F. C., Mapes, M. O., & Smith, J. (1958). Growth and organized development of cultured cells I. Growth and division of freely suspended cells. American Journal of Botany, 45(9), 693-703.

Stover, R. H. (1987). Somaclonal variation in Grand Naine and Saba bananas in the nursery and field. Banana and plantain breeding strategies, 21, 136-139.

Street, H. E. (1974). The anatomy of embryogenesis in culture. Tissue culture and plant science, 71-100.

Strosse, H., Andre, E., Sági, L., Swennen, R., & Panis, B. (2008). Adventitious shoot formation is not inherent to micropropagation of banana as it is in maize. Plant cell, tissue and organ culture, 95(3), 321-332.

Strosse, H., Schoofs, H., Panis, B., Andre, E., Reyniers, K., & Swennen, R. (2006). Development of embryogenic cell suspensions from shoot meristematic tissue in bananas and plantains (*Musa* spp.). Plant Science, 170(1), 104-112.

Swennen, R., & Ortiz, R. (1997). Morphology and growth of plantain and banana. International Institute of Tropical Agriculture (IITA).

Swennen, R., & Vuylsteke, D. (1991). Bananas in Africa: diversity, uses and prospects for improvement. Crop genetic resources of Africa, 2, 151-159.

Tan, B. C., Chin, C. F., & Alderson, P. (2011). Optimisation of plantlet regeneration from leaf and nodal derived callus of Vanilla planifolia Andrews. Plant Cell, Tissue and Organ Culture (PCTOC), 105(3), 457-463.

Tautorus, TE, Fowke, LC, & Dunstan, DI (1991). Somatic embryogenesis in conifers. Canadian Journal of Botany, 69 (9), 1873-1899.

Terzi, M., & LO SCHIAVO, F. (1990). Somatic embryogenesis In: BHAJWANI, SS (Ed.) Plant tissue culture: Apllications and limitations, Msterdam.

Thangjam, R., Maibam, D., & Sharma, J. G. (2003). A simple and rapid method for isolation of DNA from imbibed embryos of Parkia timoriana (DC.) Merr. for PCR analysis. Journal of Food Agriculture and Environment, 1, 36-38.

Tingey, S. V., & del Tufo, J. P. (1993). Genetic analysis with random amplified polymorphic DNA markers. Plant physiology, 101(2), 349.

Trigiano, R. N., & Conger, B. V. (1987). Regulation of growth and somatic embryogenesis by proline and serine in suspension cultures of Dactylis glomerata. Journal of plant physiology, 130(1), 49-55.

Tripathi, L., Tripathi, J. N., & Vroh-Bi, I. (2007). Bananas and plantains (*Musa* spp.): Transgenics and biotechnology. Transgenic Plant Journal, 1(1), 185-201.

Turgut, K., Barghchi, M., & Scott, R. (1998). Efficient shoot regeneration and somatic embryogenesis from immature cotyledons of Brassica napus L. Plant breeding, 117(5), 503-504.

Turner, D. W., Fortescue, J. A., & Thomas, D. S. (2007). Environmental physiology of the bananas (*Musa* spp.). Brazilian Journal of Plant Physiology, 19, 463-484.

Uma, S., & Sathiamoorthy, S. (2002). Names and synonyms of bananas and plantains of India. National Research Centre for Banana (ICAR).

Uma, S., Sathiamoorthy, S., & Durai, P. (2005). Banana-Indian genetic resources and catalogue. National Research Centre for Banana (ICAR).

Uma, S., Siva, S. A., Saraswathi, M. S., Manickavasagam, M., Durai, P., & Selvarajan, R. Sathiamoorthy. (2006). Variation and intraspesific relationship in Indian wild

Musa balbisiana (BB) population an evidenced by random amplified polymorphic DNA. Genet Resources Crop Evol, 53, 349-355.

Valmayor R. V., Silayoi B., Jamaluddin S. H., Kusumo S., Espino R. R. C., Pascua O. C. 2000. Banana classification and commercial cultivars in South East Asia. Information Bulletin 24.

