GENETIC ENGINEERING OF BANANA USING NONEXPRESSOR OF PATHOGENESIS RELATED GENE FOR RESISTANCE TO XANTHOMONAS WILT AND FUSARIUM WILT DISEASES

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or any other award.

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DEDICATION

This thesis is dedicated to my family and parents.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND ACRONYMS

AMP	Antimicrobial peptides
AMT	*Agrobacterium*-mediated transformation
ANOVA	Analysis of variance
AVR	Avirulence
2, 4 D	2, 4-dichlorophenoxyacetic acid
2-ip	Isopentenyl adenine
6-BAP	6-Benzylaminopurine
BBTV	Banana Bunchy Top Virus
BBTD	Banana Bunchy Top disease
BCCM	Bacterial co-cultivation medium
BRM	Bacterial re suspension medium
BSV	Banana Streak virus
BXW	Banana Xanthomonas wilt
CaMV35S	Cauliflower Mosaic virus 35S promoter
CCM	Co -cultivation medium
CIM	Callus induction medium
cDNA	complementary deoxyribonucleic acid
CTAB	Hexadecyltrimethylammonium bromide
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
E. coli	*Escherichia coli*
EC	Embryogenic cells
ECS	Embryogenic cell suspensions
EAHB	East African Highland bananas
FAO	Food and Agriculture Organization
FOC	*Fusarium oxysporum* f. sp. *cubense*
FWD	Fusarium wilt disease
GUS	beta-*glucuronidase*
HCD	Hypersensitive cell death
HR	Hypersensitive response

HRAP	Hypersensitive response assisting protein
hpt	*hygromycin phosphotransferase* gene
IAA	Indole-3-acetic acid
IITA	International Institute of Tropical Agriculture
IBA	4-Indole-3-Butyric Acid
JA	Jasmonic acid
KALRO	Kenya Agricultural and Livestock Research Organization
LB	Luria bertoni
LB	left T-DNA border sequence
LSD	Least significant difference
MB	Multiple buds
MS	Murashige and skoog
NAA	Naphthaleneacetic acid
NPR	Nonexpresser of pathogenesis related gene
NH1	*NPR1* homolog 1
OD	Optical density
NTC	Non-transgenic control
NS	No symptoms
NCW	No complete wilting
PCR	Polymerase chain reaction
PR	Pathogenesis-related
PFLP	Plant ferredoxin-like protein
PRP	Pathogenesis-related proteins
PRR	Pattern-recognition receptors
R Genes	Resistance genes
RPM	Revolutions per minute
RB	Right T-DNA border sequence
RM	Rooting medium
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase Polymerase chain reaction
SA	Salicylic acid
SAR	Systemic acquired response
SCV	Settle cell volume

SDS	Sodium dodecyl sulfate
SSC	Saline-sodium citrate
Taq	Thermus aquaticus
T-DNA	Transfer DNA
TE	Tris–EDTA
qRT-PCR	Quantitative real time reverse transcriptase PCR
USAID	United States Agency for International Development
UV	Ultra violet
V/V	Volume by volume
W/V	Weight by volume
WPI	Week post inoculation
µM	Micro molar
µFD	Micro farad
Xcm	*Xanthomonas campestris* pv. *musacearum*
X-gluc	5-bromo-4-chloro-3-indolyl glucuronide
YPG	Yeast peptone glucose medium
YTS	Yeast tryptone sucrose
YTSA	Yeast tryptone sucrose agar

ABSTRACT

Banana (*Musa* spp.) is an important fruit and staple food crop globally. It is grown in over 140 countries on more than 11 million hectares, with current production of 145 million metric tons per annum, out of which about 30 % is produced in Africa. However, its production is limited by banana Xanthomonas wilt (BXW) disease caused by bacterium *Xanthomonas campestris* pathovar *musacearum* (Xcm) and Fusarium wilt disease (FWD) caused by *Fusarium oxysporum* subspecies *cubense* race 1 (Foc). Cultivated banana varieties are sterile, triploids and have long generation period; therefore, it is difficult to develop disease resistance through conventional breeding. The use of genetic engineering is a potential option that could facilitate the development of banana cultivars resistant to a broad-spectrum of pathogens. The main objective of this study was to develop transgenic banana resistance to Xcm and Foc race 1 through over expression of non-expressor of pathogenesis related gene (*NPR/NH1*) from the rice. Embryogenic cells of farmer-preferred banana cultivars were generated, and *Agrobacterium*-mediated genetic transformation system was optemized using *beta-glucuronidase* (*gus*A) reporter gene. Two assorted groups of explants, multiple buds and male flowers, were tried for their capacity to develop EC from three locally grown cultivars of banana in Africa. Embryogenic cells of 'Gros Michel' and 'Cavendish Williams' were produced from multiple buds, and EC of 'Sukali Ndiizi' was obtained from male flowers. The EC were regenerated with efficiency of about 20,000-50,000 plantlets per ml of settled cell volume (SCV) based on cultivars. *Agrobacterium*-mediated genetic transformation was established for these cultivars using *gus*A reporter gene. Twenty to seventy transgenic events were regenerated from one ml of SCV of EC on kanamycin (100 mg/L) containing selective medium. The presence of *gus*A gene in transgenic banana plants was demonstrated by PCR. The integration and expression of *gus*A gene was further checked by dot blot, Southern hybridization and GUS histochemical assay. Banana cultivar 'Sukali Ndiizi' was transformed by the nonexpressor of pathogenesis-related gene (*NPR1*) homolog 1 (*NH1*) gene from the rice. One hundred and twelve transgenic events were regenerated in selective medium containing hygromycin B (25 mg/L). The transgenic nature of the generated events was tested by PCR analysis, dot blot, and Southern hybridization. Gene expression study was performed by RT-PCR and qRT-PCR for selected transgenic events. Potted transgenic events were evaluated against *Xanthomonas campestris* pv. *musacearum* and *Fusarium oxysporum* f. sp. *cubense* race 1 in the glasshouse. Out of twenty evaluated transgenic events, two events (10 %) exhibited no disease symptoms in all the three replicates and twelve events (60 %) showed reduced symptoms and five events (25 %) were susceptible. Fifteen transgenic events were tested for Fusarium wilt resistance in the glasshouse. Out of 15 transgenic events, three transgenic events were resistant (20 %) and seven events were tolerant (46 %) and five events (33 %) were susceptible with reduced symptoms in comparsion to control plant against Foc race 1. To understand the mechanism of disease resistance of the *NH1* gene, pathogenesis-related genes *PR1, PR2, PR3* and *PR5* were analyzed by qRT-PCR after inoculation of transgenic events with Xcm at different time intervals. The results showed that the *PR* genes were activated more in transgenic events in contrast to control non-transgenic plants at 6, 12 and 48-hour post inoculation. This study reports an efficient and reproducible protocol for genetic transformation using embryogenic cells of banana. The results of this research also provided proof of concept that the rice *NH1* can provide resistance against Xcm and Foc race 1. Overall this information will form a basis to develop Xanthomonas wilt and Fusarium wilt disease resistant transgenic banana varieties for smallholder banana farmers in Africa.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Banana (*Musa* spp.) including plantain is one of the most important staple food and cash crops in tropical and sub-tropical countries. They are grown in more than 140 countries on about 11 million hectares land area with annual production of about 145 million metric tons (mMt) (FAOSTAT, 2015). Africa alone produces about 40 mMt of banana and plantains every year (FAOSTAT, 2015). Mainly small-holder farmers grow this crop for food and domestic markets.

Uganda is the third leading producer globally, after India and China, with more than 10 mMt grown over 1.8 million hectares (CountrySTAT, 2011). However, banana yields in Uganda in the last decade have stagnated around 6 tons per hectare (CountrySTAT, 2011). Annual banana consumption in Uganda is between 220-460 kg/person, which is the world's highest consumption rate. It is the basic staple food for 13 million populations and an important source of income for smallholder farmers (Kilimo Trust Report, 2012).

Banana plants produce fruits whole year in favourable weather conditions of tropical countries, which defines it an important food security and cash crop (Jones, 2000). Many biotic and abiotic stresses are reducing the overall production of banana. The production is mainly reduced by pathogens and pests, such as the bacterial diseases mainly banana Xanthomonas wilt (BXW), fungal diseases particularly Black Sigatoka (caused by *Mycosphaerella fijiensis*), Yellow Sigatoka (caused by *Pseudocercospora musae)*, Leaf Spot disease (caused by *Pseudocercospora eumusae)* and Fusarium wilt disease also known as

Panama disease (caused by *Fusarium oxysporum* f. sp. *cubense*), viruses such as *Banana Streak virus* (BSV) and *Banana Bunchy-Top virus* (BBTV), and pests like nematodes and weevils (Jeger *et al*., 1995; Jones, 2000; Tushemereirwe *et al.*, 2004a; Tripathi *et al*., 2009a; Ploetz, 2015; Tripathi *et al*., 2016).

BXW caused by *Xanthomonas campestris* pathovar *musacearum* (Xcm), is a foremost problem to banana production in East and Central Africa (Biruma *et al.*, 2007; Tripathi *et al.*, 2009b; Blomme *et al*., 2014). It causes production loss due to premature ripening and decaying of fruits and finaly death of the diseased plant. It is endangering the source of revenue of millions of banana farmers growing banana for a staple food and income. BXW was first reported in Ethiopia on *Ensete ventricosum* also known as false banana (Yirgou and Bradbury, 1968) and further detected on banana (Yirgou and Bradbury, 1974). About 33 years later, BXW was first appeared in Uganda in Mukwono district in 2001 (Tushemereirwe *et al.*, 2004a), and it was then reported in Bugoma county of Western Kenya 2006 and spread out to other neighbouring counties (Carter *et al*., 2009; Kwach *et al*., 2013), gradually it was extended to other East and Central African countries. Currently, there are no bactericides, biocontrol agents or disease resistant banana varieties accessible to manage this disease (Biruma *et al.*, 2007; Tripathi *et al.*, 2009a). The cultivation of disease resistance banana varieties is the most effective way to manage BXW. It is difficult and time consuming to develop resistant varieties through conventional breeding since there is no known banana germplasm resistant to BXW. Transgenic approaches would offer a sustainable and effective means of controlling BXW (Tripathi *et al*., 2014a; Tripathi *et al.*, 2017).

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) is the most damaging fungal disease endangers banana production globally (Jones, 2000; Ploetz, 2015). Foc is

responsible for severe infection in banana, resulting in complete wilting of the plant and destruction of whole banana field in 2-3 years (Ploetz and Pegg, 2000). Although some sources of resistance have been identified in wild diploid bananas, the use of conventional breeding approaches to generate resistant banana cultivars is hindered by numerous problems associated with triploidy, low fertility and no seed formation in most of the banana cultivars (Lorenzen *et al*., 2010). Therefore, there is need to evaluate genetic engineering approaches to generate disease resistant cultivars by overexpression of genes conferring resistance to these diseases.

The rice nonexpressor of pathogenesis-related gene (*NPR1*) homolog 1, *NH1* gene, a key transcriptional co-regulator in plant defense responses has been reported to confer resistance to bacterial and fungal pathogens in various plant species (Bai *et al*., 2010; Chern *et al*., 2001; Parkhi *et al.,* 2010). This is a broad-spectrum regulator gene, which acts through using various transcription factors down-stream of the salicylic acid pathway. It can induce systemic acquired resistance (SAR) through activation of several pathogenesis-related (PR) genes after infection by various plant pathogens (Chern *et al.*, 2005). Therefore, this study aimed to transform banana with the rice *NH1* gene and assess the level of resistance of transgenic events to BXW and Fusarium wilt diseases.

1.2 Problem statement

Banana is an important fruit as well as food security crop and rural livelihoods mainly in Asia, Africa and Latin America and Caribbean countries. Banana Xanthomonas wilt and Fusarium wilt diseases have the potential of severely damaging banana plantations and consequently threatening the livings of millions of farmers in the East and Central Africa (Tushemereirwe *et al*., 2004b; Biruma *et. al*., 2007; Tripathi *et al*., 2009b). BXW disease affects most of the

cultivated banana varieties especially dessert banana (Ssekiwoko *et al*., 2006) resulting in economic losses of over $2 billion in a decade (Abele and Pillay, 2007). Fusarium wilt is considered the most destructive diseases in agricultural history globally (Moore *et al*., 1999; Kangire *et al*., 2001). It is affecting banana production the most of important banana growing regions in the world. The infected banana plants die before producing fruits, resulting 100 % yield losses. Current and potential impact of Fusarium wilts prove challenging due to the lack of proper reporting systems and insufficient control measures. The economic losses caused by Foc race 1 in the last century was estimated over 2.3 billion US dollars (Ordonez *et al*., 2015). Researchers have estimated that Tropical race 4 could spread up to 1.6 million hectares by 2040 if no resistant cultivars are developed. This represents 17 % of the current area under production. The annual production of this area is 36 mMt with an estimated value of around 10 billion dollars at current prices. Thus, it is assumed that such extensive spread could cause substantial socio- economic impacts on banana productions and livelihoods of banana farmer (Scheerer *et al*., 2016). According to Cook *et al*., 2015, Foc Tropical race 4 will spread over time and causing banana on farm losses exceeding $138 million per year despite a slow rate of spread and using all the preventive measures in Northern Territory, Australia.

Disease control measures are limited (Tripathi *et al*., 2009a). To develop disease and pest resistance banana varieties through conventional breeding is difficult because of the sterile nature of the crop, long generation cycle and various ploidy ranges (Lorenzen *et al*., 2010; Tripathi *et al.,* 2004). Even though sources of resistance against Foc have been reported in wild diploid bananas (Ploetz, 2005), transferring 'resistance genes' to farmer-preferred varieties by conventional breeding is challenging (Pillay *et al.,* 2001; Lorenzen *et al*., 2010). Also, the genetic variability is limited with no BXW resistant germplasm having been identified or developed to date (Tripathi *et al.,* 2004). Therefore, aim of this study was to

develop embryogenic cells of various banana cultivars and optimized regeneration and transformation system to generate transgenic banana using *NH1* gene to resistance against Xcm and Foc race 1.

1.3 Justification

Genetic engineering is considered an attractive tool to complement classical breeding techniques in developing disease and pest resistant banana. Reliable, efficient and reproducible regeneration and genetic transformation systems for farmer-preferred banana cultivars are essential for generating disease resistant banana. Embryogenic cell suspensions (ECS) are the ideal explants to be used for genetic transformation; however, development of ECS is difficult and genotype-dependent. There is an urgent need to refine protocols for development of ECS, regeneration and genetic transformation of farmer-preferred locally grown banana cultivars.

Systemic acquired resistance (SAR) is one of the strategies used for developing disease-resistant varieties through genetic engineering. SAR is a defense response in plants induced by localized infection providing resistance against a broad spectrum of pathogens (Ryals *et al*., 1997; Parkhi *et al.,* 2010). Nonexpressor of the pathogenesis-related gene (*NPR1*) is one of the key genes involved in SAR pathways. The NPR1 protein is involved in salicylic acid (SA) and jasmonic acid and ethylene signal pathways leading to SAR (Parkhi *et al.,* 2010). Salicylic acid, is a small phenolic compound that works as phytohormone involved in regulation of plant defense known as master regulator and inducing SAR against biotropic pathogens (Glazebrook, 2005). For SA, some SA-binding proteins were reported, and a recent hypothesis is that NPR3 and NPR4 proteins are SA receptors that regulates *NPR1* gene, leading to resistance against pathogens (Yan and Dong, 2014).

There are reports demonstrating that overexpression of the *NPR1* gene provides resistance against bacterial and fungal pathogens (Chern *et al*., 2005; Lin *et al.*, 2004). The *NPR1*-mediated defense response mechanism is conserved in plant kingdom (Parkhi *et al.*, 2010) and overexpression of *NPR1* gene or its homolog can provide resistance against broad-spectrum pathogens in other important crops. Therefore, there is a need to evaluate the effect of the expression of rice *NPR1* homolog, *NH1* gene in conferring resistance against Xcm and Foc in transgenic banana.

1.4 Null hypothesis

i. Regeneration and transformation of embryogenic cells of various banana cultivars are not feasible.

ii. Over-expression of the rice *NH1* gene in transgenic banana can not provide resistance to Xcm and Foc race 1.

1.5 Objectives of the study

1.5.1 General objectives

To develop transgenic banana resistant to bacterial and fungal diseases using the rice *NH1* gene.

1.5.2 Specific objectives

The specific objectives of this study were:

i. To develop the embryogenic cell suspension cultures and optimize regeneration and genetic transformation of banana cultivars ‘Gros Michel’, ‘Cavendish Williams’ and ‘Sukali Ndiizi’.

ii. To generate transgenic banana cultivar ‘Sukali Ndiizi’ using the rice *NH1* gene and determine the levels of resistance against Xcm in the glasshouse and assessment of the induction of *PR* genes.

iii. To determine the response of *NH1* transgenic banana events against Foc race 1 in the glasshouse.

1.6 Significance of the study

Optemization of *Agrobacterium*-mediated transformation (AMT) system using embryogenic cell suspension of farmer-preferred banana cultivars may provide an improve and robust technique with various banana cultivars for the further improvement of banana against other biotics and abiotic challenges. During this study generated resistant transgenic banana against Xcm and Foc may reduce production losses resulting from Banana Xanthomonas wilt disease and Fusarium wilts. This will boost the productivity of the crop and escalate the income of resource-poor farmers.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of banana and plantain

Bananas and plantains are classified under *Musaceae* family; order *Zingiberales* and genus '*Musa'*. They belong to *Liliopsida,* subclass *Zingiberidae* and division *Magnoliophyta*. The family *Musaceae* has two genera – '*Musa'* and '*Ensete'*. The genus '*Ensete'* comprises of single-stemmed herbaceous, perennial plant, thick at the base of a pseudostem, with few seeds in inedible fruits (Cheesman, 1948). This genus '*Ensete*' was coined by Cheesman in 1948 and comprises of 25 species, out of which only eight are cultivated (Novak, 1992). '*Ensete'* is grown in the Southern hilly mountainous cool environment of Ethiopia and source of starchy material that is obtained from the rhizome and pseudostem section of plants (Novak, 1992).

Carl Linnaeus gave the genus name *Musa* as an honor to Antonius Musa, physicist of Emperor Augustus (1st century BCE) or the name came from the Arabic word 'Mauz' (Janssens *et al.,* 2016). The genus '*Musa'* includes bananas and plantains with more than 50 species. The genus was classified into five sections specifically *Australimusa, Callimusa, Rhodochlamys, Eumusa* and *Ingentimusa* (Simmonds, 1987). The five sections are further catergerized according to number of the chromosomes present in each cell i.e. *Callimusa* and *Australimusa* species contain 10 chromosomes and *Eumusa* and *Rhodochlamys* species consist of 11 chromosomes. *Ingentimusa* has a single species *M. ingens* which contains 14 chromosomes (Pillay and Tripathi, 2007). *Musa ingens*, a highland banana is the biggest known herbaceous plant in Papua New Guinea. Sections *Callimusa* and *Rhodochlamys* are grown as ornamental plants. Section *Australimusa* consists of parthenocarpic edible banana (Argent, 1976).

2.2 Distribution of banana and plantain

Eumusa is widely dispersed and the most significant group of edible bananas currently cultivated globally. Most of the cultivated bananas are developed from two *Musa* species, *Musa acuminata* Colla (A genome) and *Musa balbisiana* Colla (B genome) (Cheesman, 1948; Pillay and Tripathi, 2007). *Musa acuminata* is the most common of the *Eumusa* species with its center of diversity and primary center of origin in South East Asia mainly Malaysia and Indonesia (Simmonds 1962; Horry *et al.,* 1997). Modern banana had derived from diploid *M. acuminata* (AA genome), was cultivated by farmers due to seedless nature of fruits developed as product of female plant bareness and parthenocarpy (Pillay and Tripathi, 2007). Triploid banana (AAA genome) cultivars are generated from diploids, following natural hybridization between edible diploids and *M. acuminata*, developed an assorted AAA genotypes including the dessert bananas (AAA genome) and East African Highland bananas (AAA genome-EAHB) also known as cooking banana in the secondary location of origin in East African countries (Pillay and Tripathi, 2007). These two groups of bananas very different especially in their quality of fruits (Simmonds, 1962; Pillay and Tripathi, 2007). This indicates that the A genome of different *Musa* subspecies are also diverse in other subspecies. In most Southeast Asian countries triploids (AAA genome) have substituted the cultivated AA diploids because of their better seedless fruits. However, diploid banana (AA genome) remain very important for banana breeding progam and several varieties is available in Papua New Guinea (Simmonds, 1962).

2.3 Origin of banana and plantain

Banana and plantain are monocotyledonous perennial herbaceous plants, which grow well in tropical countries of the world. The earliest evidence to banana is about 500 BC. Some banana researchers believe that bananas were the first fruit on the planet and were originated to the Southeast Asia in Malaysia, Indonesia and Philippines (Simmonds, 1966; Simmonds, 1987).

M. acuminata is an innate of the Malaysia and neighouring countries and *M. balbisiana* is mainly cultivated in India (Simmonds, 1966). Several wild diploid banana cultvars still grow in the forests of Southeast Asian countries. Primary centers of origin of the bananas are Southeast Asia and the Western Pacific regions (Simmonds, 1962; Robinson, 1996; Jones, 2000, Janssens *et al.*, 2016) and they are mainly distributed and cultivated in the tropical and subtropical countries. Bananas and plantains are assumed to have spread from Asia throughout the humid tropics (Valmayor, 2000) mainly by migrating people carrying banana suckers (Simmonds, 1962). The history of banana farming is, therefore, related to the early migration of human populations. Banana cultivation results in the development of a many triploids (AAB) bananas, which are grown in the Pacific Island countries (Simmonds, 1962). In 15th century Arabian merchants brought banana from India to Africa through Madagascar (Simmonds, 1962).

Banana and plantain are grown in the different ecological regions of the sub-Saharan Africa (Swennen and Vuylsteke, 1991). Plantains (AAB) are mainly grown in West and Central Africa, whereas cooking and beer bananas (AAA) are cultivated in the East African countries. Farmers in these ecological regions grow several varieties of banana and plantains. Africa is known as secondary center of origin of banana, which has more than 100 banana and plantain cultivars (Lescot, 2000 and 2008).

2.4 Importance of banana and plantain

Bananas are important and familiar fruits all over the world, with an annual yield of 145 million metric tons (mMt). They are grown in 140 countries and island territories on about 11 million hectares of cultivated area and have financial worth of about 40 billion US Dollars (FAOSATAT 2015). The highest banana producing countries are India about 27 mMt, China

with 12 mMt, Uganda with 9.5 mMt followed by and Phillippines 8.6 mMt and Brazil 6.9 mMt (FAOSTAT, 2012). Bananas including plantains are one the most essential food crops globally after maize, wheat and rice (Tripathi *et al*., 2014b).

The sweet banana is also called the dessert banana. The banana fruits contain high percentage of water and sugars. The sweet banana fruits are rich source of minerals and vitamins (Robinson, 1996). The ripe banana also contains neuro-hormones like serotonin, dopamine, and norepinephrine. Banana peels and pulp comprise phenolic compounds, biogenic amines and carotenoids. These neuro-hormones have a positive influence on the human brain and act as a mood stabilizer (Robinson, 1996; Pereira and Maraschin, 2015).

2.5 Improvement of banana through conventional breeding

Current banana breeding programs mainly focus on developing hybrid banana and plantain with resistance to pests and diseases, high-yielding varieties, short stature of the pseudostem of the plant, early flowering and maturity and fruit better quality (Pillay and Tripathi, 2007). The earliest banana breeding programs were aimed at developing disease-resistant banana for export after the epidemic of Fusarium wilt in 'Gros Michel' in Central America (Rowe and Rosales, 1993). 'Gros Michel' has been replaced with 'Cavendish Williams', cultivar which was prone for fungal pathogen *Mycosphaerella fijiensis,* which cause black Sigatoka, at that time, was newly introduced to Central America. Banana breeders attempted to develop cultivars with resistance to Fusarium wilt and black Sigatoka and keeping other traits comparable to 'Gros Michel' (Sathiamoorthy and Balamohan, 1993). Priority traits included high-yield, good fruit quality, flavor, equal ripening of fingers, and reduced plant height (Stover and Simmonds, 1991). The initial challenge for banana breeders was to find suitable diploids with a combination of disease resistance and acceptable agronomic traits, and tetraploids with

seed fertility for use in banana breeding. Most of the agronomic important traits are more common in diploid parents (Tenkouano *et al.,* 1999). Therefore, the major banana breeding programs focused on the production of improved diploid parents, which can be used for crossing (Ortiz and Vuylsteke, 1996). Various improved diploid bananas have been developed by banana breeders and listed in the public domain successfully, and disseminated globally (Rowe and Rosales, 1993; Tenkouano *et al.,* 2003). Banana breeders have developed diploid bananas that have been used as a mother plants of tetraploid hybrids bananas in Fundacion Honduran de Investigacion Agricola, Honduras (Rowe and Rosales, 1993).

Tetraploid hybrids of plantains (PITA-14) and bananas (BITA-3) have developed by IITA and few of them show resistance to fungal disease and have good banana bunch sizes (Vuylsteke *et al.,* 1993; Ortiz and Vuylsteke, 1998a and 1998b; Jones, 2000). These hybrid bananas have been worldwide evaluated by banana breeders and farmers for adoption as new cultivars and have been shown to be high-yielding. Ploidy manipulation techniques have been performed to reduce the chromosome number to triploid to develop sterile male hybrids (Ortiz, 1997). Most important achievements in improving quality of fruits was reinstating parthenocarpy in the triploid banana (Tenkouano *et al.,* 1998). For example, triploid hybrids with black Sigatoka resistance have been developed by crossing from diploid parent with tetraploid hybrids (Ortiz *et al.,* 1998c). Various banana breeding program have been investigated through conventional and innovative breeding techniques and developed improved banana from diploids, triploids, tetraploids (Silva *et al.,* 2001; Tenkouano *et al.,* 2003).

Some important *Musa* cultivars like ‘Cavendish Williams’ which is main commercial cultivar, remain recalcitrant to conventional breeding. Several levels of ploidy, low genetic variability, long generation times and seedless nature of edible banana cultivars have hindered the

improvement of bananas for disease-resistant through conventional breeding. Most of the cultivated edible bananas are triploids which produce minimum number of seeds due to low fertility (Silva *et al*., 2001; Vuylsteke *et al.,* 1993; Lorenzen *et al.,* 2010). There are very few seeds develop in banana fruits and their germination are also very difficult due to hardy outer structure of the seeds (Silva *et al*., 2001) Banana plants also adopt 14-18 months to bear the fruits. (Pillay and Tripathi, 2007), which prolong banana breeding efforts compared to early maturing crops. *In vitro* tissue culture and recombinant DNA technology can be use for improving banana cultivars, which are not possible to be improved through conventional breeding.

2.6 Improvement of banana through genetic engineering

In order to meet the food demand of the growing population on our planet, there is an urgent need to increase food production through increased resistance/tolerance to biotic and abiotic stresses, in a timely and cost saving way using novel technologies. Genetic engineering is an alternative way for the improvement of banana varieties not responsive to breeding technology (Pillay and Tripathi, 2007). These include the dessert 'Cavendish' banana and the 'False Horn plantains' (Jones, 2000). With progress in plant biotechnology, plant tissue culture and the availability of various techniques for transfer of any gene construct into a plant species becomes attractive (Tripathi *et al.,* 2009a). In theory, genes from any organism, once identified as valuable, cloned, may be transferred to a plant (Tripathi *et al.,* 2009a). The sterility of many cultivars makes banana transformation particularly important as gene flow is negligible. Although no transgenic bananas and plantains have been released for commercial production yet, their production should be encouraged and supported through scientific knowledge and risk assessment studies.

Many genes are currently available which can provide protection against viral, fungal and bacterial pathogens and several pests. Developing modified banana using these genes requires robust regeneration and transformation protocol (Tripathi *et al.*, 2009a). Two systems for the genetic transformation of bananas have been established. Micro-projectile bombardment technique has been used for the genetic modification of the cooking banana cultivar 'Bluggoe', the plantain 'Three Hand Planty' and dessert banana cultivar 'Grand Nain' using embryogenic cells (EC) (Sagi *et al.*, 1995; Becker *et al.*, 2000). *Agrobacterium*-mediated transformation (AMT) system, which is another way of introducing genes, offers many benefits in comparison to particle bombardment. AMT technique inserts only a few copies genes of interest at higher frequency with minimal rearrangements (Hansen and Wright, 1999; Shibata and Liu, 2000). Transgene insertion into EC using AMT technique has been reported for the banana cultivars 'Rasthali', 'Lady finger', 'Sukali Ndiizi' 'Gonja Manjaya' 'Gros Michel', and 'Cavendish Williams' (Ganapathi *et al.*, 2001; Khanna *et al.*, 2004; Tripathi *et al.*, 2010; Namukwaya *et al.*, 2012; Tripathi *et al.*, 2012, Tripathi *et al*., 2015).

Many researchers have tried to develop EC from several banana cultivars using different types of explants. Novak *et al.* (1989) have developed EC from leaf sheaths and rhizome 'Grand Nain'. For 'Bluggoe' variety of banana mainly used for cooking, shoot tip were used to produce EC (Dheda *et al*. (1991). Embryogenic cells have also been developed from zygotic embryos (Escalant and Teisson, 1989; Marroquin *et al.*, 1993). However, immature male flowers were found to be better for production of EC and their regeneration into complete plantlets of 'Grand Nain' and 'Rasthali' (Novak *et al.*, 1989; Dheda *et al.*, 1991; Escalant *et al.*, 1994; Cote *et al.*, 1996; Navarro *et al.*, 1997; Becker *et al.*, 2000; Ganapathi *et al.*, 2001). Recently, Tripathi *et al.* (2012) have reported the development of embryogenic cells using multiple buds from a plantain cultivar 'Gonja Manjaya'.

Embryogenic cells are the preferred starting material for genetic transformation; though, most of the protocol for developing embryogenic cells are laborious, time-consuming and depend on genotype of banana cultivars (Tripathi *et al*., 2009b) Therefore, other regeneration systems have also been developed using shoot tips and intercalary meristems from different cultivars (May *et al.,* 1995; Tripathi *et al.,* 2005; Tripathi *et al.,* 2008a). Shoot tips are apical meristematic tissue isolated from *in vitro* grown banana plantlets. The intercalary meristems are densely packed meristematic tissues just beneath the apical region in the axil of leaf sheath. The transformation techniques using shoot tips and intercalary meristems are applicable to several cultivars (Tripathi *et al.,* 2003; Tripathi *et al.,* 2005; Tripathi *et al*., 2008a).

Several transgenes available currently for improvement of banana have been isolated from other plant species, insects, and microbes (Pillay and Tripathi, 2007). There is need to identify and isolate disease resistance genes from *Musa* germplasm including wild type bananas and transfer to farmer-preferred disease-susceptible banana cultivars. The use of trait specific transgene may allow the production of transgenic plants resistant to fungi, bacteria, viruses and nematodes with genetic modification techniques rather than using classical breeding (Tripathi *et al*., 2009b).

2.7 Banana Xanthomonas wilt

Banana Xanthomonas wilt (BXW) disease is the biggest biotic stress for cultivation of banana in East and Central Africa (Tushemereirwe *et al.,* 2003; Biruma *et al*., 2007; Tripathi *et al.,* 2009b). The disease causes death of the infected plants due to wilting and decaying of fruits. It affects the living of millions of banana growers. BXW was first spotted in the Southern part of Ethiopia on *Ensete ventricosum* (Yirgou and Bradbury, 1968) and gradually dispersed in banana (Yirgou and Bradbury, 1974). The pathogen infects the most banana cultivars grown in

East Africa except some resistance in diploid cultivar *Musa balbisiana* (Ssekiwoko *et al.,* 2006). The economic importance of BXW is because of complete production losses and death of the banana plants that produce sucker plants for the next cropping generation (Tripathi *et al.,* 2009b). Total, monetary damages were projected at US $ 2-8 billion in ten years (Abele and Pillay, 2007). The impact on production in the Great Lakes region in Africa is shocking. The average production losses are highest in Democratic Republic of Congo (83 %) and Uganda (71 %), and in other East African countries like Burundi, Kenya, Rwanda and Tanzania is about 39 to 51 % (Ainembabazi *et al*., 2016).

2.7.1 Causal organism

The causal organism is a rod-shaped, motile, Gram-negative bacterium and named as *Xanthomonas campestris* in the 1970s and renamed *X. campestris* pathovar *musacearum* (Xcm) in the 1980s (Yirgou *et al.,* 1968; Yirgou *et al.,* 1974; Young *et al.,* 1991). Xcm belongs to family *Xanthomonadaceae,* and order Xanthomonadale. The bacteria produce yellow, dome-shaped mucoid, circular colonies (Figure 2.1), when cultured under laboratory in YPGA medium (Tripathi *et al.,* 2007). The DNA sequence and fatty acid data of bacterium revealed that strains of Xcm are very much like strains of *Xanthomonas vasicola* and therefore, the name *X. vasicola* has been proposed for Xcm (Aritua *et al.,* 2008).

2.7.2 Disease symptoms

Infected banana plants show symptoms like yellowing, drooping and drying of leaves, and untimely ripening of fruits with further rottening (Tushemereirwe *et al.,* 2004a). Majority of the diseased plants in the infected banana fields completely die in short time (45-60 days) after infection (Figure 2.2A). Symptoms on banana fruits and inflorescence include early and uneven ripening with sometimes complete rotting of entire bunch of fruits and contracting and rotting

of the inflorescence containing only male flowers after appearance of all the fruits (Figure 2.2B). Transverse-section of the pseudostem and inflorescence rachis of the diseased plant shows yellowish bacterial exudations appearing in 30 min after cutting (Figure 2.2C and D). Wilting symptoms spread very fast in the humid and hot weather and visible within 21 to 30 days in cultivated fields and 14 to 21 days after inoculation in the glasshouse conditions (Tripathi *et al.,* 2008b). Banana cultivar, growth stages and mode of infection affect banana bacterial wilt symptoms progression and further spread of disease to other plants. (Tripathi *et al*., 2009b). Flowering banana plants attract insects which infect the flowers. From infected flower, Xcm spread to fruits and finally to leaves and pseudostem. Fruits show premature ripening and leaves turn yellow and brown and later whole plants completely collapse and die. Contaminated farming tools also infect banana plants before flowering. Soil-borne infection can also occur through the damaged roots (Mwangi *et al.,* 2007). In infected leaves, Xcm spread from leaf tip to petiole and finally in pseudostem. Water-soaking symptoms appear red brown patches on the leaves in rainy season (Tripathi *et al*., 2009a).

Figure 2.1: Pure culture of *Xanthomonas campestris* pathovar *musacearum* on Yeast tryptone sucrose agar medium in petri dish. (Scale bar 1cm)

Figure 2.2: Banana Xanthomas wilt disease symptoms. **A)** Infected banana field showing completely wilted plants, **B)** Rotten bunch of banana and dried inflorescence, **C)** Pseudostem of infected plant containing yellow bacterial exudation of Xcm, **D)** Yellow bacterial exudation of Xcm on rachis. Scale bar 30 cm and 1 cm. Source- BXW infected banana plantation in Uganda

2.7.3 Management of Banana Xanthomonas Wilt

Management strategy is similar in bacterial diseases of banana prevelent globally like Moko disease (caused by *Ralstonia solanacearum),* which is originated in Latin America, Blood disease caused by the Blood disease bacterium, which is widespread in Indonesia (Eden-Green and Sastraamadja, 1990; Supriadi, 2003), and Bugtok disease caused by *Pseudomonas solanacearum*, which is predominantly in the Philippines (Thwaites *et al.*, 2000), including BXW. Control of BXW depends on the prevention of spread of the disease, reduction of impact, and the rehabilitation of affected areas by growing alternative crops. The inflorescence of the banana plant presents the entry point of Xcm infection. Therefore, early cutting of the male

buds from banana plants avoids the insect transmission of Xcm from diseased plants to healthy plants. Removal of male bud after completion of fruiting, by twisting the male bud to break it, is one of the ways for avoiding the spread of the disease through contaminated cutting tools. This is preventive measure of BXW to check the spread of disease through de-budding. However, application of de-budding has been unreliable (Kagezi *et al.*, 2006). Banana farmers believe that de-budding reduces the quality of the juice-producing banana (Bagamba *et al.*, 2006). A crop-free period of few months is required to prevent re-infection of newly planted banana with from soil-borne Xcm (Tripathi *et. al*., 2013).

The most desired approach to manage BXW disease is to use disease resistant varieties. So far, there is no banana variety with complete resistance to Xcm. A few cooking banana cultivars such as ‘Mbwazirume’ and ‘Nakitembe’, which have attached bracts with inflorescence while fruiting of banana bunches may prevent getting infection by insect vectors contaminated with Xcm (Tripathi *et al.,* 2008). Currently no disease-resistant banana is available for farmers therefore control measures to prevent the disease is awareness about BXW to reduce the impact through cultural practices, has been recommended. Currently, no biological or chemical control method is available to prevent BXW. Transgenic approaches are highly desired to develop BXW resistant varieties.

2.8 Fusarium wilt disease in banana

Fusarium wilt of banana (Panama disease) caused by a soil born fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) (Snyder and Hansen) (Figure 2.3) (Ploetz and Pegg, 2000; Michielse and Rep, 2009; O’Donnell *et al*., 2009; Ordóñez *et al.,* 2015).

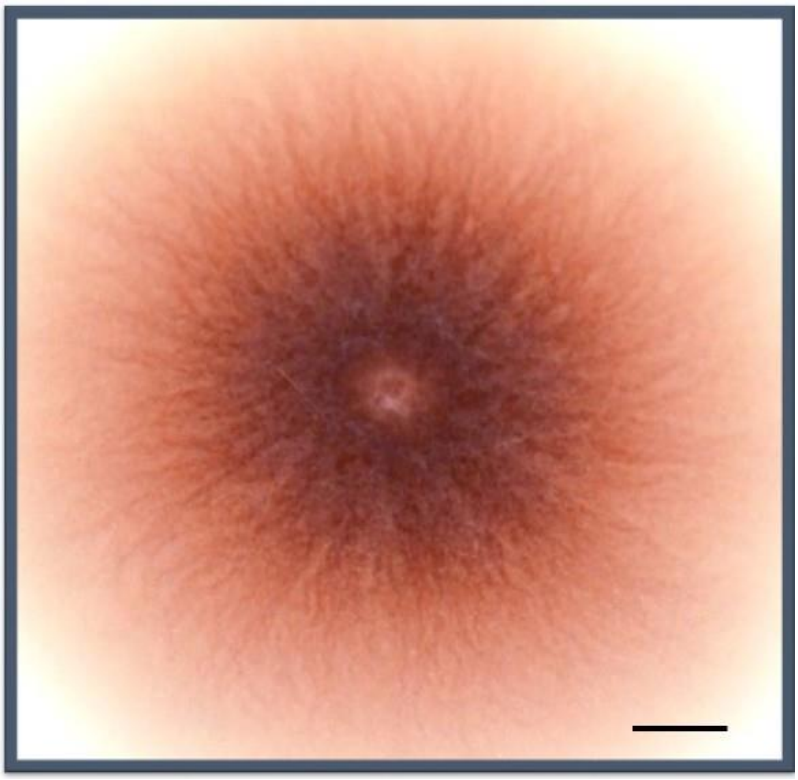


Figure 2.3: Plant pathogen *Fusarium oxysporum* f. sp. *cubense* race 1 in petri dish cultured on Potato Dextrose Agar medium. Scale bar 1 cm.

It was first identified in 1874 in Australia (Stover, 1962) but currently, it is prevalent in several bananas producing countries. Foc is classified into tropical races (TR) 1 to 4 according to disease severity and banana cultivars, which they basically cause severe infection. A highly virulent form of the fungal pathogen, 'tropical race 4', has caused great losses to both small-holder farmers and big commercial companies since it infects 'Cavendish' cultivars which were previously assumed to be resistant and a replacement for susceptible-cultivar 'Gros Michel' in commercial plantation (Buddenhagen, 1990; Hwang and Ko, 2004). Foc 'tropical race 1' and 'tropical race 2' has been reported in East and Central Africa (Tushemereirwe and Bagabe, 1998), whereas Foc 'tropical race 4' spreads in South Africa (Viljoen, 2002).

2.8.1 Life cycle and disease symptoms of *Fusarium oxysporum*

Fusarium wilt also recognized as deadly vascular disease causes complete vascular blockage of water and nutrients movement which leads to yellowing and wilting of leaves, cracking of pseudostem and finally death of complete plants. The pathogen enters the plant through damage root tips and moves through the xylem vessels and colonizes inside the rhizome and pseudostem. Initial symptoms appear on damage roots, which turn brown reddish. At this stage reddish brown discolorations of vascular system can be seen. Fungal spores germinate until the entire xylem is completely blocked (Ploetz and Pegg, 2000). In completely wilted and dried plant, the fungus develops chlamydospores (Nelson *et al.,* 1983; Ploetz, 2015) (thick-walled spores) which are discharged into the soil when the plant dies (Stover, 1962; Nelson *et al*., 1983) The pathogen spreads through infected planting materials, soils, rain water, farming tools and grazing animals and human migration (Jones, 2000, Ploetz, 2006). The disease symptoms include yellowing and browning of leaves, the longitudinal splitting at the base of the pseudostem which extend gradually upward, internal reddish-brown discoloration of pseudostem and rhizome (Figure 2.4A, B and C) and finally the complete wilting of the plant (Jones, 2000; Ploetz, 2006). Sometimes, symptoms are not seen in the fruit except the bunch size becomes smaller (Thangavelu and Mustaffa, 2010). Most times the plant dies without developing any fruit.

2.8.2 Management of Fusarium wilt in banana

Currently, several control methods are available such as the use of clean planting materials developed through tissue culture, use of pathogen-free soil and removal of infected plant material from banana-growing fields (Moore *et al.*, 1995). To circumvent spread of the fungal pathogens, application of strict quarantine measures has been introduced for the rapid detection of Foc TR 4 using PCR (Dita *et al.*, 2010).