Van Den Houwe, I. (1998). Elimination of endophytic bacteria from banana tissue culture.

Van Den Houwe, I., Panis, B., and Swennen, R. (2000) The in vitro germplasm collection at the *Musa* INIBAP Transit Centre and the importance of cryopreservation. In: Cryopreservation of tropical plant germplasm: Current research progress and application (Engelmann F and Takagi H eds.). Tokyo, Japan: IPGRI-JIRCAS, pp. 255–260.

Vasil, I. K. (1987). Developing cell and tissue culture systems for the improvement of cereal and grass crops. Journal of plant physiology, 128(3), 193-218.

Vendrame, W. A., Kochert, G., & Wetzstein, H. Y. (1999). AFLP analysis of variation in pecan somatic embryos. Plant cell reports, 18(10), 853-857.

Venkatachalam, L., Sreedhar, R. V., & Bhagyalakshmi, N. (2007). Genetic analyses of micropropagated and regenerated plantlets of banana as assessed by RAPD and ISSR markers. In Vitro Cellular & Developmental Biology-Plant, 43(3), 267-274.

Verdeil, J. L., Alemanno, L., Niemenak, N., & Tranbarger, T. J. (2007). Pluripotent versus totipotent plant stem cells: dependence versus autonomy? Trends in plant science, 12(6), 245-252.

Verdus, M. C., Dubois, T., Dubois, J., & Vasseur, J. (1993). Ultrastructural changes in leaves of Cichorium during somatic embryogenesis. Annals of Botany, 72(4), 375-383.

Vidal, M. D. C., & De García, E. (2000). Analysis of a *Musa* spp. somaclonal variant resistant to yellow Sigatoka. Plant Molecular Biology Reporter, 18(1), 23.

Vishnoi, R., Raj, S. K., & Prasad, V. (2009). Molecular characterization of an Indian isolate of Banana bunchy top virus based on six genomic DNA components. Virus Genes, 38(2), 334-344.

Vuylsteke, D. (1989). Shoot-tip Culture for the Propagation. Conservation and Exchange of *Musa* germplasm. Practical Manual for Handling Crop Germplasm in vitro 2, IBPGR, Rome, Italy.

Vuylsteke, D. R. (1998). Field performance of banana micropropagules and somaclones. In Somaclonal variation and induced mutations in crop improvement (pp. 219-231). Springer, Dordrecht.

Vuylsteke, D., Swennen, R., & De Langhe, E. (1991). Somaclonal variation in plantains (*Musa* spp., AAB group) derived from shoot-tip culture. Fruits, 46(4), 429-439.

Vuylsteke, D., Swennen, R., Wilson, G. F., & De Langhe, E. (1988). Phenotypic variation among in-vitro propagated plantain (*Musa* sp. cultivar 'AAB'). Scientia Horticulturae, 36(1-2), 79-88.

Wiggans, S. C. (1954). Growth and organ formation in callus tissues derived from Daucus carota. American Journal of Botany, 321-326.

Williams, E. G., & Maheswaran, G. (1986). Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. Annals of Botany, 57(4), 443-462.

Williams, E. G., Taylor, N. L., Bosch, J., & Williams, W. M. (1990). Registration of tetraploid hybrid clover germplasm from the cross of Trifolium ambiguum× T. repens. Crop Science, 30(2), 427.

Wong, W. C. (1986). In vitro propagation of banana (*Musa* spp.): initiation, proliferation and development of shoot-tip cultures on defined media. Plant cell, tissue and organ culture, 6(2), 159-166.

Worobetz, K. (2000). The growth of the banana industry in Costa Rica and its effect on biodiversity. Department of Biological Sciences, University of Alberta: Edmonton, Alberta, Canada.

Wright, N. S. (1983). Uniformity among virus-free clones of ten potato cultivars. American Potato Journal, 60(6), 381-388.

Yang, X., & Zhang, X. (2010). Regulation of somatic embryogenesis in higher plants. Critical Reviews in Plant Science, 29(1), 36-57.