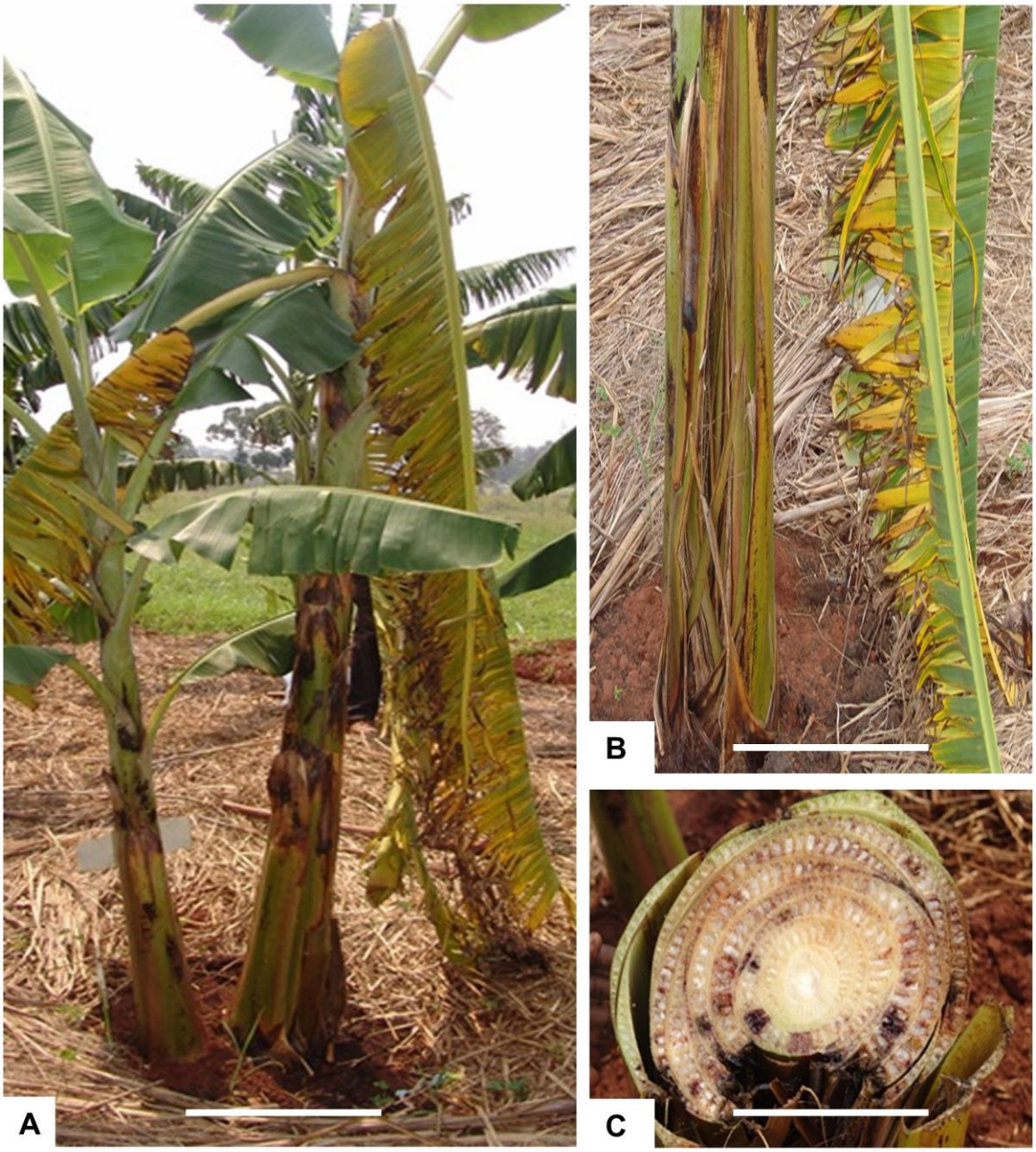


Figure 2.4: Fusarium wilt infected banana plants showing disease symptoms. **A)** Infected plant with yellow leaves, **B)** Pseudostem splitting due to blockage of plant vascular system with *Fusarium oxysporum* f. sp. *cubense* race 1 **C)** Internal reddish purplish brown discoloration of cut pseudostem. Scale bar 30 cm. Fusarium wilt infected banana plantation in Uganda. (Source: Photographed by me).

Chemical control through fungicides and fumigants are also in application but are not very sustainable or environmentally safe for small-holder farmers (Pei *et al*., 2005). Various

fungicides are used to control Foc like prochloraz, propiconazole and Benomyl inhibited mycelial growth at minimal concentrations (Nel *et al*., 2007). Surface sterilants mainly Farm cleanse and Domestos, Sporekill are also used to prevent germination of microconidia (Meldrum *et al*., 2013). Biocontrol inoculants mainly *Bacillus amyloliquefaciens* NJN-6, *Sphingobium*, *Dyadobacter*, and *Cryptococcus* reduce proliferation of Foc in infected soil (Fu *et al.,* 2017). Management of this disease is largely based on barring of the pathogen from infection free lands and the use of resistant cultivars (Ploet, 2006). The perennial production and long generation period of the banana crop, the multi-cyclic nature of fungal disease and long survival period of the fungal spores impede the introduction of realistic management technology. Effective chemical, biological and traditional measures are not accessible, even though various literatures are available on disease management practices (Ploetz, 2015). Disease resistance transgenic banana varieties are highly required to manage the deadly disease like Foc.

2.9 Transgenic strategies for resistance to bacterial and fungal pathogens

Plants have developed a multifactorial mechanism for protecting themselves from invading pathogens. This includes several proteins produced before infection or during pathogen attack (Patil *et al*., 2012). Plants develop a first line active defense system to identify various pathogens using pathogen-associated molecular patterns (PAMP), which are recognized by pattern recognition receptors (PRR) (Boller and Felix, 2009; Schwessinger and Ronald, 2012; Zipfel, 2014). Plants also protect themselves from pathogens using various defence-related mechanisms, mainly cell wall strengthening by callose deposition and accumulation of phytoalexins, synthesis of SA and H_2O_2 and the activation of the pathogenesis-related (PR) genes and several other unknown genes (Kachroo *et al*., 2003; Bai *et al.,* 2010; Doughari, 2015). A hypersensitive reaction is started with the several defence responses which prevent

the pathogen growth at the infection site (Durrant and Dong, 2004). Differential expression profiles of the resistant cultivar 'Yueyoukang 1' and the disease-susceptible 'Brazilian' banana infected with Foc TR4 showed that genes related to several PR proteins, transcription factors and lignification of cell walls were induced in the resistant cultivar 'Yueyoukang 1 (Bai *et al*., 2013). This suggests that these genes are important to provide resistance in banana against Foc TR4 (Bai *et al*., 2013). Using green florescent protein (GFP), Foc TR 4 was consistently confirmed to colonize in a susceptible banana cultivar within 15 days but not a resistant cultivar (Chunyu *et al*., 2011).

2.9.1 Antimicrobial peptides

Disease resistant transgenic plants can be generated by using various genes encoding fungal hydrolytic proteins (Lorito *et al.*, 1998), antimicrobial proteins (Cary *et al.*, 2000; Li *et al.*, 2001) and by inducing plant defense-related genes (Keller *et al.*, 1999). Antimicrobial peptides (AMP) and its analogues are proteins that have antimicrobial activity against pathogens like bacteria, viruses, and fungi (Jacob and Zasloff, 1994; Rajasekaran *et al*., 2001; Tripathi *et al.*, 2016), yet most are safe for plants and mammalian cells. These includes magainin, cecropins, attacins, thionins and defensins (Hultmark *et al.*, 1983; Boman and Hultmark, 1987; Zasloff, 1987; Molina *et al.*, 1993; Broekaert *et al.*, 1995).

2.9.1.1 Magainins

The antimicrobial proteins, magainins, were first extracted from the skin of African clawed frog (*Xenopus laevis*) (Zasloff, 1987). Protection against both bacterial as well as fungal plant pathogens was demonstrated by expression of a magainin analogue, Myp-30 and MSI-99, in genetically engineered tobacco plants (De Gray *et al.*, 2001; Li *et al.*, 2001). MSI-99 has been expressed in the banana cultivar 'Rasthali' and conferred enhanced resistance against *Fusarium*

oxysporum f. sp. *cubense* (Chakrabarti *et al.,* 2003). It also demonstrated resistance against *Alternaria alternate*, *Botrytis cinerea* and *Sclerotinia sclerotiorum* in tobacco (Chakrabarti *et al.,* 2003).

2.9.1.2 Cecropins

Cecropins are antibacterial proteins found in the hemolymph of silk moth (*Hyalophora cecropia*). They can provide resistance to broad-spectrum of pathogenic bacteria by destroying the cell membranes (Durell *et al.,* 1992; Kaduno-Okuda *et al.,* 1995).

2.9.1.3 Attacins

Attacins is a bactericidal protein found in *H. cecropia* pupae (Hultmark *et al.,* 1983). Attacins expressed in potato confers resistance to *E. carotovora* (Arce *et al.,* 1999). Genetically engineered pear and apple harbouring the attacins gene, shows resistance to *E. amylovora* (Norelli *et al.,* 1999; Reynoird *et al.,* 1999). The stability of expression of attacin E gene in the several events of the transgenic apple cultivar 'Galaxy' was preserved yet conferred resistance to *E. carotovora* which causes fire blight in apple. This gene did not affect fruit quality or plant morphology and was observed for more than a decade in the confined field trial (Borejsza-Wysocka *et al*., 2010). Transgenic orange cultivars (*Citrus sinensis*) with attacin A gene shows reduction in disease severity against *Xanthomonas citri* subsp. *citri* which causesAsian Citrus Canker (ACC) disease (Cardoso, *et al*., 2010).

2.9.1.4 Thionins

Thionins are sulphur and cysteine-rich proteins found mainly in higher plants. These proteins show toxicity against several bacterial and fungal pathogens. Thionins are involved in protection against phyto-pathogens by attacking the cell walls directly by inducing pores that

release potassium and calcium ions causing death of the microbes (Molina *et al.*, 1993). The α-thionin gene which was isolated from barley, confers resistance to *P. syringae* in transgenic tobacco (Carmona *et al.* 1993). Transgenic tomato and *Arabidopsis* with *Thionin 2.1* gene from *Arabidopsis* demonstrated resistance to several pathogens (Chan *et al*., 2005; Epple *et al*., 1997). The protein thionin 2.4 collects in the cell walls of *Fusarium graminearum* and *Arabidopsis thaliana*, suggesting that it plays an important role to prevent fungal spore entry at the plant cell wall and inducing pores in the cell membrane of fungal cells (Sano *et al*., 2013). Transgenic rice having oat thionin *Asthi1* confers enhanced resistance against *Burkholderia glumae and B. plantarii* (Iwai *et al.*, 2002). The thionin SE60 protein isolated from soybeanshows bactericidal activity against *Pseudomonas syringae* (Choi *et al.*, 2008). Transgenic sweet potato with barley *α-hordothionin* show a resistance to *Ceratocystis fimbriata* which causes Black Rot disease (Muramoto *et al*., 2012). Recently, Hao *et al.* (2016) reported that over-expression of endogenous citrus thionin in transgenic citrus plants confers resistance to Citrus Canker and Huanglongbing.

2.9.1.5 Plant defensins

Plant defensins are another type of antimicrobial proteins which inhibit growth of various plant pathogens. Transgenic banana plants with a defensin gene showed resistance against *Fusarium oxysporum* f. sp. *cubense* race 1 (Ghag *et al.*, 2014). Transgenic banana plants expressing petunia floral defensins (Ghag *et al*., 2012), and Ace-AMP1 (Mohandas *et al.*, 2013) also confer significant resistance against *Fusarium oxysporum*. Ntui *et al*. (2010) has demonstrated that 'Egusi' melon (*Colocynthis citrullus* L.) transformed with wasabi defensin gene provides resistance to Alternaria leaf spot and Fusarium wilt disease. Transgenic rice harbouring *Rs-AFP 2* gene shows resistance to fungal infection (Jha and Chattoo, 2010). Transformed Tobacco plant with *Nmdef* 02 gene confers resistance against fungal disease (Portieles *et al.*,

2010). Plant defensin from radish (Rs-AFP1, 2, 3, 4) are conserved in several plant species, including members of the *Brassicaceae* family, and prevent the growth of a broad-spectrum of plant pathogens (Broekaert *et al.*, 1995). Plant defensin genes from *B. campestris* and *B. oleracea* confers reduced symptoms to bacterial leaf blight of rice (Kawata *et al.*, 2003; Tripathi *et al*., 2004). The plant defensin genes induced by ethylene and jasmonic acid (Thomma *et al.*, 2002; Broekaert *et al.*, 2006;).

2.9.2 Resistance genes

Various disease resistance (R) genes have been well-characterized, cloned and demonstrated enhanced resistance against pathogens in different plant species. Many R-gene related proteins have common structural motifs and provide disease resistance to various group of pathogens indicating that they may have similar mechanism (Bent, 1996). *Arabidopsis RPS4* gene shows resistance against *P. syringae* pv. tomato (Gassmann *et al.*, 1999). The *Pto* gene that encodes serine/threonine protein kinase, expressed in tomato plants provided resistance to bacterial pathogens *P. syringae, X. campestris* and *Cladosporium fulvum* (Kim *et al.*, 2002; Mysore *et al.*, 2003). The pepper *Bs*2 gene demonstrated resistance against *X. campestris* species (Tai *et al.*, 1999). Transgenic tomato with the pepper *Bs2* gene had improved resistance to *Xanthomonas* species in multi-year field trials in a confined field trial (Horvath *et al.*, 2012). The maize *Rxo1*gene provides resistance against bacterial streak disease in rice (Zhao *et al*., 2004). *Rxo1* also shows resistance to bacterial disease *Burkholderia andropogonis*, which infects sorghum and maize. It also confers resistance against diverse ranges of bacterial pathogens. *Rxo1* gene also showed resistance in transgenic rice and demonstrated the prospect of non-host R-gene transfer from maize to rice (Zhao *et al*., 2004).

The *A. thaliana RRS1* gene confers resistance to several strains of *R. solanacearum*, causative agents of Moko disease of banana (Deslandes *et al*., 1998). The rice *Xa21* gene has conferred resistance against many isolates of Xoo (Song *et al.*, 1995; Wang *et al.*, 1996). Transformation of *Xa21* gene in rice makes it resistant to *Xanthomonas oryzae* as well as resistant against bacterial infections in transgenic citrus fruits and transgenic tomatoes (Afroz *et al*., 2011; Mendes *et al.,* 2010). *In vitro* transgenic bananas with the *Xa21* gene also shows resistance to Xcm. These resistant plants were further grown in the glasshouse, where their resistance was tested again on 90-day old plants. The resistance of the transgenic plants varied, but 58 % of plants were fully resistant (Tripathi *et al.,* 2014b).

2.9.3 Receptors

Plants use pattern-recognition receptors (PRR) to recognize conserved microbial signatures also known as microbial associated molecular patterns (MAMPs) (Zipfel and Oldroyd, 2017). This recognition triggers immune responses in plants. Several PRRs have been identified in plants. The first study was flagellin sensing 2 (FLS2), a leucine-rich repeat receptor kinase (LRR-K) which recognize N-terminal, 22 amino acid flg22 of bacterial flagellin peptide (Gomez -Gomez *et al*., 1999; Beck *et al.,* 2014). Flagellin is the protein related to bacterial flagellum and its recognized in many plant species (Ranf, 2018). Plants activate their defense mechanism by sensing the PAMPs or MAMPs recognized by PRRs (Schoonbeek *et al.,* 2015). EF-Tu is another type of PRR, which binds to thermo-unstable elongation factor and plays an important role in plant defense. Unlike flagellin, which is recognized by plants belonging to varied families, EF-Tu is recognized only by *Brassicaceae* family (Zipfel, 2014). The over-expression of *Arabidopsis* EF-Tu receptors in wheat enhance the bacterial elongation factor (elf18) responsiveness and shows resistance against *Pseudomonas syringae* pv. *oryzae* (Schoonbeek *et al.,* 2015). *Nicotiana benthamiana,* tomato and rice expressing *Arabidopsis*

EF-Tu receptor showed high resistance against pathogenic bacteria (Lacombe *et al*., 2010; Lu *et al*., 2015; Schwessinger *et al*., 2014).

Several other PRRs which have been studied include Xa21 protein from rice which perceives type I secreted sulfate protein Ax21 (Pruitt *et al.,* 2015) and EIX1 and EIX2 which detect fungal xylanase, chitin elicitor binding protein which binds to fungal chitin and glucan binding protein which binds to *β*-glucans oomycete (Fliegmann *et al.,* 2004). Fungal chitin is also bound by lycin motif receptor like kinase CERK1 is binding protein (Gimenez-Ibanez, *et al*., 2009; Mazzotta and Kemmerling, 2011; Boutrot and Zipfel, 2017). Plants also possess immune receptors that recognize variable pathogen effectors. These effectors are proteins produced by the pathogens during infection. These receptors are also known as R-proteins which are coded by plant resistance genes (R-genes). These genes encode nucleotide binding, leucine rich repeat (NB-LRR) domains protein products which are specific for a given effector protein. When an effector protein is detected by its specific counter R-protein, then signaling response is activated. Unlike PRRs, which confer a broad-spectrum resistance, R-genes are race specific (Lacombe *et al*., 2010, Rodriguez-Moreno *et al*., 2017). PRRs should be further explored as their transfer to disease-susceptible plants can provide broad spectrum resistance against bacterial plant pathogens.

2.9.4 Non-expresser pathogenesis-related genes

Transgenic plants expressing non-expresser pathogenesis-related **(***NPR1)* gene isolated from *Arabidopsis* shows enhanced disease resistance to various pathogens (Cao *et al.*, 1998). NPR1 is a regulator of systemic acquired resistance and can provide resistance against broad-spectrum pathogens. Over-expression of the rice *NPR1* homolog (*NH1*) showed high resistance against *X. oryzae* pv. *oryzae* (causal agent of bacterial blight disease), indicating the presence

of a parallel defence pathway in rice (Chern *et al.*, 2005). The expression of *NH1* gene also confers resistance to the fungal pathogen *Magnaporthe grisea* (Yuan *et al.*, 2007; Quilis *et al*., 2008; Feng *et al.,* 2011). Two *NPR1* homologues, *MNPR1A* and *MNPR1B*, have been cloned from *Musa* (Yocgo *et al*., 2008). Phylogenetic analysis of these genes confirms that both *Musa* genes are closely related to other monocot *NPR1* genes. These *Musa* genes, when expressed in *Arabidopsis*, shows resistance to several pathogens (Yocgo *et al.,* 2012). *NPR1* gene (*MdNPR1*) has also been isolated from the *Musa* cultivar 'Dongguan Dajiao', which is resistant to *F. oxysporum* f. sp. *cubense* race 4 (Zhao *et al*., 2009). This gene can be transferred to Foc-susceptible cultivars of banana. *AtNPR1* transgene confers resistance to the bacterial disease Huanglongbing (HLB) in transgenic orange cultivars 'Hamlin' and 'Valencia' even after 3 years of planting in a hot spot of disease (Dutt *et al*., 2016).

2.9.5 Plant defense genes

The plant defense genes are known to enhance the hypersensitive response (HR) in plants upon infection with by bacterial and fungal pathogens through the release a protein elicitor. As elicitor targets a broad range of plant pathogens, defense genes may be a noble idea for developing resistance for both bacterial and fungal pathogens (Lin *et al.,* 1997). The *Capsicum annuum* L. ferredoxin-like amphipathic protein (PFLP) and hypersensitive response-assisting protein (HRAP) are plant proteins, which induces HR (Lin *et al.*, 1997; Chen *et al.*, 2000). PFLP also has iron depletion antibiotic action in addition to HR reaction. These *Pflp* and *Hrap* transgenes induce the hypersensitive responses in plants after infection from various bacterial pathogens like *Erwinia, Pseudomonas*, *Ralstonia* and *Xanthomonas* in plants including tobacco, tomato, broccoli, rice, orchids and potato (Tang *et al.*, 2001; Lu *et al.*, 2003; Huang *et al.*, 2004). The expression of the *Hrap* or *Pflp* gene in banana also resulted in high level of

resistance against Xcm (Tripathi *et al.,* 2010; Namukwaya *et al.,* 2012; Tripathi *et al.,* 2013 Tripathi *et al.,* 2014a; Tripathi *et al*., 2017).

2.9.6 Thaumatin-like protein gene

In the past various transgenes have been used to develop genetically modified banana and many conferred the significant levels of resistance to fungal pathogens. Transgenic banana with rice Thaumatin-like protein (TLP) or PR-5 gene showed significant resistance against *Fusarium oxysporum* (Mahdavi *et al*., 2011). TLP induce in transgenic rice because of pathogen infection, abiotic stresses, and developmental signals (Datta *et al*., 1999) showed enhanced resistance in a glass house challenge. Thaumatin-like proteins cause damage of fungal cell wall leading to inhibit fungal growth and multiplication (Tobias *et al*., 2007). The over-expression of defense-related genes engaged in the phenylpropanoid and salicylic acid (SA) pathway were highly induced in the Foc TR 4- resistant banana cultivar (Nongke No. 1) in comparison to the Foc TR 4- susceptible banana cultivar (Cavendish). It has been demonstrated that SA has an important role in plant-microbe interactions (Wang *et al*., 2015).

2.9.7 Anti-apoptosis related genes

The genetic modification of banana through plant cell death technology is a potential technology for providing resistance to several pathogens (Dickman *et al*., 2001; Lincoln *et al*., 2002; Li and Dickman, 2004; Paul *et al*., 2011; Magambo *et al*., 2016;). Transgenic banana harboring the anti-apoptosis-related gene shows resistance against Foc race 1 (Paul *et al*., 2011; Magambo *et al*., 2016).

There is always the risk that pathogens will find ways to overcome the mechanism of resistance used against them. To develop durable resistance in plants, it is important to identify broad-

spectrum resistance genes, which provide better options for control of diseases like Fusarium wilt and BXW. Currently, many genes isolated from various sources proven to be effective resistance to bacterial and fungal diseases are available. Plant genes are the first choice for genetic engineering of banana due to several ethical and biosafety related issues with animal genes and their products, therefore, transgenic plants with plant genes may be more acceptable for farmers, consumers and biosafety regulators.

2.10 Systemic acquired resistance pathways

Systemic acquired resistance (SAR) is innate immunity, which is development of resistance response triggered by pathogens after an initial infection (Ryals *et al.*, 1997; Durrant and Dong, 2004). It is important for plants to resist biotic and abiotic stresses. It can be induced by a wide range of pathogens therefore, SAR is considered broad spectrum. It activates several PR genes, and the induction of SAR is mainly through the accumulation of endogenous salicylic acid (SA). In addition to SA, Jasmonic acid (JA) and ethylene (ET) signaling molecules are also major players in the regulation of SAR pathways (Figure 2.5) that are involved in induced defense responses against pathogens (Pieterse and van Loon, 1999).

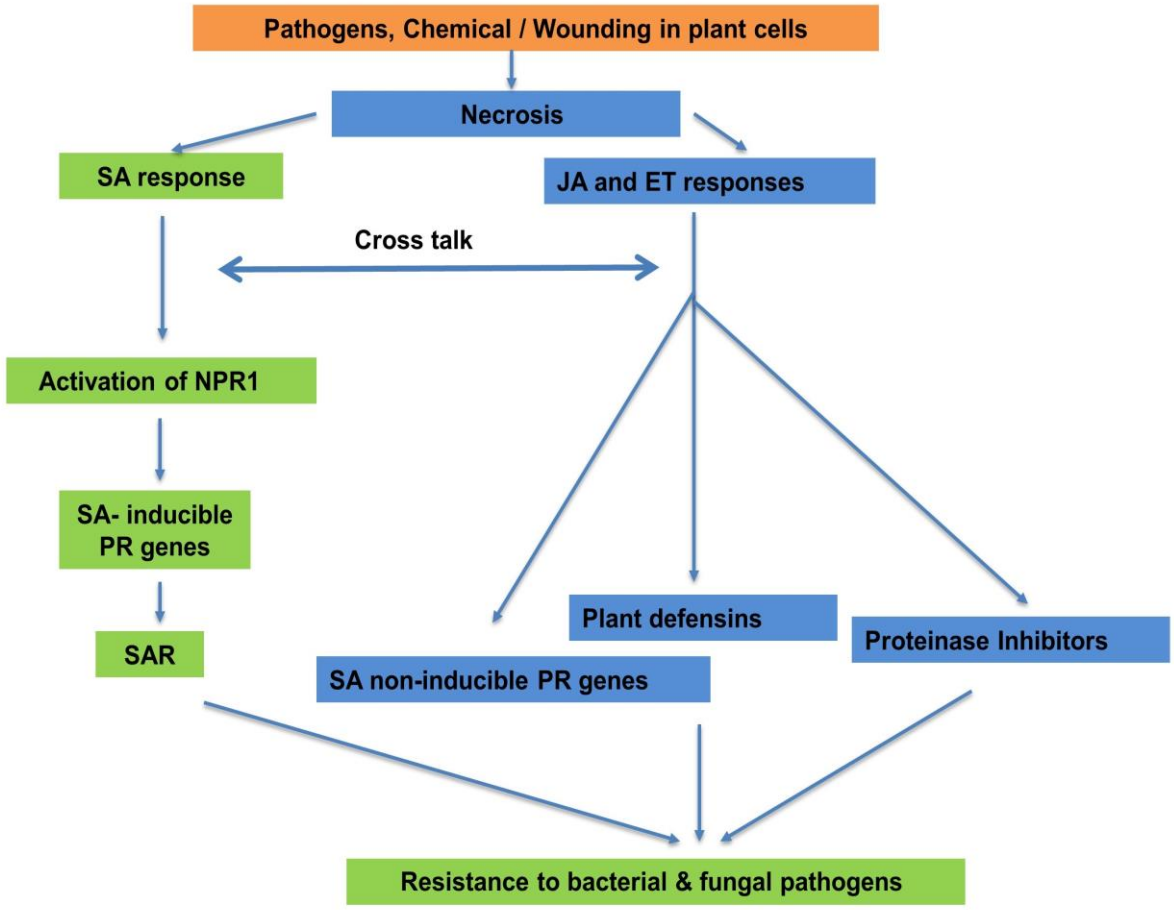


Figure 2.5: Simplified depiction of systemic acquired pathways (SAR) induced by signally compounds. Salicylic acid (SA), Jasmonic acid (JA) and Ethylene (ET) are induced by plant pathogens, various chemicals and wounding respectively (Pieterse and Loon, 1999).

The pathogen-induced SA and its methylated derivative activate a molecular signal transduction pathway involving NPR1 and NH1 in *Arabidopsis* and rice respectively (Cao *et al*., 1994; Chern *et al*., 2005; Jones and Dangl, 2006; Park *et al*., 2007).

2.10.1 Salicylic acid

Salicylic acid (SA) is a small phenolic signaling compound which is induced in plant cells by localized infection by pathogens (Clarke *et al*., 2000; Glazebrook, 2005). These compounds activate the plant defense machinery through NPR proteins which are found in cytoplasm as

oligomeric form (Kinkema *et al*., 2000). Upon activation, oligomeric form become monomeric and enter the nucleus. These monomeric NPR1 proteins bind to transcription factors which induce several pathogenesis related genes. Therefore, NPR1 protein works as co-activator of transcription factors and it is also known as master regulator of plant defense (Mou *et al*., 2003).

2.10.2 Jasmonic acid

Jasmonic acid (JA) is also a phytohormone that works during abiotic stresses and regulates several plant growth mechanisms (Glazebrook, 2005; Jones and Dangl, 2006). It plays a key role in plant defense pathway, which is not dependent on SA (Figure 2.5). Cross-talk between the SA-dependent and the SA-independent pathways provides several resistance mechanisms in varying combinations in plant defense system (Rojo *et al*., 1999***;*** Pieterse and van Loon, 1999). JA induces defense responses mainly due to necrotrophic infection using JA receptors to activate various transcription factors like MYB2, MYB3 and MYB4 (Browse, 2009; Fernandez-Calvo *et al*., 2011).

2.10.3 Ethylene

Ethylene (ET) is a phytohormone which is involved in a very complex pathway involving different feedback loops for synthesis of antimicrobial compounds and regulatory transcription factors (Figure 2.5) (Lamb and Dixon, 1997***;*** Denoux *et al*., 2008; Merchante *et al*., 2013). It also regulates many aspects of growth and development of plant life cycle like seed germination, root development, flower senescence, abscission, and fruit ripening (Johnson and Ecker,1998; Lumba *et al*.,2012). It was reported that wounding and elicitors such as systemin and JA, induces ethylene generation in tomato cell culture (O'Donnell *et al.,* 1996).

CHAPTER THREE

REGENERATION AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF EMBRYOGENIC CELLS OF VARIOUS BANANA CULTIVARS

3.1 Introduction

Banana and plantain is an important staple food and cash crop for East and Central Africa and major fruit crop globally. However, its production is severely affected by many pests and diseases. Therefore, there is an urgent requirement to develop diseases and pests resistance banana cultivars. The improved and disease resistance banana cultivars can be produced either through conventional breeding or genetic modification of banana genome. The banana breeding is difficult due to prolong life cycle of banana plant 16 to 18 months, restricted genetic variations, seedless sterile crop and different level of ploidy ranging from diploids to tetraploids and lack of disease or pest resistance in *Musa* germplasm (Silva *et al.*, 2001; Pillay and Tripathi, 2007; Lorenzen *et al*., 2010). Genetic transformation provides an alternative tool for improvement of banana. The efficient, robust and reproducible regeneration and transformation system are a basic requirement for production of transgenic plants (Hansen and Wright, 1999; Khanna *et al*., 2004; Tripathi *et al.*, 2012; Tripathi *et al*., 2015).

Transgenic plants can be generated using several techniques like *Agrobacterium*-mediated transformation (AMT), gene gun or biolistic particle bombardment method or electroporation. AMT is the most preferable method due to integration of a minimal copy number of the transgenes and the insertion of large pieces of transgenes (Lindsey, 1992; Gelvin, 2003). This is also very economical and cost saving method for genetic modification of any crop due to not requiring any expensive equipment like gene gun. Several techniques for AMT are tested and refined using various explants like embryogenic cells (Ganapathi *et al*., 2001; Khanna *et al*.,

2004; Kosky *et al.*, 2010; Tripathi *et al*., 2012, Tripathi *et al*., 2015) and apical meristematic tissues (May *et al*., 1995; Tripathi *et al*., 2005 and 2008a). Most preferred starting material for AMT of banana is embryogenic cells; but generation of cell cultures is difficult, time consuming, labor intensive and highly cultivar dependent. Only few cultivars respond to available protocol to generate embryogenic cells. Therefore, development of embryogenic cells (EC) and establishment of AMT system for each farmer-preferred cultivar are essential preconditions for genetic modification. Production and regeneration of EC of banana have been have been reported from leaves, corm sections, meristematic tissues, zygotic embryos and male flowers (Novak *et al*., 1989; Dheda *et al*., 1991; Marroquin *et al.*, 1993; Escalant *et al*., 1994; Côte *et al*., 1996; Navarro *et al*., 1997; Becker *et al*., 2000; Grapin *et al*., 2000; Strosse *et al*., 2006; Tripathi *et al*., 2012, Tripathi *et al*., 2015).

The availability of an AMT system for banana cultivars is essential to produce several transgenic events. Here, regeneration and transformation through EC of banana cultivars ‘Gros Michel’, ‘Cavendish Williams’ and ‘Sukali Ndiizi’ has been described. The EC of ‘Gros Michel’, ‘Cavendish Williams’ and ‘Sukali Ndiizi’ were developed and regenerated into complete well-rooted plants. This is the first study where both explants immature male flowers and highly proliferated multiple buds (scalps) were used to produce EC of banana. Application of these EC for regeneration and AMT system was studied to provide an optimized regeneration and transformation system to develop transgenic banana plants at regular basis by using gene of economically important traits.

3.2 Materials and methods

3.2.1 Plant materials

Inflorescence (male buds) of the farmer-preferred banana cultivars especially ‘Cavendish Williams’ (dessert, AAA), ‘Gros Michel’, (dessert, AAA), ‘Ngombe’ (AAA-EAHB) and ‘Sukali Ndiizi’ (AAB, known as apple banana) were received from field collection at KALRO, Thika. Tissue culture generated plantlets of ‘Mpologoma’ (AAA-EAHB), ‘Cavendish Williams’ and ‘Gros Michel’ were obtained from tissue culture laboratory of IITA-Nairobi. These plantlets were used to develop multiple buds also known as scalps.

3.2.2 Generation of multiple buds

The tissue culture plantlets of ‘Mpologoma’, ‘Cavendish Williams’ and ‘Gros Michel’ were micro-propagated and subcultured on Murashige and Skoog (MS) media containing with 6-Benzylaminopurine (6-BAP) 2.5 mg/L every 6-8 weekly interval as reported (Tripathi *et al.* 2012; Tripathi *et al*., 2015). Several small cremish-white buds were regenerated at the junction of pseudostem and roots of each small shoot. These buds were transferred to multiple bud induction medium (MBI, Table 3.1) at 26 ± 2 ^{o}C in the complete dark. The induced multiple buds were subcultured regularly on new MBI medium at 4 –5 weekly intervals until densely packed white small buds appeared. Good quality of multiple buds was induced and proliferated with monthly regular subculture in 3 to 4 months depending on quality of starting explants. These multiple buds were observed for presence of any contamination regularly and contaminated multiple buds were discarded.

Table 3.1: Composition of various media used in the banana transformation experiments

Medium	Composition
Proliferation medium (PM)	MS salts and vitamins, myo-inositol (100 mg/L), ascorbic acid (100 mg/L), BAP (2.5 mg/L), sucrose (30 g/L) and gelrite (3 g/L), pH 5.8
Multiple bud induction (MBI)	MS salts and vitamins, myo-inositol (100 mg/L), sucrose (30 g/L), ascorbic acid (100 mg/L), BAP (24 mg/L) and gelrite (3 g/L), pH 5.8
Callus induction medium for multiple buds (CIM1)	MS salts and vitamins, myo-inositol (100 mg/L), ascorbic acid (10 mg/L), 2,4-D (1 mg/L), zeatin (0.2 mg/L), sucrose (30 g/L) and gelrite (3 g/L), pH 5.8
Callus induction medium for immature flowers (CIM2)	MS salts and vitamins, biotin (1 mg/L), IAA (1 mg/L), 2, 4-D (4 mg/L), NAA (1 mg/L), sucrose (30 g/L) and gelrite (3 g/L), pH 5.8
Embryo development medium (EDM)	SH salts, MS vitamins, glutamine (100 mg/L) malt extract (100 mg/L), proline (230 mg/L), lactose (10 g/L), zeatin (0.05 mg/L), kinetin (0.1 mg/L), NAA (0.2 mg/L), 2iP (0.2 mg/L), sucrose (45 g/L) and gelrite (3 g/L), pH 5.8
Embryo maturation medium (EMM)	MS salts and vitamins, myo-inositol (100 mg/L), ascorbic acid (100 mg/L), sucrose (30 g/L) and gelrite (3 g/L), pH 5.8
Germination medium (GM)	MS salts, Morel vitamins, IAA (2 mg/L), BAP (0.5 mg/L), sucrose (30 g/L), and gelrite (3 g/L), pH 5.8
Rooting medium (RM)	MS salts and vitamins, myo-inositol (100 mg/L), ascorbic acid (10 mg/L), IBA (1 mg/L), sucrose (30 g/L) and gelrite (3 g/L), pH 5.8
Yeast extract broth (YEB)	Yeast extract (0.1 %), beef extract (0.5 %), peptone (0.5 %), sucrose (0.5 %) and MgSO4 (0.04 %)
Bacterial culture resuspension medium (BRM)	MS salts and vitamins, biotin (1 mg/L), malt extract (100 mg/L), glutamine (100 mg/L), proline (230 mg/L), ascorbic acid (40 mg/L), PVP10 (5 g/L), cysteine (200 mg/l), IAA (1 mg/L), NAA (1 mg/L), 2,4-D (4 mg/L), acetosyringone (200 µM) and sucrose (85.5 g/L), pH 5.3

(Murashige and Skoog, 1962; Morel and Wetmore1951; Schenk and Hildebrandt, 1972)
Source: Tripathi *et al.*, 2015

3.2.3 Sterilization and isolation of immature male flowers

Male buds had been collected from the field grown banana plants within a month period after emerging of complete fruiting bunches of banana cultivars ('Cavendish Williams', 'Gros Michel', and 'Ngombe' and 'Sukali Ndiizi'). The outer upper most part (2/3) of the inflorescence was dissected and removed, the inner part of the bud 3-4 cm which stored highly immature male flowers was surface sterilized by dipping into absolute ethanol (70 % v/v, Sigma Aldrich) for 2-5 min maximum. The isolated inner part of the male bud was rinsed in autoclaved distilled water 3 times at interval of 5 min. Finally, bud was cut into 2 to 3 cm in length by removing bract one by one using fine scalpel blade under laminar hood to avoid any contamination.

3.2.4 Callus induction using multiple buds

Creamish-white small thin cross section of multiple buds was dissected by fine scalpel blade and cultured on Callus Induction Medium (CIM1) for 3-5 months and incubated at 26 ± 2 ^{o}C in complete dark chamber covered by thick black coloured cloth (CIM1, Table 3.1) for initiation of embryogenic calli as reported previously (Tripathi *et al*., 2012; Tripathi *et al*., 2015). Three hundred pieces of multiple buds of each banana cultivars were isolated and incubated in complete dark on CIM1 in each experiment. About nine hundred pieces of multiple buds were cultured in total in three experiments on CIM1. The cultures were incubated in the dark till friable calli initiated without transferring to fresh media. These cultures were observed regularly by naked eye as well as under microscope for initiation and development of friable embryogenesis. Cultures were also monitored for any bacterial or fungal contaminations. Contaminated cultured were carefully discarded after autoclaving.

3.2.5 Callus induction using immature male flowers

From surface sterilized male bud, immature male flowers were dissected under Labomed stereomicroscope (Model Luxeo 4D from Fremont, California, USA) and transferred to Callus Induction Medium (CIM2). Approximately 6-9 highly immature male flowers were cultured per 90 mm petri dish containing 25 ml of CIM2 medium and total of three hundred immature male flowers were cultured for callus induction in total in three experiments for each banana cultivar. The petri dish containing cultures were incubated at 26 ± 2 ^{o}C in the dark chamber until initiation of friable embryogenic calli without changing any medium. The cultures were monitored for 3-5 months under stereomicroscope regularly for progression of friable embryogenic calli.

3.2.6 Production of embryogenic cells

Creamish-white friable embryogenic calli of each cultivar were recognized and isolated from group of calli under stereo-microscope and cultured into a 25 ml Erlenmeyer flask (conical flask) containing Liquid Callus Induction Medium (LCIM1 or LCIM2 depending upon the type of explants either multiple buds or male flower). At first, minimal volume of medium about 5 ml was used for culture initiation in 25 ml Erlenmeyer flask for one week and then medium was added up to 10 ml in 3 weeks. On the 4th week, fine granular cells were cultured into a new 25 ml Erlenmeyer flask. After 8 weeks of sub-culture, fine white granular cells were transferred into 250 ml Erlenmeyer flask containing 30 to 50 ml medium depending on concentration of embryogenic cells for proliferation and the big nodular and dead clumps were discarded by using sterile forceps. Culture was incubated in complete dark chamber on rotatory shaker at 90 rpm for fast multiplication and maintenance. Every 7–14 days, these embryogenic cells were washed and new medium (LCIM1 or LCIM2) was added after removing 80 % of old medium. This step was repeated regularly in 7–14 days depending on quantity and quality and

rate of multiplication of embryogenic cells (Tripathi *et al*., 2012; Tripathi *et al.*, 2015). Large quantity of proliferating EC cultures need frequent changing and adding of new medium.

3.2.7 Assessment of regeneration capacity of embryogenic cells

The concentration of newly developed rapidly proliferating embryogenic cells (EC) was maintained to 3-5 % settled cell volume (SCV) with LCIM1 or LCIM2 (depending on the explant) and incubated in dark chamber for 3 to 4 days on orbital shaker at 90 rpm to refresh the cells in new culture medium. The diluted EC (about 1 ml of SCV) were plated on sterile nylon mesh using 1 ml autoclaved tip with pippete and dried on sterile paper towel and incubated on Embryo Development Medium (EDM, Table 3.1) in 90 mm Petri dishes for 2 months in complete dark chamber at 28 °C. Medium was changed every 2 weeks interval. After two months, white tiny embryos were cultured on Embryo Maturation Medium (EMM), which was hormone free medium, for 1 month in dark. Later, these embryos were cultured on Embryos Regeneration medium (ERM) for shoot development for 1-2 months in dark. The small shoots were cultured in proliferation medium in light as reported (Tripathi *et al*., 2012; Tripathi *et al*., 2015). Number of plantlets generated per ml SCV of EC of each cultivar was recorded as regeneration efficiency. The regeneration experiments were performed three times with different lines of EC to check the reproducibility of the systems.

3.2.8 Binary vector and *Agrobacterium* strain

The plant binary vector pCAMBIA2301 having β-*glucuronidase* (*gus*A) as a reporter gene and the *neomycin phosphotransferase* (*npt*II or kanamycin-resistance) as the selectable marker gene, driven by Cauliflower Mosaic Virus 35S constitutive promotor and NOS terminator, was used for *Agrobacterium*-mediated transformation (AMT) experiments (Figure 3.1). The *gusA* gene was from *E. coli* and contained intron to confirm that expression of *glucuronidase* activity

is derived from higher plant cells, due to presence of catalase intron it was not expressed in surviving *Agrobacterium* cells during transformation.

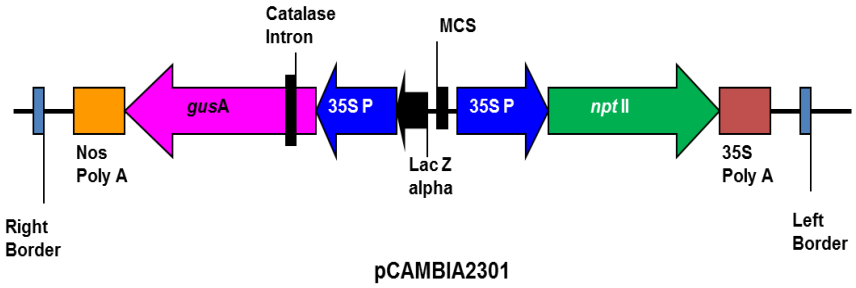


Figure 3.1: Map of binary vector pCAMBIA2301 containing the *neomycin phosphotransferase* (*npt*II) gene as the selectable marker, β-*glucuronidase* (*gus*A) gene as a reporter gene, Cauliflower Mosaic Virus 35S promotor and NOS as terminator.