Youmbi, E., Ella, B., & Tomekpe, K. (2006). Effect of Thidiazuron on In-Vitro Proliferation Capacities of Some Banana (*Musa* spp.) Cultivars with Weak Multiplication Potential. Akdeniz üniversitesi ziraat fakültesi dergisi, 19(2), 255-259.

Zaffari, G. R., Kerbauy, G. B., Kraus, J. E., & Romano, E. C. (2000). Hormonal and histological studies related to in vitro banana bud formation. Plant Cell, Tissue and Organ Culture, 63(3), 187-192.

Zietkiewicz, E., Rafalski, A., & Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics, 20(2), 176-183.

Zimmerman, J. L. (1993). Somatic embryogenesis: a model for early development in higher plants. The plant cell, 5(10), 1411-1423.

NAME OF CANDIDATE : MAISNAM AKBAR SINGH

DEGREE : MASTER OF PHILOSOPHY

DEPARTMENT : BIOTECHNOLOGY

TITLE OF DISSERTATION : *In vitro* somatic embryogenesis of *Musa acuminata* (AAA) cv. Vaibalhla

DATE OF PAYMENTS OF ADMISSION : 06.08.2019

COMMENCENENT OF SECOND SEM/DISSERTATION :

(From conclusion of end semester exam)

APPROVAL OF RESEARCH PROPOSAL

BOS : 05.05.2020

SCHOOL BOARD : 12.06.2020

REGISTRATION NO. & DATE : MZU/M.Phil./627 of 12.06.2020

DATE OF SUBMISSION :

EXTENSION (IF ANY) :

Head

Department of Biotechnology

Head
Deptt. of Biotechnology
MIZORAM UNIVERSITY
AIZAWL - 796004

BIO-DATA

Name : **Maisnam Akbar Singh**

Father's name : **Maisnam Mangol Singh**

Permanent Address : **Ngaikhong Khullen Awang Leikai, Bishnupur, Manipur-795126**

Phone : **9108367655**

Email : **masingh2018@gmail.com**

EDUCATIONAL QUALIFICATION

Sl. No.	Examination passed	Year	Board/University	Percentage
1	HSLC	2011	CBSE	76%
2	HSSLC	2013	CBSE	77.5%
3	B. Sc	2016	Bangalore University	68.12%
4	M. Sc (Biotechnology)	2018	Bangalore University	69.76%

Papers presentation/participation in conference/symposiums:

1. Presented a paper title "In vitro shoot induction of *Musa acuminata* (AAA) cv. Vaibalhla for mutation breeding and flow cytometric analysis of different stages of cell cycle in different developmental stage" in National conference on Microbes in health, agriculture & Environment. Organized by Department of Biotechnology, School of Life Science, Mizoram University, Aizawl – 796004 during 20th & 21st June, 2019
2. Presented a paper title "Determination of LD50 in gamma irradiated aseptically raised *Musa acuminata* (AAA) cv. Vaibalhla inflorescence explants" in International Seminar on Recent Advanced in Science and Technology (ISRAST). Organized by North East (India) Academy of Science and Technology (NEAST), Mizoram University, Aizawl -796004, Mizoram (India) during 16th – 18th November.

National Conference on
Microbes in Health, Agriculture & Environment
Organized by:
Department of Biotechnology
School of Life Sciences
Mizoram University, Aizawl – 796004, Mizoram, INDIA

Certificate

Certified that Mr./Ms./Dr./Prof. Maisnam Akbar Singh *of* Department of Biotechnology (MZU, Aizawl) *participated and presented a paper entitled* In vitro multiple shoot induction of Musa acuminata (AAA) Cv. Vaibalhla for mutation breeding and flow cytometric analysis of different ploidy of cell cycle in diploid/triploid *in the National Conference on "Microbes in Health, Agriculture & Environment " organized by Department of Biotechnology, School of Life Sciences, Mizoram University, Aizawl - 796004 during 20th & 21st June, 2019.*

Dr. Thangjam Robert Singh
Professor
Department of Biotechnology
Mizoram University
Aizawl - 796004, India