Agrobacterium tumefaciens strain EHA 105 containing pCAMBIA2301 was harvested in LB broth medium with selective antibiotics (rifampicin 50 mg/L and kanamycin 50 mg/L) for 48 h at 28 °C with shaking at 220 rpm. The culture was used for AMT as reported by Tripathi *et al*. (2012). Bacterial culture was refreshed by growing over-night at similar conditions. The density of bacterial culture was adjusted to 0.5-0.8 with adding bacterial culture resuspension (BRM) medium supplemented with acetosyringone (200 µm).

3.2.9 *Agrobacterium*-mediated transformation and regeneration of transgenic events

Embryogenic cells (EC) of the 'Cavendish Williams', 'Gros Michel' and 'Sukali Ndiizi' were co-cultivated using refreshed *Agrobacterium* culture EHA105 harboring pCAMBIA2301 for 45 min at orbital shaker with slow speed approximately 30 to 45 rpm. To enhance the transformation efficiency, EC and bacterial mixtures were centrifuged for 10 min at 1000 rpm twice. After co-cultivation, EC was plated on semi-solid BRM medium in petri-dishes and incubated at 22 °C oven for 72 hours. Excess *Agrobacterium* culture was removed by washing of EC with liquid CIM containing cefotaxime (300 mg/L) thrice. *Agro*-infected EC was spread

on mesh (size 4 cm^2) and excess media was dried on autoclaved and oven dried paper towel. These meshes with *Agro*-infected EC were cultured on 90 mm petri-dishes containing semi-solid EDM with cefotaxime (300 mg/L) incubated in complete dark at 28 °C. After a week, transformed EC was transferred to selective medium containing kanamycin (100 mg/L) and cefotaxime (300 mg/L) (Tripathi *et al.,* 2012; Tripathi *et al*., 2015). EDM media were changed every 15 days for 8-12 weeks. Further transformed embryos were transferred to EMM for 4 weeks. Matured embryos were transferred to ERM for 4-8 weeks. Small plantlets regenerated on ERM were cultured to proliferation medium (PM, Table 3.1) for micro-propagation using 6-8 weekly subculture on new medium. Efficiency of genetic transformation was estimated as number of kanamycin resistant and PCR positive transgenic events developed per 1 ml SCV of EC of each banana cultivar (Table 3.2). Experiments were performed thrice to check the reproducibility of the transformation system. Rooted transgenic and non-transgenic control plantlets were weaned in sterile soil in small deposable cup in humid chamber. After 6 weeks, these plants were transferred to big plastic pots in glasshouse. Plants were watered manually every alternate day. Plant morphology was monitored regularly for check any abnormality.

3.2.10 Histochemical GUS assay

Transient GUS assay was performed with embryogenic cells (EC) after 48 hours of co-cultivation and stable GUS assay with kanamycin resistant randomly selected transgenic shoots as mentioned (Jefferson, 1987; Tripathi *et al*., 2012, Tripathi *et al.*, 2015). GUS substrate solution was used to performed GUS assay (5-Bromo-4-chloro-3-indolyl-β -D-glucuronic acid (1 %), potassium-ferricyanide (50 mM), potassium-ferrocyanide (50 mM), NaOH buffer (1M) (pH 7.0), Na-EDTA buffer (100 mM) (pH 8.0), and Trition-X100 (1 %). Chlorophyll was removed with leaves tissue by using 50 % ethanol. Transient expression of *gus*A gene was inspected in *Agro*-infected EC after 12 hours of GUS assay, however stable expression of the

*gus*A gene was detected after 24 h of GUS assay with leaves, petioles, pseudostem and roots. Non-transformed EC, leaf and roots of non-transgenic *in-vitro* plants were also included in experiment. Experiments were repeated three times to check transformation efficiency and reproducibility of AMT system. SMZ1500 stereomicroscope (Carlsbad, CA, USA) attached with high zoom Nikon camera was used to capture all the microscopic pictures.

3.2.11 Polymerase chain reaction analysis of transgenic events

Plant genomic DNA was extracted from 100 mg freshly detached leaves tissue stored in liquid nitrogen from transgenic plants and non-transgenic control plant using a DNeasy kit (Qiagen) Quantity and quality of DNA was checked by Nanodrop2000 and gel electrophoresis. Polymerase chain reaction (PCR) was done using *gus*A gene specific primers, F: 5′-TTTAACTATGCCGGGATCCATCGC -3′ and R: 5′-CCAGTCGAGCATCTCTTCAGCGTA -3′. PCR was performed in a total volume of 25 µl each reaction, containing 50 ng gDNA, 2.5 µl 10X PCR buffer with 15 mM $MgCl_2$, 0.5 µl of 10 mM dNTP, 0.5 µl of 10 µM of each primer, 0.2 µl of HotStar*Taq* DNA polymerase (Qiagen, Germany). The PCR program were: initial denaturation at 95 ^oC for 5 min, 35 cycles of denaturation at 94 ^oC for 15 s, annealing at 62 ^oC for 45 s, extension at 72 ^oC for 50 s and final extension at 72 ^oC for 7 min and final storage at 4 ^oC. The amplified PCR products were resolved by electrophoresis on 0.8 % (w/v) agarose gel containing GelRed™ (Biotium) fluorescent DNA stains (1:10000 ratio). Ten-fold diluted plasmid DNA as a positive and non-transgenic control plant DNA as a negative control were included in each PCR experiment. 1 Kb plus DNA ladder (Thermo Fisher Scientific) was used to determine the size of amplified product. All the gel pictures were taken by gel documentation system attached with camera and computer.

3.2.12 Dot blot and Southern blot analysis

Dot blot and Southern blot hybridization was performed to confirm the integration of *gus*A gene into the genome of transformed banana. Genomic DNA (gDNA) for dot blot analysis was extracted from randomly selected PCR positive transgenic events using a Plant DNeasy kits (Qiagen, GmbH, Germany). 200 ng of gDNA with 0.4 M NaOH in triplicates of each transgenic line was denatured for 10 min at 98 °C on Applied Biosystems Thermocycler 9700 followed by promptly chilled on ice for 5 min and transferred on a positively charged nylon membrane (Roche Applied Sciences, Mannheim, Germany) using a Biorad dot blotter micro-filtration equipment following the manufacturer's guidelines. To fix the transfer gDNA on membrane UV cross-linker was used. A *gusA* gene specific probe was labelled with DIG-dUTP using PCR DIG probe synthesis kits (Roche Applied Sciences, Mannheim, Germany). Pre-hybridization, hybridization, stringency washes and detection were carried out using a DIG Luminescent Detection Kit for nucleic acid (Roche Diagnostics, UK) according to the manufacturer's instructions.

For Southern blot large scale plant gDNA was extracted from leaves tissue (1-2 gm) of transgenic and control non-transgenic plantlets using cetyltrimethylammonium bromide (CTAB) method with some modifications (Gawel and Jarret, 1991). About 20 µg of gDNA of transgenic events and non-transgenic control plant were restricted using enzyme *Hin*dIII (New England Biolabs, USA). Plasmid DNA was also restricted to use as a positive control. Restricted DNA was resolved on 1 % agarose gel containing 1 µl GelRed™ fluorescent DNA stains (1:10000 ratio) for 5 hours at 50 Volts. Gel was treated by depurination solution for 15 min, denaturation buffer for 45 min and neutralization buffer for 45 min. Restricted DNA was transferred on positively charged membrane by capillary action for 16 hours. Plant DNA was fixed on membrane by UV cross linker. After that prehybridization was performed for 2 h at

42 °C in hybridization oven and followed by hybridization for overnight. Washing of membrane and detection of signals was carried out as described in DIG manual.

3.2.13 Statistical analysis

All the transformations and regeneration experiments were performed three times and data were evaluated using Minitab 14. The means and standard deviation were calculated for regeneration and transformation experiments. One-way analysis of variance (ANOVA) was performed and interactions between means were separated by least significant difference (LSD) at $P < 0.05$.

3.3 Results

3.3.1 Development of embryogenic cells

Banana cultivars mainly 'Ngombe', 'Sukali Ndiizi', 'Gros Michel' and 'Cavendish Williams' were selected to isolate immature and smallest male flowers, whereas 'Mpologoma', 'Gros Michel' and 'Cavendish Williams' were selected to induce fine multiple buds without having any leafy tissue (Figure 3.2 and Table 3.2). White creamish friable calli were induced from immature male flowers of 'Ngombe', 'Sukali Ndiizi', 'Gros Michel' and 'Cavendish Williams', within 4-6 months post culture (Figure 3.2). 850 incubated explants of 'Cavendish Williams' developed calli which were creamish-yellowish in colour and non-embryogenic but 30 calli turned into ideal friable embryogenic calli. For 'Gros Michel', out of 630 calli, only 22 calli developed into EC. Similarly, with 'Sukali Ndiizi', out of 45 calli, only 3 turned in to EC. Fine granular and creamish white embryogenic cells were recovered after 1–2 months from 'Sukali Ndiizi' cultivar after transferring of the ideal friable embryogenic calli to LCIM2 (Figure 3.3). The volume of these granular EC increased gradually, and good quality cell

culture was recovered within 4 months of culture in LCIM2 by regular subculturing and adding new LCIM2.

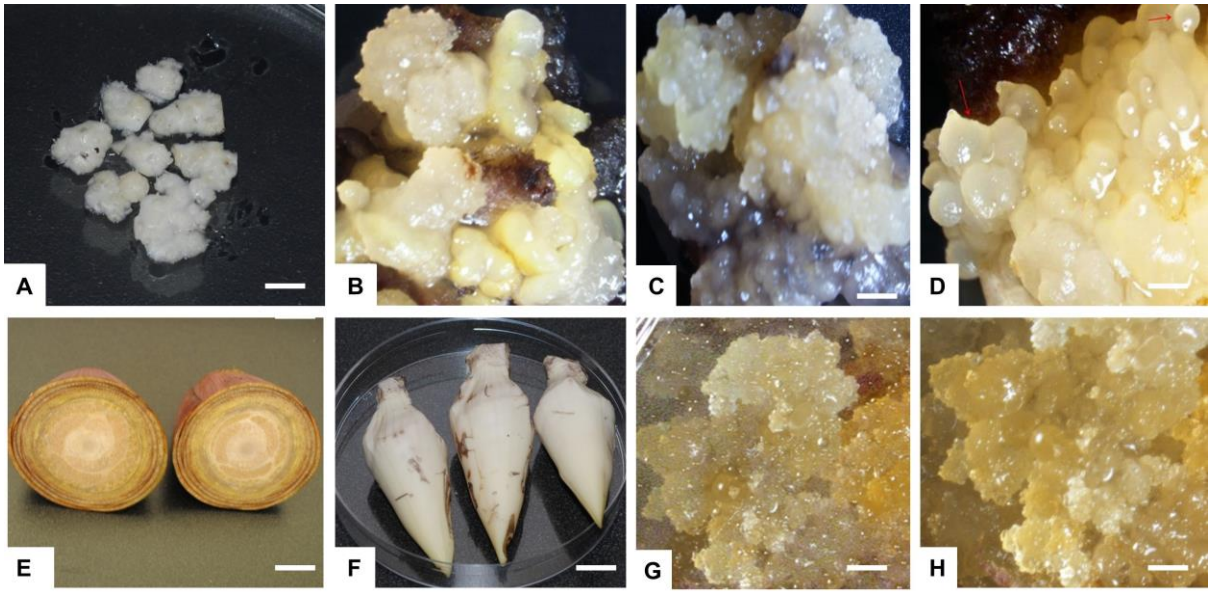


Figure 3.2: Induction of embryogenic calli through multiple buds and immature male flowers of banana cultivars. **A)** Multiple buds, **B)** Embryogenic calli of 'Cavendish Williams', **C**) 'Gros Michel', **D)** 'Mpologoma', **E and F)** Banana inflorescences, **G**) Embryogenic calli of 'Sukali Ndiizi' and (**H**) 'Ngombe'. Scale bar 1cm in **A** and **E**, and 100 µ in **B, C, D, G and H**. Source -Tripathi *et al*., 2015.

Immature male flowers of 'Ngombe', 'Gros Michel' and 'Cavendish Williams' did not produce any embryogenic cell. It was observed that calli was induced from immature flowers of these cultivars, but none were ideal embryogenic calli to generate embryogenic cells.

For cultivars 'Mpologoma', 'Gros Michel' and 'Cavendish Williams', friable embryogenic calli were obtained in 3-5 months of culture of multiple buds in CIM1 (Figure 3.2). When the calli were transferred to LCIM1, fine EC developed in about 1-2 months. Within 4 months of regular subculture of EC of 'Gros Michel' and 'Cavendish Williams' in LCIM1, uniform EC were obtained (Figure 3.3). No EC was obtained for 'Mpologoma' after transferring the friable

calli to LCIM1 as the calli turned black due to excess secretion of phenolic compounds in liquid medium.

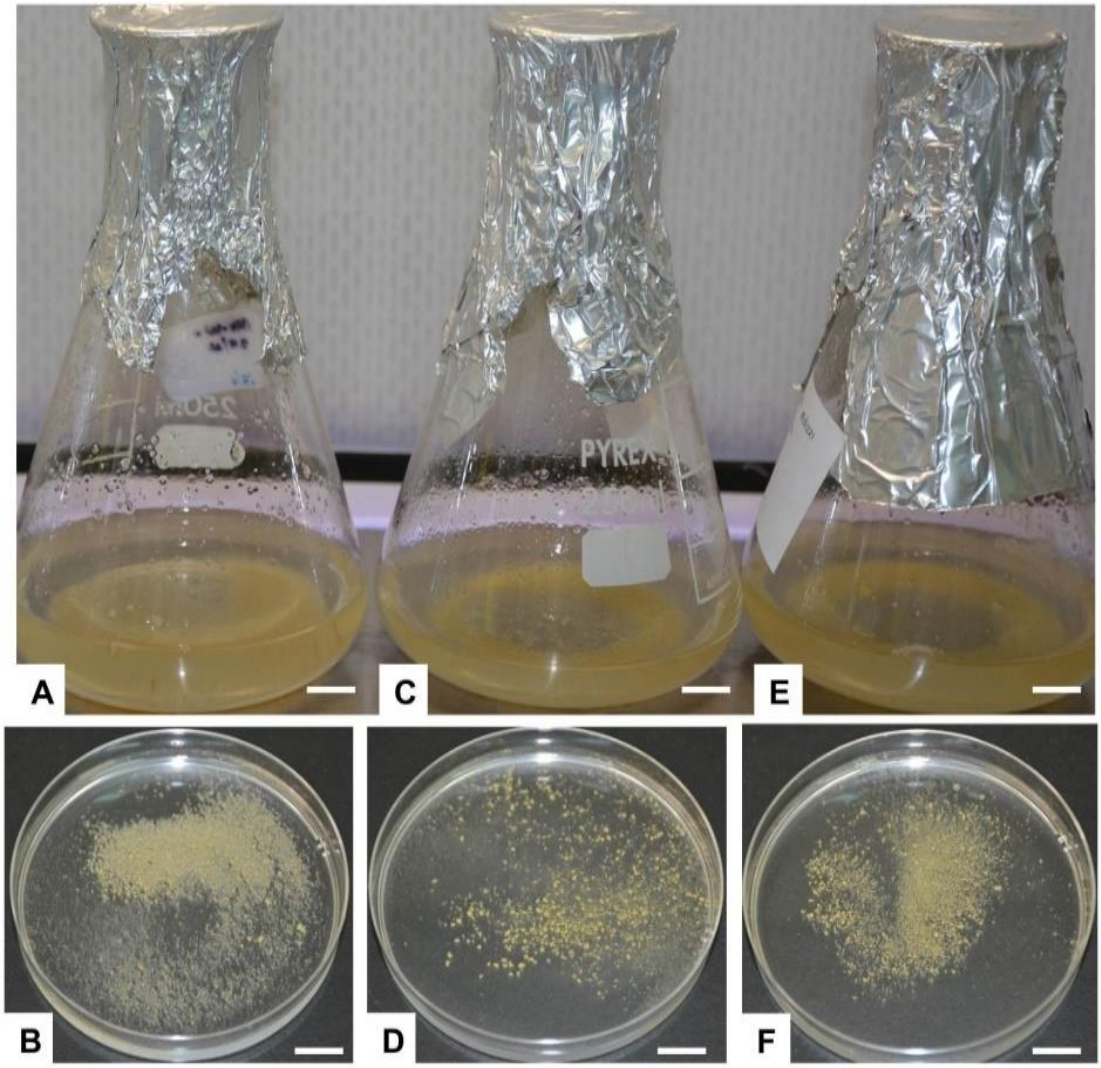


Figure 3.3: Embryogenic cells of banana cultivars. **A)** 'Cavendish Williams' in liquid, **B)** 'Cavendish Williams' on plate, **C)** 'Gros Michel in liquid, **D)** 'Gros Michel' on plate, **E)** 'Sukali Ndiizi' in liquid, **F)** 'Sukali Ndiizi' on plate. Scale bar 1 cm. Source -Tripathi *et al.,* 2015.

Table 3.2 Banana cultivars used for production of friable calli and embryogenic cells

Banana cultivars	**Explants (multiple buds/ male flowers)**	**No. of explants used**	**No. of explant that developed calli**	**No. of callus that developed embryogenic cells***	**Remarks**
'Cavendish Williams' (genome AAA)	multiple buds	900	850 (94%)	30 (3.5%)	Embryogenic cells developed.
'Cavendish Williams' (genome AAA)	male flowers	300	36 (12%)	All calli turned black.	-
'Gros Michel' (genome AAA)	multiple buds	900	630 (70%)	22 (3.4%)	Embryogenic cells developed.
'Gros Michel' (genome AAA)	male flowers	300	30 (10%)	All calli turned black.	-
'Mpologoma' (genome-EAHB)	multiple buds	900	432 (48%)	All calli turned black.	-
'Sukali Ndiizi' (genome- AAB)	male flowers	300	45 (15%)	3 (6.6%)	Embryogenic cells developed.
'Ngombe' (AAA-EAHB)	male flowers	300	26 (8.6%)	All calli turned black.	-

* Only microscopically selected friable creamish yellow and translucent calli were used to develop embryogenic cell suspension. Source- Tripathi *et al.*, 2015.

The development of EC was found to be cultivar dependent. The embryogenic cells were developed using either highly immature male flowers or fine sections of multiple buds (scalp) depending upon cultivar used. The cells were maintained for 12-18 months by regular subculturing every 7-14 days. The quantity of EC increased 2-3 folds every 2 weeks, but the regeneration ability of EC decreased with time and, they are very prone to contamination due to regular changing of media.

3.3.2 Assessment of regeneration capacity of embryogenic cells

The regeneration of fine, uniform EC (8-12 weeks old) of banana cultivars 'Sukali Ndiizi', 'Gros Michel' and 'Cavendish Williams' was investigated for generation into complete plantlets and their regeneration efficiencies were estimated (Figure 3.4 and 3.5). After transferring the EC from liquid callus induction medium (LCIM) to EDM (Table 3.1), tiny white embryos developed within 6-8 weeks. These embryos were then cultured on EMM (Table 3.1) for maturation for 4 weeks. Mature white embryos were further transferred to EGM (Table 3.1) for shoot regeneration. These embryos started germinating into small white shoots with tiny hairy roots in 4-8 weeks. These shoots were further cultured on proliferation medium for development of complete plantlets (Figure 3.4). The cultures were moved to light with photoperiod of 16 h in light and 8 h in dark cycle, and after 2 weeks of incubation in light, the shoots turned green with small roots. It was observed that regeneration of complete banana plantlets from these embryogenic cells was very similar not depending upon the type of plant tissues (male flowers or multiple buds) as source of development of cells.

The fully developed shoots were cultured on rooting medium where they produced well-developed roots in 3- 4 weeks. Most of the rooted plantlets were successfully weaned in humid chamber and later acclimatized in big pots in glasshouse. All the potted plants were

morphologically similar with no abnormalities were observed. The regeneration of complete plantlets from EC took about 13-15 months.

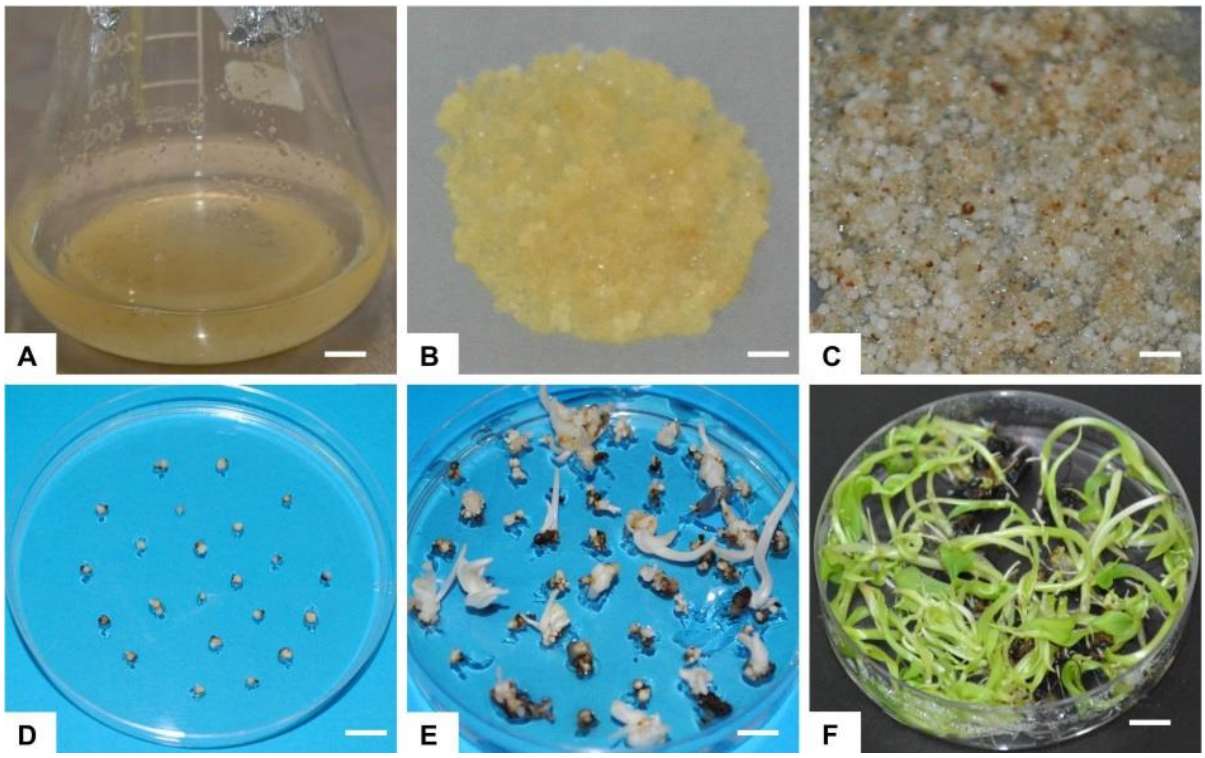


Figure 3.4: Regeneration of embryogenic cells. **A)** Embryogenic cells **B)** Embryogenic cells plated on nylon mesh cultured on embryo development media, **C)** Regenerating white embryos on embryo development media, **D)** Embryos on embryo maturation media, **E)** Embryos on germination media, **F)** Regenerated shoots on proliferation media. Scale bar 1 cm. Source - Tripathi *et al.*, 2015.

20,000-50,000 shoots were produced from 1 ml settle cell volume (SCV) of EC (Figure 3.5). The EC of 'Sukali Ndiizi' produced approximately 50,000 plantlets per ml SCV, however 'Gros Michel' produced only about 20,000 shoots per ml SCV. EC of 'Cavendish Williams' produced about 30000-40000 plantlets per ml SCV. This indicated that regeneration efficiency in banana is highly based on cultivar. About 3-5 % of somaclonal abnormalities such as retarded growth of plants, mosaic and abnormal leaves were observed in cultivar 'Cavendish Williams' under glasshouse conditions.

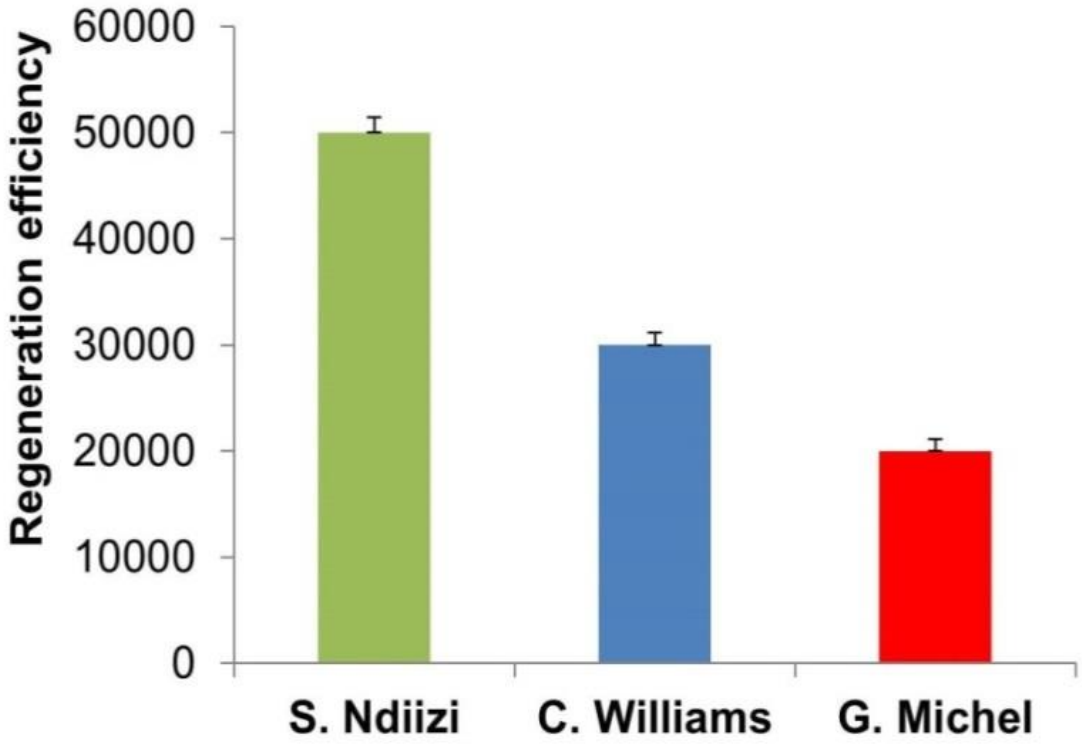


Figure 3.5: Regeneration efficiency of embryogenic cells of banana cultivars. Regeneration efficiency as number of shoots regenerated from 1 ml of settled cell volume of embryogenic cells. Values were presented mean ± s.e. Source - Tripathi *et al.,* 2015.

3.3.3 *Agrobacterium*-mediated transformation and regeneration of transgenic events

EC of 'Sukali Ndiizi', 'Gros Michel' and 'Cavendish Williams' were co-cultivated with virulent *Agrobacterium tumefaciens* strain EHA 105 harboring the plasmid pCAMBIA2301. Transformed EC proliferated on selective EDM containing kanamycin (100 mg/L), however the control non-transformed EC turned brown and black. Further on this selective medium, embryos were generated from transformed EC in 4-8 weeks (Figure 3.6A). These transformed embryos became big in 4 weeks after transferring on EMM (Figure 3.6B). The big globular embryos were picked with the help of forceps and transferred to EGM for germination. The putative transformed shoots started developing within 8 weeks (Figure 3.6C). The small shoots which were initially white with few leaves were cultured on proliferation medium (PM) and

shifted to light at 16/8 h cycle (Figure 3.6D and 3.6E). Fully developed green plantlets of ‘Sukali Ndiizi’ and ‘Cavendish Williams’ produced roots upon transferring to rooting medium within 3 to 4 weeks. It was observed that regeneration and development of shoots for ‘Gros Michel’ cultivar was very problematic. Shoots cultured on proliferation medium supplemented with 6-BAP turned into complete nodular structures instead of proper shoots with leaves. To solve this problem the shoots germinated on EGM were cultured into hormone free medium without 6-BAP for 4 weeks and then transferred to proliferation medium. This adjustment in proliferation medium provided complete plantlet from ‘Gros Michel’.

Figure 3.6: *Agrobacterium*-mediated transformation of embryogenic cells. **A)** *Agro*-infected cells proliferating on selection media, **B)** Embryos on embryo development media, **C)** Embryos on embryo maturation media, **D)** Shoots germination on embryo germination medium (scale bar 1 cm), **E)** Complete shoots in proliferation media, **F)** Potted transgenic plants in the glasshouse. Scale bar 10 cm. Source- Tripathi *et al.*, 2015.

Regeneration of transformed EC on selective medium containing kanamycin produced about 20-70 transgenic events (depending upon cultivar used) / ml SCV in 7-9 months after co-cultivation with *Agrobacterium*. Transformed EC of 'Sukali Ndiizi' provided the highest number of transgenic events (60-70) / ml SCV, followed by 'Cavendish Williams' with approximately 30-40 transgenic events /ml SCV and then 'Gros Michel' with only 20-30 transgenic events/ ml SCV (Figure 3.7).

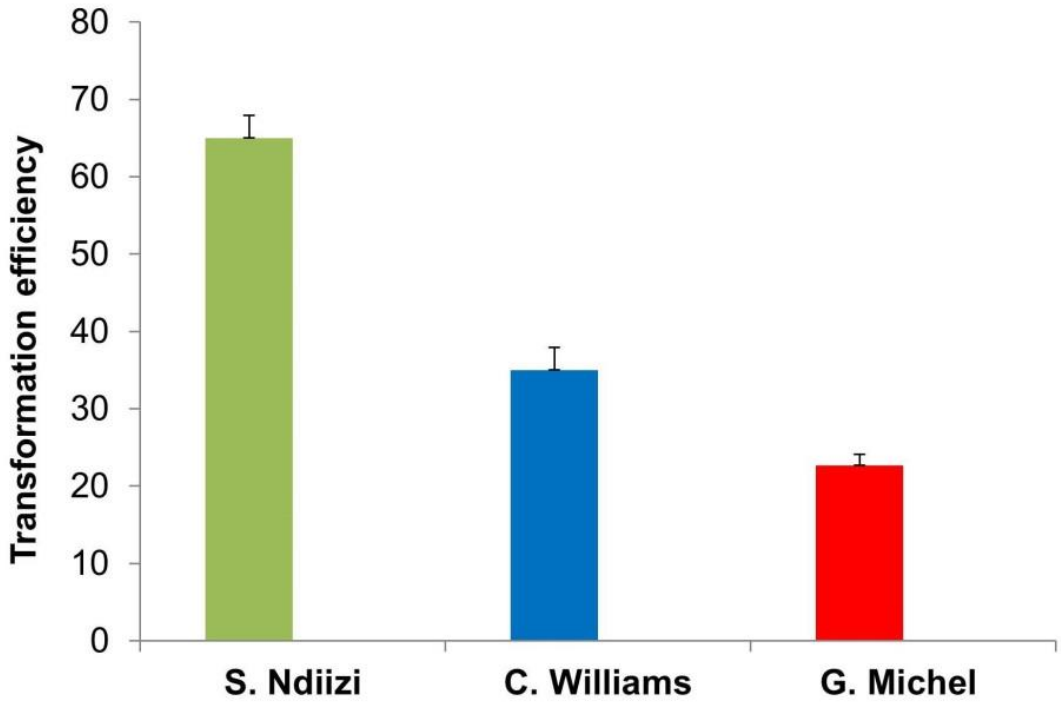


Figure 3.7: *Agrobacterium*-mediated transformation efficiency of banana *cultivars* using embryogenic cells. Transformation efficiency calculated as number of transgenic events generated from 1 ml of settled cell volume of embryogenic cells. Values were presented mean ± s.e. Source- Tripathi *et al.*, 2015.

To confirm the robustness and reproducibility of the generation of transgenic events, the transformation experiments were performed thrice using different cell events of embryogenic cells. The complete transgenic plantlets with proper roots were planted into plastic disposable cup containing sterile soil and kept in humid chamber. About 96-100 % plants were successfully grown as indicated by development of new leaves (Figure 3.6F). After six weeks

these plants were transferred to bigger pot with sterile soil. All the transgenic events were morphologically like control non-transgenic plants. The same protocol was used for transformation and complete plant regeneration from EC developed from both types of explants either male flower or multiple buds. A stepwise protocol for transformation and regeneration is described in flow diagram (Figure 3.8).

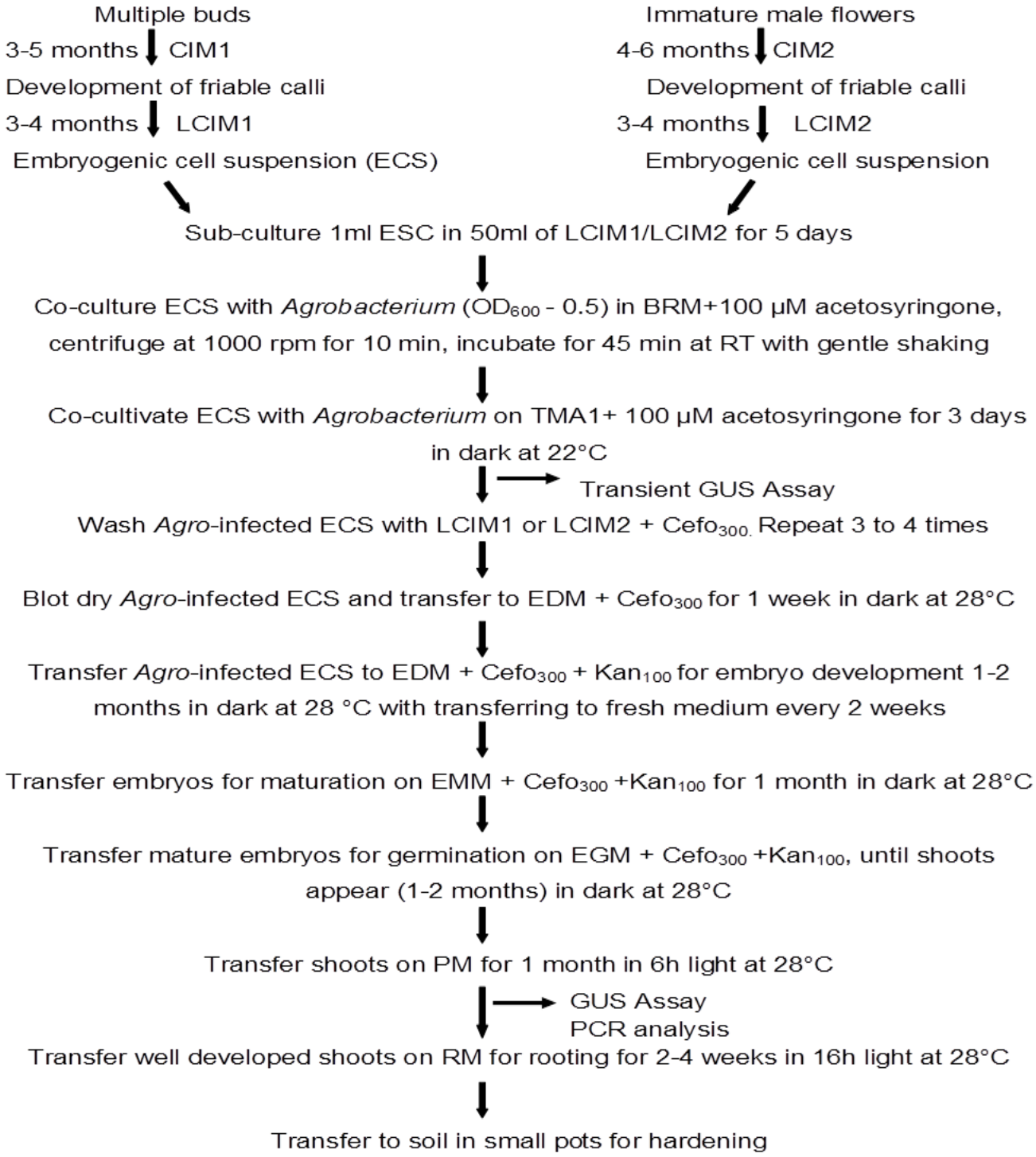


Figure 3.8: Flow diagram showing several stages of *Agrobacterium*-mediated genetic transformation of banana using embryogenic cells (EC) derived from multiple buds or immature male flowers. CIM- callus induction medium, LCIM-liquid callus induction medium, BRM- *Agrobacterium* resuspension medium, EDM- embryo development medium, EMM- embryo maturation medium, EGM- embryo germination medium, RM- rooting medium, $Cefo_{300}$- cefotaxime (300 mg/L), Kan_{100}- Kanamycin (100 mg/L). Source − Tripathi *et al*., 2015.

3.3.4 Histochemical GUS Assay

Transient expression of the *gus*A gene was checked with EC after 72 hours of co-cultivation with *Agrobacterium* and blue coloration was observed (Figure 3.9A). The stable expression of the *gus*A gene was confirmed in germinating shoots, pseudostems, leaves, petioles and roots of transgenic plantlets (Figure 3.9B-F). Blue coloration of GUS staining was seen in all the transformed tissues tested. The blue coloration in various tissues of transgenic events showed uniform presence of the protein. GUS activity was not detected in non-transformed control EC (Figure 3.9G), and in leaves and roots of NTC plantlets (Figure 3.9H-I).

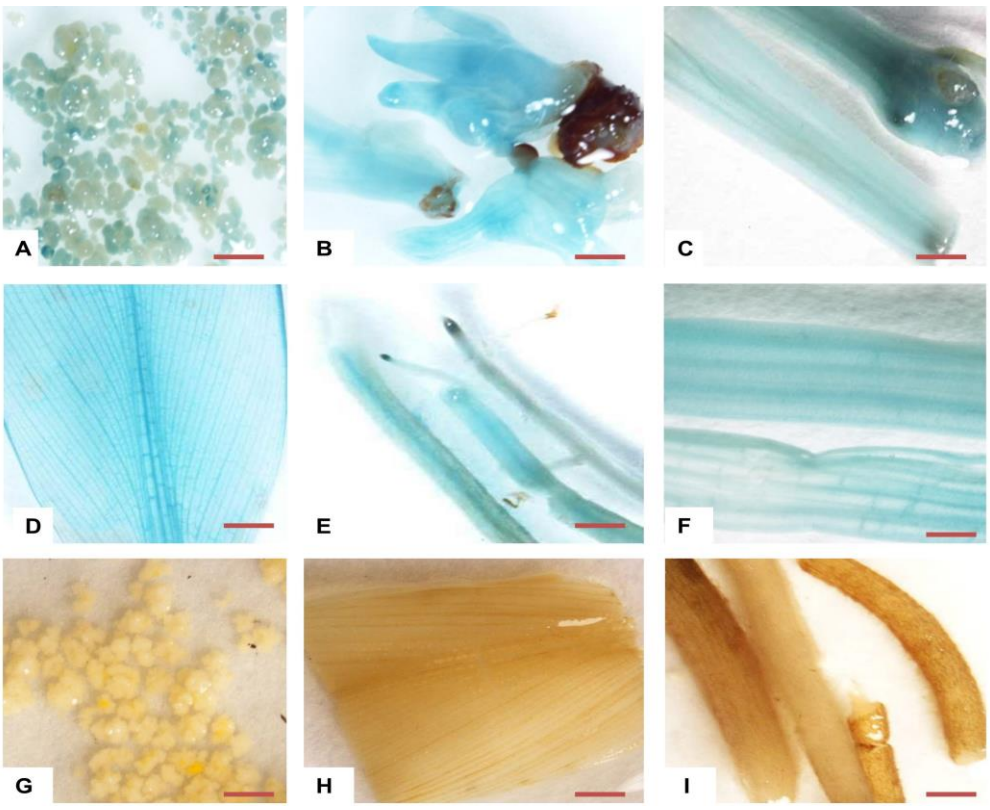


Figure 3.9: GUS histochemical assay. **A)** Transient expression of *gus*A gene in embryogenic cells after 72 h of *Agro*-infection, **B)** Stable expression of *gus*A gene in regenerating embryos, **C)** Pseudostems, **D)** Leaf, **E)** Roots and **F)** Leaves petioles **G)** Non- transformed embryogenic cells, **H)** non-transgenic control leaf, **I)** Non-transgenic control plant roots. All the photographs were taken by Nikon SMZ 1500 microscopic camera attached with computer. (Scale bar 100µ) Source- Tripathi *et al*., 2015.

3.3.5 Molecular characterization of transgenic events

Randomly selected transgenic events of 'Sukali Ndiizi', 'Gros Michel' and 'Cavendish Williams' was tested by PCR analysis to confirm the presence of *gus*A gene. All the transgenic events showed amplified DNA fragment corresponding to the *gus*A gene (528 bp) in all the transgenic events tested for banana cultivars (Figure 3.10). DNA from NTC plants showed no PCR product.

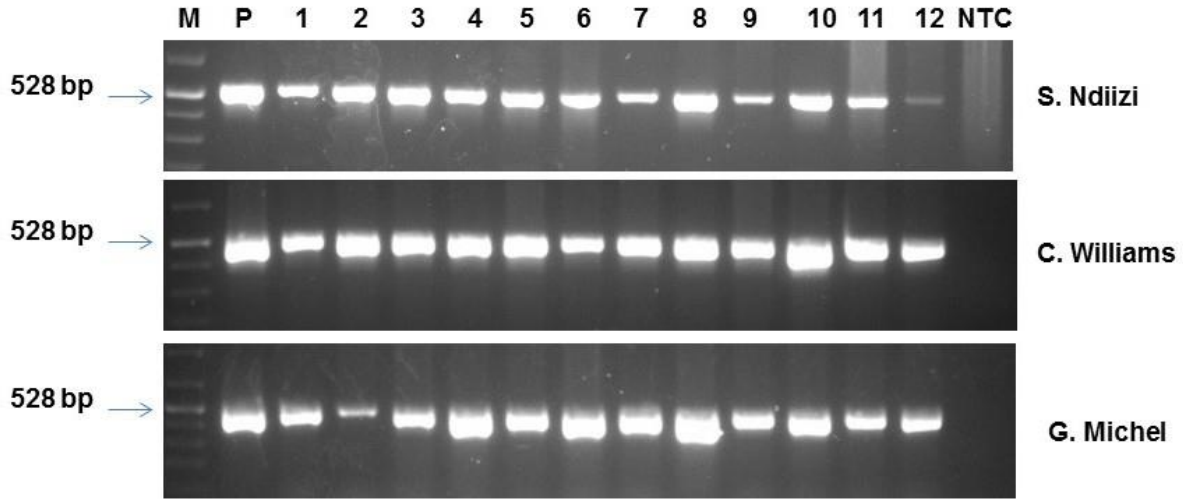


Figure 3.10: Polymerase chain reaction analysis of transgenic events of banana cultivars containing *gusA* transgene using *gusA* gene specific primers. Transgenic events, lanes 1-12 (Cultivars- S. Ndiizi, C. Williams and Gros Michel) M- 1 Kb plus DNA molecular weight marker, P- plasmid DNA, NTC- non-transgenic control plant. 0.8 % agarose gel was used for electrophoresis. Source -Tripathi *et al.*, 2015.