(Dr. Thangjam Robert Singh)
Department of Biotechnology
Convenor

(Prof. S.K. Mehta)
Dean, School of Life Sciences
Mizoram University

(Prof. K.R.S. Sambasiva Rao)
Vice Chancellor
Mizoram University

NEAST

2nd Annual Convention of
North East (India) Academy of Science and Technology (NEAST)
&
International Seminar on Recent Advances in Science and Technology (ISRAST)
(16th -18th November 2020)
(Virtual)

Certificate

11215150238

This is to certify that

Mr./Ms./Dr./Prof. Maisnam Akbar Singh
Department of Biotechnology, School of Life Sciences, Mizoram University, Aizawl–796004, India

has attended and presented an Oral Presentation entitled, "Determination of LD50 in Gamma Irradiated Aseptically Raised Musa acuminata (AAA) cv. Vaibalhla Inflorescence Explants" in the 2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST) & International Seminar on Recent Advances in Science and Technology (IRSRAST) during 16th-18th November 2020 (Virtual) organized by NEAST, Mizoram University, Aizawl-796004, Mizoram (India).

Dr. Thangjam Robert Singh
Professor
Department of Biotechnology
Mizoram University
Aizawl - 796004, India

Prof. Diwakar Tiwari
General Secretary, NEAST

Prof. R. Lalthantluanga
President. NEAST

North East (India) Academy of Science & Technology (NEAST), Mizoram University, Aizawl-796004 (India)
Reg. No.: MSR 903 under Mizoram Societies Act-2018

ABSTRACT

In vitro somatic embryogenesis of *Musa acuminata* (AAA) cv. Vaibalhla

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

MAISNAM AKBAR SINGH

MZU REGN NO: 1905390

M.PHIL REGN NO: MZU/M.Phil./627 of 12.06.2020

DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF LIFE SCIENCE

MARCH, 2022

ABSTRACT

Banana (*Musa* spp.), including plantains and dessert varieties are large perennial monocotyledonous herbaceous plants which belong to the order *Zingiberales* in the family *Musaceae*. The family *Musaceae* comprises of two genera *Musa L* and *Ensete* where *Musa* differs from *Ensete* in producing suckers and small. The *Musa* genus consists of approximately 50 species while the genus *Ensete* has 9 species. The genus *Musa*, on the basis of basic chromosomal number and phenotypic characteristic, has been divided into four different sections namely *Rhodochlamys*, *Eumusa*, *Callimusa* and *Australimusa*. *Eumusa* constitute the largest, most widely distributed, highly diversified and the most important section containing all the edible bananas. Mainly cultivars in the *Eumusa* section are mainly derived from two species namely *Musa acuminata* having A genome and *M. balbisiana* having B genome. The species, *Musa acuminata* is the most widespread of the *Eumusa* section. The selection of seedless diploid and triploid parthenocarpy hybrids thereafter helped in the process of its wide distribution. India has 70% of the total diversity and distributed in Southern and North-Eastern States. Both the species, *M. acuminata* and *M. balbisiana* serves as progenitor for majority of the cultivated bananas, which may be diploid (2n=22), triploid (2n=33) or tetraploid (2n=44). Most cultivated *Musa* are triploids (2n=33) with characteristic genome constitutions while diploids and tetraploids are lesser in number. Somatic embryogenesis technique used in the genus *Musa* is aimed at two main objectives-the development of high performance micropropagation and regeneration system useful for genetic improvement. Embryogenesis is regarded as a model for testing the totipotency of crop tissues. Analysis of the impacts of different medium, growth conditions, and modifying factors, particularly quantities and types of plant growth regulators, is important for the improving of high frequency tissue culture responses. As basic material for genetic manipulation, research groups emphasized on the utilization of protoplasts or embryogenic cells as well as meristems of banana in the early stage of genetic transformation of banana. The present study was carried out for the initiation and standardization of aseptic cultures; to develop an efficient and reproducible in vitro plant regeneration system through somatic embryogenesis using male flower bud and standardization and acclimatization of the hardened plantlets.