PCR positive transgenic events were further subjected for dot blot and Southern blot analysis to check the transgene integration and copy number of transgene. Dot blot analysis was performed with 24 PCR positive transgenic events. The DNA from NTC plant and plasmid DNA of pCAMBIA2301 was used as negative and positive control, respectively. Each sample was used in triplet. All the tested transgenic plants showed positive dot signals and NTC plant showed no signal. Plasmid DNA showed various intensity of dot signal depending on

concentration of plasmid DNA (Figure 3.11). Plasmid DNA concentration varies from 10, 5 and 1 ng.

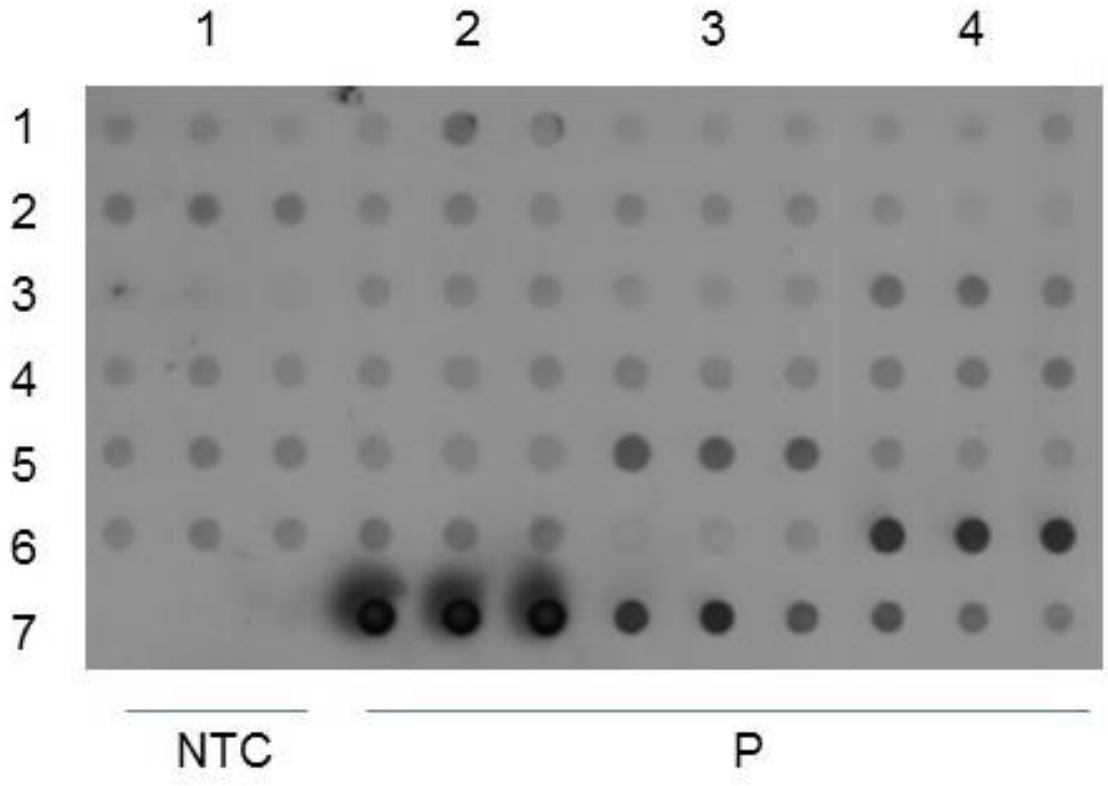


Figure 3.11: Dot blot analysis of 24 transgenic events harboring *gusA* transgene (1- 4) and 1-6 in triplicates) to confirm integration of transgene in banana genome, pCAMBIA2301 plasmid as positive control (P) in triplet's P (10, 5, 1 ng) and control non-transgenic plant (NTC) in lower lane 7. Source- Tripathi *et al.,* 2015.

Southern analysis was performed with genomic DNA of randomly picked transgenic events to confirm copy number of transgenes. Genomic DNA was restricted with *Hin*dIII restriction enzyme as it has only a single restriction site in the binary vector pCAMBIA2301. Transgenic plants showed variable hybridization band patterns, however NTC plant showed no signal (Figure 3.12). Copy number of *gusA* transgene varied from 1-4 copies in all the tested transgenic plants.

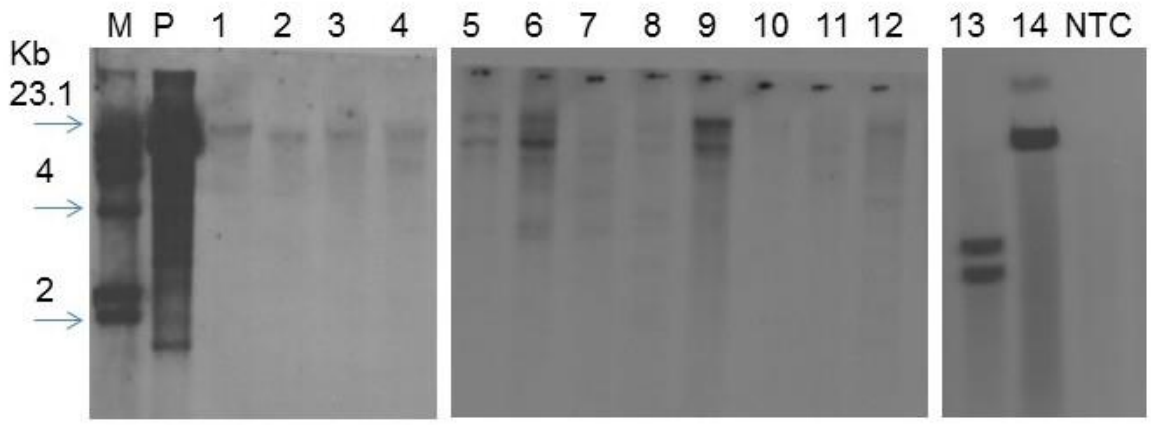


Figure 3.12: Southern analysis of genomic DNA of transgenic events restricted with *Hin*dIII restriction enzyme. Lanes (1–14) transgenic plants, NTC- non-transgenic control plant, M-DIG-labeled molecular weight marker, P- plasmid DNA digested with *Hin*dIII. Source-Tripathi *et al*., 2015

3.4. Discussion

Genetic modification of banana is an essential technique in modern plant biotechnology to develop new banana varieties with improved agronomic traits, such as resistance against bacteria (Tripathi *et al.*, 2014a), fungi (Paul *et al*., 2011; Dale *et al.*, 2017), viruses (Shekhawat *et al*., 2012; Elayabalan *et al.*, 2013) and pests like nematodes (Tripathi *et al.*, 2015; Tripathi *et al.*, 2017), and abiotic stresses like drought and salinity, and other traits like enhance nutrients and fruit quality, reduce flowering duration, reduce size of pseudostem, increasing photosynthetic effectiveness, strong rooting system to survive in high winding conditions and hold the weight and of big bunches and uniformity of all the banana (Pillay *et al*., 2002; Bakry *et al*., 2009). Developing improved banana varieties using traditional breeding technique is difficult due to low genetic variability in *Musa* germplasm, polyploidy, long generation cycle, sterility of most of the commonly grown cultivars (Silva *et al*., 2001). Therefore, genetic engineering remains an important alternative option for banana improvement (Ganapathi *et al*., 2001; Peraza-Echeverria *et al*., 2007; Roux *et al*., 2008; Santos *et al.*, 2009, Tripathi *et al.*, 2014a; Dale *et al.*, 2017). For genetic modification of banana, it is necessary to optimize

regeneration and transformation systems which can be applicable to several *Musa* species using embryogenic cells (Tripathi *et al.*, 2015).

In this study, two types of plant tissues like multiple buds and immature male flowers were compared for their ability to produce EC in various banana cultivars. EC were obtained from both explants depending upon cultivars (Table 3.2). However, multiple buds are the better option as these can be developed and multiplied in the laboratory using tissue culture plantlets. To collect male buds of banana cultivars, researchers and students need to have access to field grown banana plants, which is possible only in banana growing regions. Besides, availability of male buds is seasonal and hundreds of male buds are required to develop EC of any banana cultivar at regular basis. It was reported previously that EC of 'Cavendish Williams' could be developed using male flowers (Xu *et al*., 2005; Ghosh *et al*., 2009; Youssef *et al*., 2010; Chong-Pe´rez *et al*., 2012). However, it was not possible to obtain EC of 'Cavendish Williams' from immature male flowers, but it developed from multiple buds efficiently.

In this study it was further demonstrated that complete plantlets were regenerated from EC in 13-15 months for dessert banana cultivars, similar to previous report (Tripathi *et al*., 2012). The results of this study confirmed that efficiency of regeneration through EC highly depends on the cultivar of banana as reported in previous publications. Regeneration of 25,000–30,000 shoots per 1 ml of SCV of EC was reported for plantain cultivars (Tripathi *et al*., 2012; Strosse *et al*., 2006).

Somaclonal variation was observed in few plants (3-5 %) of 'Cavendish Williams' under glasshouse conditions. To minimize somaclonal abnormalities in transgenic plants, EC were used only up to 12-18 months after their generation, for transformation experiments. The EC

older than 18 months is not recommended for transformation. Production of EC and their regeneration is time intensive and cultivar dependent. In earlier reports, 14—42 months and 18-27 months were required for regeneration of banana and plantains, respectively, initiating from callus induction (Strosse *et al*., 2006).

Several AMT protocols of banana have been previously reported (Ganapathi *et al*., 2001; Khanna *et al*., 2004; Tripathi *et al*., 2012, Tripathi *et al.*, 2015). AMT frequency of various farmer-preferred cultivars is still very low, and it is difficult and time-consuming process, and only a few cultivars have been successfully genetically transformed and evaluated up to confined field trial study (Tripathi *et al.*, 2014a, Dale *et al.*, 2017). Regeneration of transgenic banana plants depend on several characteristics such as variety used, type and age of initial explants, virulency of *Agrobacterium* strains, plasmid constructs, the selective antibiotics and regeneration conditions (Hansen and Chilton, 1994; Cheng *et al*., 2004; Hiei *et al.*, 1997, Tripathi *et al*., 2012, Tripathi *et al.*, 2015). Therefore, there is need to develop the EC and improve the protocol for each economically important farmer-preferred variety. A robust and efficient regeneration and transformation system is needed for generation of several transgenic events with various gene constructs in any crop. This study describes an efficient regeneration and AMT system for various cultivars of banana.

Transgenic events were regenerated on selective medium containing kanamycin. However, inhibition effect of kanamycin on regeneration of transformed shoots was reported in previous studies (Yao *et al*., 1995; Tripathi *et al*., 2012; Tripathi *et al.*, 2015). Thus, in this study no antibiotic selection was used in proliferation and rooting of transgenic plants. To avoid escape of transgenic plants, regeneration was performed on kanamycin selective medium and freshly prepared kanamycin stock was used for medium preparation.

Transformation efficiency of EC with *Agrobacterium* was dependent on regeneration efficiency of EC used for transformation. The higher regeneration capacity of EC provides the more number of transgenic events as shown by regeneration transformation efficiency of 'Sukali Ndiizi' (Figure 3.5 and 3.7). Regeneration efficiency of transgenic events also depends on quality and age of EC used for AMT. It was also observed that newly proliferated fine EC produce more number of transgenic events in comparison to older cells. Big nodules like structure of EC are not good for transformation as they turn black. These nodular structures need to be discarded during subculturing of EC. The transformation efficiencies (approximately 20-70 transgenic events per ml SCV) attained in this study was related well to earlier published reports. Ganapathi *et al*. (2001) obtained about 40 transgenic events of banana cultivar 'Rasthali' (AAB) per 0.5 ml SCV of EC. It was also reported that banana cultivars 'Cavendish' and 'Lady Finger' germinated 25-65 transgenic events per 50 mg of SCV of EC, whereas 'Dwarf Cavendish' produced much higher, about 600 events from 50 mg of SCV of EC (Khanna *et al*., 2004; Ghosh *et al*., 2009; Chong-Pe´rez *et al*., 2012).

In this study, a protocol was optimized for transformation and regeneration of various cultivars of banana especially 'Sukali Ndiizi', 'Gros Michel' and 'Cavendish Williams' using EC. Genetically transformed EC of banana have the potential to generate large number of genetically modified plants in 1 ml SCV of EC can generate approximately 20-70 of genetically engineered banana plantlets with the expected quality. The protocol established here is an essential and important step for development of transgenic banana with various agronomic traits of interest.

CHAPTER FOUR

EXPRESSION OF THE RICE *NH1* GENE IN BANANA CONFERS RESISTANCE AGAINST *XANTHOMONAS CAMPESTRIS* PV. *MUSACEARUM*

4.1 Introduction

Banana including plantain (*Musa* spp.) is major source of income and food, providing more than 25 % of the energy requirements to millions of people in the tropical region of the world (Tripathi *et al*., 2012; Tripathi *et al*., 2013). Diseases and pests can hamper banana production altogether and are considered the most serious constraints to production under adverse environmental conditions. Major diseases of banana are caused by bacteria, fungi, nematodes, and viruses (Ploetz, 2004). Banana Xanthomonas wilt (BXW), caused by *X. campestris* pv. *musacearum* (Xcm), is the most destructive and serious disease in East and Central Africa (Tripathi *et al*., 2014a). Pathogens spread extremely rapidly into new fields by insects, infected planting materials, cutting tools and splashes of rain during the rainy seasons (Tripathi *et al*., 2009a). Once Xcm spreads out in fields, disease control measures become very challenging (Eden-Green, 2004). Currently, there are no resistant varieties or anti-microbial and bio-control agents available to the farmers for controlling this disease. BXW can be managed by applying phytosanitary practices, but those practices are labour intensive and difficult to sustain in rural farming systems (Biruma *et al*., 2007; Tripathi *et al*., 2009a).

Resistant plants can survive pathogen attack by engaging various defense strategies. Upon recognition of the foreign organism, several defense responses are triggered that can prevent pathogen proliferation in the infected tissues through hypersensitive response (HR). During the HR, reactive oxygen species (ROS) are induced, phytoalexins accumulate, plant cell walls are reinforced and salicylic acid (SA) is synthesized. From the point of entry of pathogens, the

defense response is then signaled to other distant parts of the plant through a process called systemic acquired resistance (SAR), which activates expression of various pathogenesis-related (PR) genes, leading to a durable enhanced resistance against many pathogens (Durrant and Dong, 2004; Feng *et al.,* 2011). One of the key factors connecting SA signaling to PR genes expression is a nonexpressor of pathogenesis-related gene (*NPR1*) (Mou *et al*., 2003). The *Arabidopsis NPR1* gene is a regulator of SA mediated SAR (Cao *et al*., 1994; Delaney *et al.*, 1995; Glazebrook *et al*., 1996; Ryals *et al*., 1997; Shah *et al*., 1997).

The over-expression of *NPR1* in transgenic *Arabidopsis* showed resistance to several bacterial and fungal pathogens (Cao *et al*., 1998). Over-expression of the *AtNPR1* gene and its homologs have demonstrated resistance against bacterial, fungal and viral pathogens in various economically important crops including wheat, grape, apple, citrus, and tobacco (Le Henanff *et al*., 2009; Makandar *et al*., 2006; Malnoy *et al*., 2007; Meur *et al.,* 2008; Yuan *et al*., 2007; Zhang *et al*., 2010). Expression of *Arabidopsis NPR1* in rice showed resistance against *Xanthomonas oryzae* and *Magnaporthe grisea* (Chern *et al*., 2001; Yuan *et al*., 2007; Quilis *et al*., 2008), supporting the presence of a parallel defense pathway in rice. The *NPR1* gene has also shown resistance in tomato against bacterial, fungal and viral diseases (Lin *et al*., 2004). Over-expression of the *Arabidopsis NPR1* in transgenic carrots demonstrated resistance to necrotrophic and biotrophic pathogens (Wally *et al.,* 2009). Even though transgenic *Arabidopsis* plants over-expressing *NPR1* became more sensitive to benzothiadiazole (BTH) and salicylic acid (Friedrich *et al*., 2001), they displayed no phenotypical defects and showed activation of *PR* gene expression by inducers or following infection by pathogens (Cao *et al*., 1998). The rice *NPR1* homolog, *NH1* has also shown resistance against bacterial pathogens in rice (Chern *et al*., 2001, 2005). Yuan *et al*. (2007) over-expressed *OsNPR1, OsNPR2* and *OsNPR3* in rice, and observed that only *OsNPR1* conferred enhanced resistance to Xoo.

Development of disease resistant banana varieties is a sustainable and cost-effective approach to manage banana Xanthomonas wilt and other biotic constraints, especially for low-income farmers. However, traditional breeding methods require more than decades to achieve a usable and acceptable product due to sterility, triploids of the most of edible cultivars and the lack of genes for resistance in available cultivated germplasm (Tripathi *et al*., 2014a). For these reasons, transgenic approaches as an alternative and rapid method to improve banana against bacterial pathogen, was explored.

In the present study, the rice homolog of *NPR1* (*NH1*) gene was introduced through *Agrobacterium*-mediated transformation system into the banana cultivar 'Sukali Ndiizi' (AAB genome). Stably transformed transgenic plants were regenerated in selective medium. Molecular characterization of regenerated plants confirmed that regenerated plants were transgenic harboring *NH1* gene. Furthermore, well-rooted potted plants were evaluated for resistance against Xcm in the glasshouse.

4.2 Materials and methods

4.2.1 Plant material

Embryogenic cells (EC) of the farmer preferred cultivar 'Sukali Ndiizi' (*Musa* spp., AAB genome) also known as apple banana in East Africa was initiated and developed using immature male flowers. EC were multiplied and maintained by regular sub-culture in new culture medium every 10 to 14 days as previously reported by Tripathi *et al*., (2015). These EC were used to generate transgenic banana plants (Figure 3.3).

4.2.2 Binary vector and *Agrobacterium* strain

The plasmid construct (pCAMBIA1300-Ubi: NH1) used in this study was provided by Prof. Pamela Ronald, UC Davis. The binary vector having rice *NH1* gene regulated by a maize constitutive *ubiquitin* promoter and the *hygromycin phosphotransferase* (*hpt*) regulated by Cauliflower Mosaic Virus (CaMV 35S) promoter (Figure 4.1) was used for transformation of EC of banana. The plasmid vector was transferred to highly virulent *Agrobacterium tumefaciens* strain EHA 105 (Hood *et al.*, 1993) by electroporation. Engineered *Agrobacterium* strain 105 was cultured on LB agar medium [Tryptone (1.0 %), yeast extract (0.5 %) and NaCl (0.5 %, Agar (1.5 %)], containing rifampicin (25 mg/ L) and kanamycin (50 mg/ L) at 28 ℃ for 48 h. Single colony was picked and inoculated to LB broth (same as LB agar but without agar) containing rifampicin (25 mg/ L) and kanamycin (50 mg/ L) at 28 ℃ with shaking at 200 rpm for 48 h. The bacterial culture was refreshed by growing new culture overnight at similar conditions until the optical density (OD600) reached 0.5 to 0.8.

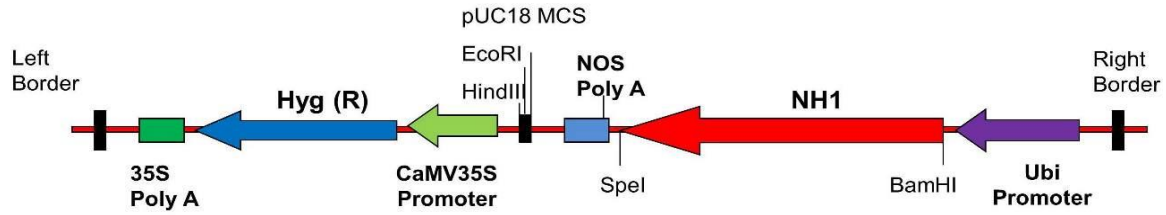


Figure 4.1: Map of the binary vector pCAMBIA1300-Ubi: NH1 showing T-DNA region containing rice *NH1* gene driven by a maize constitutive *ubiquitin* promoter and *hygromycin phosphotransferase* (*hpt*) as selection marker under CaMV 35S promoter. This map also shows single restriction site for *Hin*dIII restriction enzyme.