Screening of *Musa acuminata* (AAA) cv. Vaibalhla for the presence of banana bunchy top virus (BBTV) in the *Musa* samples selected were carried out using primer specific to BBTV DNA-R genome. MS media was used as the basal medium for the invitro somatic embryogenesis induction of the male flowers supplemented with different concentrations of plant growth regulators viz. NAA, BAP, TDZ and IBA. The morphogenic changes observed on the respective PGR supplemented medium were recorded and studied. The regenerated plantlets were then transplanted and hardened in the greenhouse for further use in the study. The genetic fidelity test of the regenerated plantlets through invitro somatic embryogenesis was carried out using the UBC ISSR primers. PCR amplifications was carried out to check the

reproducibility within the regenerated samples and only the distinct and well resolved fragments were scored. Furthermore, the regenerated plantlets of *Musa acuminata* (AAA) cv. Vaibalhla were screened for banana bunchy top virus (BBTV) using the same primer specific to BBTV DNA-R genome and the results were studied.

The selected *Musa acuminata* (AAA) cv. Vaibalhla samples were successfully screened of banana bunchy top virus (BBTV) and found free from BBTV. PCR amplification result using the BBTV DNA-R primer of the samples showed the no band which confirms the absence of BBTV in the samples selected. Establishment of aseptic culture of *Musa acuminata* (AAA) cv. Vaibalhla using male flowers through invitro somatic embryogenesis was successfully achieved in the current study. Male flower buds surface sterilized with 70% ethanol was found effective in successful regeneration of plantlets through somatic embryogenesis. In vitro somatic embryogenesis and plantlet regeneration of the explants were successfully achieved with varied morphogenic responses. Media supplemented with 0.1 mg/L of NAA, 1 mg/L of BAP and 1mg/L TDZ showed the maximum percentage of bulging (70.11). Maximum white body like structure (WBL) was observed in media supplemented with 0.1 mg/L of NAA, 1 mg/L of BAP and 1 mg/L TDZ while minimum was observed in media supplemented with 0.1 mg/L of NAA and 2 mg/L TDZ. Somatic embryogenesis was observed from the proembryonal globular protuberances which were developed from the WBLs after 10 week of inoculation giving rise to embryos through formation of heart, torpedo and cotyledonary stages. Maximum embryo induction per explant (33.81) and maximum explants generating embryos (48.84) was observed on media supplemented with 0.1 mg/L of NAA, 1 mg/L of BAP and 1 mg/L TDZ. Shoot formation of the cultured embryos was observed on the 22^{nd} week after inoculation. Media supplemented with 2 mg/L BAP and 1 mg/L of TDZ showed superior response of 44.04% of the total explants showing shoot formation with highest number of 25.81 per explant and longest shoot length of 13.36 cm. Leaf formation from the regenerated shoots was observed on the 25^{th} week after inoculation. Media supplemented with 2 mg/L BAP and 1 mg/L of TDZ was found most effective with the highest percentage of shoot showing leaf induction (38.24%) with maximum number of leaves per shoot (11.32) and longest leaf length (11.87 cm). Regenerated young plantlets showed high percentage of root induction with 13.93±0.14 per explant and maximum root length of 16.44±0.06 when transferred in media supplemented with 1 mg/L of IBA. ISSR primers was successful in evaluation of genetic fidelity in the regenerated *M. acuminata* cv. Vaibalhla samples. A total of 15 ISSR primers were used for the screening out of which 10 primers showed clear, sharp and reproducible bands. Only monomorphic bands with a maximum of 6 (UBC-886) and a minimum of 2 (UBC-899) was observed in the primers tested and no polymorphic band was found among the samples tested. Fully regenerated plantlets through somatic embryogenesis were screened for BBTV using the DNA-R primer. PCR amplification of the regenerated plantlet samples showed no band

which indicates the absence of BBTV, thereby confirming the successful regeneration of disease-free plantlets through somatic embryogenesis.

In conclusion, the present study can be useful in efficient utilization of invitro somatic embryogenesis for used as a biotechnology tool in establishment of asceptic culture and furthermore utilization of *Musa acuminata* (AAA) cv. Vaibalhla in crop improvement programs.