4.2.3 Genetic transformation and regeneration of transgenic plants

EC of 'Sukali Ndiizi' were transformed through *Agrobacterium* as explained in Chapter 3, section 3.2.9. The putative transgenic plantlets were regenerated on hygromycin B antibiotic

(25 mg/L) selection in 7 to 9 months (Tripathi *et al*., 2015). Putative transgenic shoots generated on selective medium were transferred to proliferation medium (MS medium supplemented with 2.5 mg/L, 6-BAP) for maintenance and multiplication through regular sub-culture in new medium every eight weeks. Transgenic shoots were cultured on rooting medium (MS medium containing 1 mg/L IBA) (Tripathi *et al*., 2012). Rooted plantlets were planted in plastic disposable cups (10 cm diameter) having sterile and autoclaved soil for weaning and hardening. The plants were grown in shaded and humid conditions for 4 weeks. The hardened plants were then shifted to big pots (30 cm diameter) in sterile and autoclaved soil. Plant growth parameters (plant height, girth at base of pseudostem, total number of developed green leaves, length and width of third leaf) were documented on transgenic and NTC plants at 90 days after weaning of plants in the glasshouse. Total green leaves area of plant was estimated using the formula (Kumar *et al.*, 2002; Tripathi *et al.*, 2014b).

$$Total\ leaves\ area\ = 0.8 * L * W * N$$

Where: Total green leaves area (cm^2)

L = length of third fully developed middle leaf (cm); W = width of same leaf (cm); N = Total number of green leaves in a plant.

4.2.4 Molecular analysis

4.2.4.1 PCR analysis

Total plant genomic DNA was isolated from 200 mg fresh leaves of all the generated putative transgenic banana plants using DNeasy kit (Qiagen). PCR analysis was performed using *Ubi: NH1* and *hpt* gene specific primers. The primers used were a maize *ubiquitin* promoter and *NH1* gene specific with the *Ubi* forward primer being 5’ TGATATACTTGGATGATGGCA 3’ and the *NH1* reverse primer being 5’ GGACGGCGATGCGCGCGTC 3’ (Chern *et al*.,

2005). For the *hpt* gene, the forward primer 5` GATGTTGGCGACCTCGT 3` and the reverse primer 5` GTGTCACGTTGCAAGACCTG 3` were used. The PCR was performed in 0.2 mL microfuge tubes in a Gene Amp® Applied Biosystem (ABI) 9700 machine. PCR reactions were set up in 25 µL volumes containing 50 ng of plant DNA template (in 2 µL nuclease free water, and 23 µL of master mix, which consisted of 15 mM $MgCl_2$ containing PCR buffer (Qiagen), 10 µM deoxy ribonucleotides (dNTP) (0.5 µl), the gene-specific primers at a final concentration of 0.5 µM and 0.2 µL HotStar *Taq* DNA polymerase (Qiagen). For *Ubi: NH1* primers, PCR conditions were set at 95 ℃ for 10 min for denaturation and 35 cycles of amplification of T-DNA at 94 ℃ for 30 s, 60 ℃ for 90 s, and 72 ℃ for 60 s, followed by 72 ℃ for 7 min for extension of the amplified product. For *hpt* primers, PCR conditions were 95 ℃ for 5 min for denaturation and 35 cycles of amplification of T-DNA at 94 ℃ for 30 s, 58 ℃ for 40 s, and 72 ℃ for 60 s, and 72 ℃ for 7 min for extension of the amplified product. Positive controls (plasmid DNA) as well as one negative control (DNA of control non-transgenic plant) were included in each experiment. The amplified PCR products were electrophored on 1 % (w/v) agarose gel stained with Gel Red® (Biotium). The gel was run at 90 V for 2 hours and thereafter visualized using Syngene gel documentation system and photographed was taken.

4.2.4.2 Dot blot

To check the transgene integration of many transgenic events, initial molecular analysis was performed by dot blot using (200 ng) genomic DNA extracted as described in section 4.2.4.1. DNA samples were denatured by 200 µL of 0.4 M Sodium Hydroxide (0.4 N) at 95 °C for 5 min and denatured DNA samples were incubated in ice bucket for 5 min. A positive control (plasmid DNA) and negative control (non-transformed plant DNA) was also included in experiment. Bio-Rad dot blotter was used to transfer denatured DNA on positively charged membrane (Roche) using a vacuum pump. Transfer DNA on membrane was fixed by UV cross

linker. PCR labelled DIG probe was used. The hybridization was performed using a DIG Luminescent Detection Kit for Nucleic Acids (Roche). To remove unspecific hybridization signals and excess probe from the membrane, stringency washes were performed at a room temperature and higher temperature (68 ^{0}C) in hybridization chamber at salt concentration: 2X SSC and 0.1 % SDS, twice for 5 min, followed by 0.5X SSC and 0.1 % SDS, twice for 15 min. For signal detection a ready to use CDP star chemiluminescent substrate was used. X-ray film was developed using developer and fixer manually at various time interval to get optimal result.

4.2.4.3 Southern blot analysis

Genomic DNA was extracted from 2 g leaf tissues of PCR positive transgenic plantlets using a modified hexadecyl trimethyl ammonium bromide (CTAB) extraction method (Gawel and Jarret, 1991). For Southern analysis, genomic DNA (10 µg) of transgenic plants, non-transgenic control plant and the plasmid vector DNA (10 pg) were digested with *Hin*dIII restriction enzyme which has only one restriction site in plasmid vector DNA. Site of restriction digestion was shown on Figure 4.1. The restricted DNA was separated on an agarose gel (0.8 % w/v) at low voltage (50 V) for 5 hours transferred to a positively charged nylon membrane (Roche Diagnostics, Hertfordshire, UK) by capillary blotting technique and covalently linked to the membrane by UV crosslinker at 1200X100 µJoule/cm^2. Detection probes were labeled with digoxigenin-dUTP (Roche) by PCR. The primers used were *NH1* gene and a maize *ubiquitin* promoter with the *Ubi* forward primer being 5' TGATATACTTGGATGATGGCA 3' and the *NH1* reverse primer being 5' GGACGGCGATGCGCGCGTC 3' (Chern *et al*., 2005). The hybridization was performed using a DIG Luminescent Detection Kit for Nucleic Acids (Roche) according to the manufacturer's instructions. To remove unwanted hybridization signals and back ground due to excess probe, more stringent membrane washes were performed at a higher temperature (68 $^{\circ}$C) and lower salt concentration: 2X SSC and 0.1

% SDS, twice for 5 min, followed by 0.5X SSC and 0.1 % SDS, twice for 15 min. For detection of signal on the membrane CDP star chemiluminescent substrate was used according to the manufacturer's instructions (Roche). Twenty µL of CDP star chemiluminescent substrate was dissolved and mixed on 1 mL of detection buffer which was used as detection substrate. X-ray films were developed in developer and fixer at various time intervals of exposure to get perfect results.

4.2.5 Disease evaluation against *Xanthomonas campestris* pv. *musacearum* in glasshouse

Based on PCR and Southern analysis results, twenty transgenic events containing the rice *NH1* gene were used for disease resistance under the glasshouse conditions. Non-transgenic control (NTC) plants were also comprised in the experiment. Each transgenic event with three replicates and the NTC plants were used for the disease evaluation study. 90-day old plants were artificially infected by injecting 100 µL of a bacterial culture of Xcm (10^8 cfu/mL) using an insulin syringe into the midrib of the second fully opened mature leaf (Tripathi *et al*. 2010; Tripathi *et al*., 2014b). Bacterial culture of Xcm used in this study was obtained from National Agricultural Research Laboratory NARL, Uganda in 2012. Injected plants were observed daily up to 60 days for development of wilting symptoms of BXW disease, with initial symptoms of yellowing and browning of the leaves and final symptoms of complete dying of the plants. Data were recorded for each plant based on the appearance of the first disease symptoms, number of days to complete wilting and after 60 days of inoculation (dpi). All the photographs were taken after 2 months of inoculation. The experiment was repeated twice with the same transgenic events. The plants which showed no symptoms of disease were observed for a longer time up to six months. The resistance of transgenic plants to Xcm was determined at 60 dpi based on the reduction in wilting in assessment with non-transgenic control plants (Tripathi *et al*., 2010; Tripathi *et al*., 2014b) as follows:

$$\text{Resistance } \% = \frac{\text{Reduction in wilting in transgenic plant}}{\text{Total number of leaves wilted in control plant}} \text{X } 100$$

Where, reduction in wilting = total number of leaves in transgenic plants - number of wilted leaves in transgenic plant.

All the challenged plants were divided into three categories 1) resistant there was no symptoms in the injected plant, except natural yellowing and drying of 1-2 older leaves due to the aging process, In banana older leaves gradually turn yellowish and start drying as new leaves appear on the top of the pseudostem, 2) partial resistance if a plant showed reduction in symptoms in comparison to the non-transgenic control plant 3) susceptible if a plant developed symptoms in all the leaves and completely died after inoculation like non-transgenic control plants (Tripathi *et al*., 2010; Tripathi *et al*., 2014b).

4.2.6 Expression profile of pathogenesis related genes

4.2.6.1 Plant materials

Three transgenic events S15, S47 and S100 were selected which showed significant resistance to Xcm in the glasshouse disease evaluation. Six-week-old *in-vitro* plantlets having 4 or 5 fully opened leaves were used for inoculation. The leaf petiole of the second youngest fully opened leaf of transgenic and NTC plants was inoculated with 100 µl of 10^6 cfu culture of Xcm or distilled water for mock inoculation using ultra fine 31-gauge insulin syringes. Injected leaf samples were taken at each time point 0, 6, 12, 24 and 48 h post inoculation (pi), and samples were quickly frozen in liquid nitrogen. Leaves from infected transgenic plants as well as NTC plantlets were collected for total RNA extraction from two biological replicates per treatment at each time point.

4.2.6.2 RNA extraction and cDNA synthesis

Total plant RNA was extracted using Qiagen RNeasy mini-kits (UK) with slight modifications from freshly collected leaf sample (100 mg) ground to a fine powder in liquid nitrogen using a sterile chilled pestle and mortar. The RNase Inhibitor, murine (NEB, UK) was used during RNA extraction to prevent RNA degradation. Total RNA was treated with DNase I (NEB, UK) at 37 °C for 1 h. The cDNA was synthesized with 2 µg of DNase treated RNA using the Maxima First Strand cDNA Synthesis kits and oligo-dT primers according to manufacturer's instruction with slight modifications to optimize the reaction. Program used for cDNA synthesis was 25 °C for 10 min, 50 °C for 35 min and reaction was terminated at 85 °C for 5 min. ABI 7500 (Applied Biosystems) thermocycler was used for cDNA synthesis.

4.2.6.3 Quantitative real-time (qRT-PCR) analysis

The cDNA (5 µL) was diluted 10-fold using nuclease free water for qRT-PCR. Primers were designed from genes encoding the *PR1, PR2, PR3,* and *PR5* genes (Table 4.1). The *Musa 25S* ribosomal RNA was used as an endogenous control. qRT-PCR was performed through the 7500HT Real-Time PCR machine using a Maxima SYBR Green/ROX qPCR master mix. The reaction mixture contained 2 µl of 10-fold diluted cDNA, 0.3 µl of 10 µM of forward and reverse primers, 10 µl Maxima SYBR-Green/ROX master mix, and remaining nuclease-free water in total volume 20 µl was added into respective wells of a 96 well micro-titer plate and qRT-PCR was performed. NTC plant cDNA was used for comparison study. Non-template control, containing nuclease free water were also included into the analysis. For each sample reaction was set up in triplet using target gene primers as well as endogenous gene primers. qRT-PCR conditions were as follows: pre-incubation for 5 min at 95 °C, followed by 40 cycles consisting of a denaturation step at 94 °C for 15 s, primer annealing at 60 °C for 45 s and primer extension at 72 °C for 50 s. Specificity of PCR amplification was confirmed by melting curve

analysis (60 °C to 95 °C). After completion of the cycling reactions, melt curve analysis (60–95 °C every 0.5 °C for 1 s) was conducted to remove the effects of primer dimer formation and non-specific amplified products.

Relative gene expression was determined with comparative CT method (Livak and Schmittgen, 2001).

$2^{-\Delta\Delta CT}$; with $\Delta\Delta CT = \Delta CT_{TG}$ (CT target gene – CT reference gene) – ΔCT_{CG} (CT non—transgenic control plant with target gene - CT reference gene).

Table 4.1: List of primers used for pathogenesis-related (*PR*) genes expression study

Target gene	Description	Forward primer	Reverse primer	Product size
PR1	Pathogenesis-related protein 1	GGACTTCGTGAG CCC CCACAA	GCTGGTTGGCGTAG TTCTG	121
PR2	β-1,3-glucanase	TTGGATGGACCT GTACGTGA	TCACGCATTTGTGCC TATGTG	137
PR3	Chitinase protein IV	GGCTCTGTGGTT CTGGATGA	CCAACCCTCCATTGA TGATG	149
PR5	Thaumatin-like protein	TTGGATGGACCT GTACGTGA	TCACGCATTTGTGCC TATGTG	102
Musa 25S ribosomal	Endogenous gene	ACATTGTCAGGT GGGGAGTT	CCTTTTGTTCCACAC GAGATT	102

Source- NCBI: Reference sequence NC_025209.1 and NC_025210.1; Yocgo *et al.*, 2008 and 2012; Tripathi *et al*., 2014b

4.2.7 Statistical analysis

Data were scrutinized using the statistical program Minitab 16 (Minitab 16 statistical software, 2012). The means and standard error (SE) presented were for three replicates in each experiment. One-way Analysis of Variance (ANOVA) performed with a Minitab 14 identified those means that were different from the corresponding value for the non-transgenic control (NTC) plants. Tukey`s family error rate was calculated.

4.3 Results

4.3.1 Banana embryogenic cells

EC were the preferred material for banana transformation. Actively dividing uniform EC of banana cultivar ‘Sukali Ndiizi’ were used for generating transgenic events (Figure 3.3F).

4.3.2 Genetic transformation and plant regeneration

EC of Sukali Ndiizi were genetically modified through *Agrobacterium tumefaciens* strain EHA105 containing the plasmid vector pCAMBIA1300-Ubi: NH1 in which the rice *NH1* gene was regulated by the maize *Ubiquitin* constitutive promoter. Only successfully transformed EC survived, proliferated and multiplied on hygromycin B selective medium, whereas untransformed EC completely turned black and died (Figure 4.2A). Only 5 to 10 % regenerating embryos turned into complete shoots on selective medium (Figure 4.2B and 4.2C). This might be due to a high concentration of antibiotic hygromycin B (25 mg/L) in selective medium.

The genetically modified EC were regenerated on selective medium, and about 32–38 transgenic events were generated from 1 mL settle cell volume of EC on selective media added with hygromycin B (Figure 4.2D). In total, 112 individual putative transgenic events were generated in three *Agrobacterium* mediated transformation experiments over the period on 7 to 11 months. The regenerated independent transgenic shoots were clonally micro-propagated in proliferation medium and further cultured in rooting medium for root development. All transgenic shoots produced roots within 4–6 weeks. The plantlets with fully developed roots of the transgenic events were planted in soil to disposable plastic cups for hardening and

acclimatization in the humid chamber contained in the glasshouse (Figure 4.2E). Further after six weeks these plants were up rooted and replanted into bigger pots.

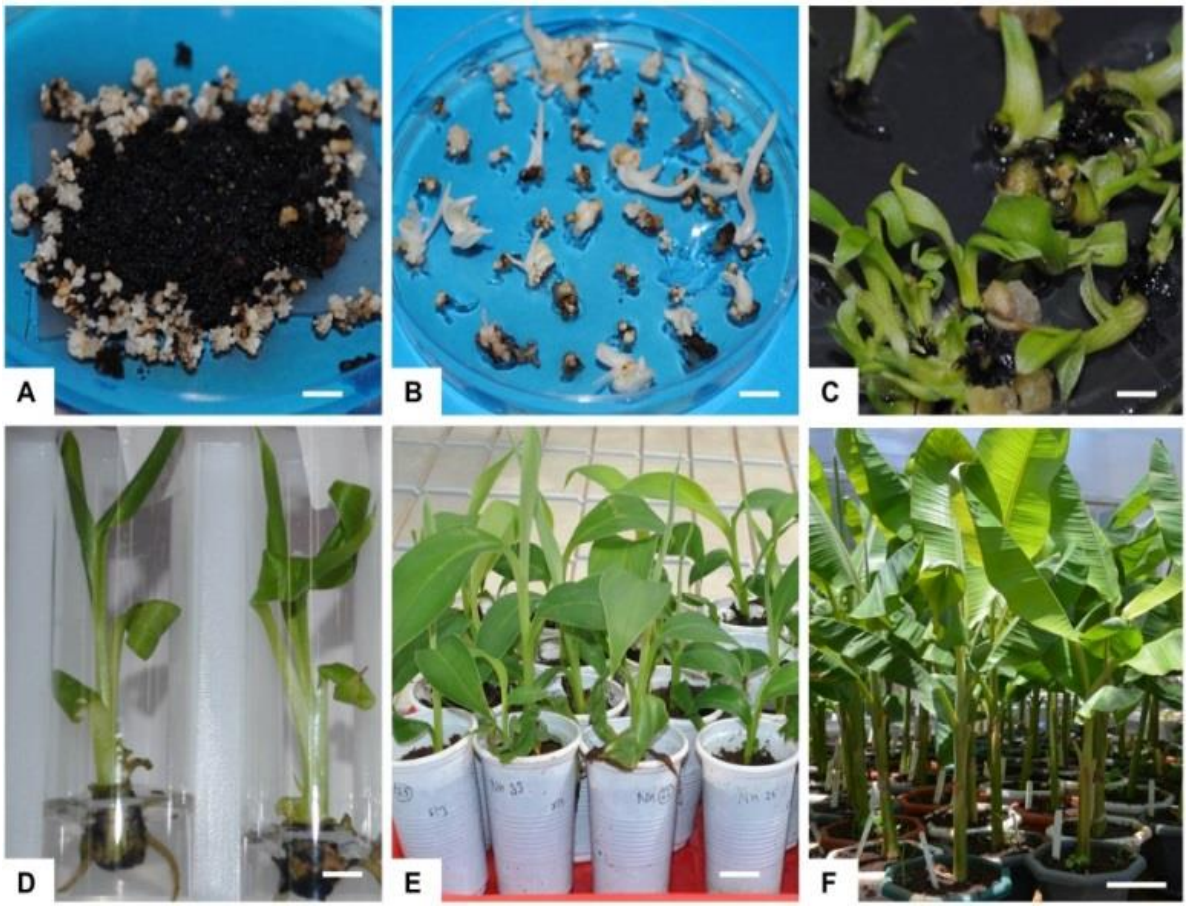


Figure 4.2: Transgenic plants regeneration and acclimatization in glasshouse. **A)** Transformed white embryos regenerating in hygromycin B selective medium, **B)** Transformed white embryos turning into small white shoots in hygromycin B selective medium in dark, **C)** Transformed shoots became green in light, **D)** Shoots with small roots, **E)** Weaning of transgenic plants in small cups (scale bar 1 cm). **F)** Transgenic plants planted in the pots in the glasshouse (scale bar 10 cm).

4.3.3 Molecular analysis of transgenic plants

To confirm the presence of transgene in the regenerated plants preliminary molecular characterization was performed by PCR and dot blots. The presence of the *NH1* gene was investigated in all transgenic events by PCR using promoter ubiquitin (Ubi) and transgene (*NH1*) specific primers. All transgenic events have shown the amplified product of 350 bp

confirming the existence of the transgene. NTC plant showed no amplified product. (Figure 4.3). PCR was also done using primers specific selective marker *hygromycin phosphotransferase gene* (*hpt*) and an amplified product of 415 bp was seen in all tested transgenic events (Figure 4.3). These results confirmed the presence of both transgenes *NH1* and *hpt* in all the transgenic events.

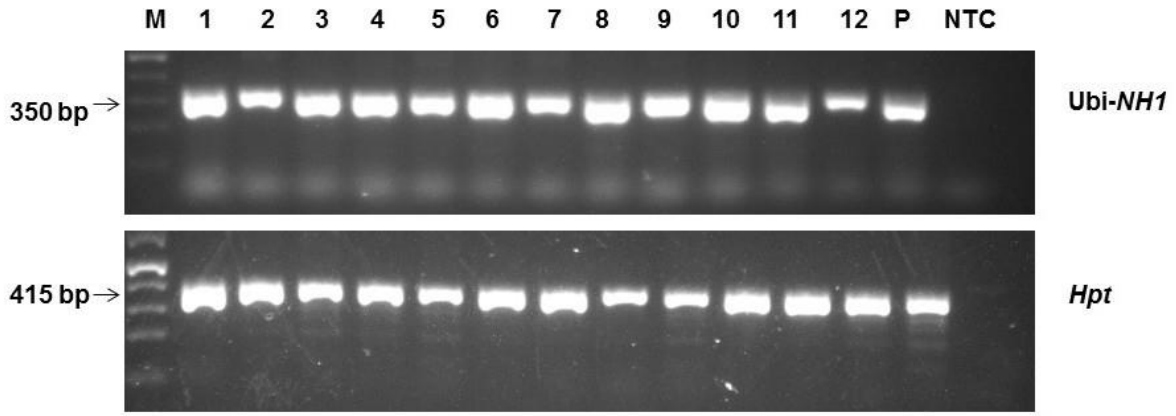


Figure 4.3: Representative pictures of PCR analysis of transgenic events (lanes 1-12). PCR results showed presence of amplified products of both Ubi-*NH1* and *hpt* gene, P- positive control, NTC-Non-transgenic control plant (NT), M- 1Kb plus DNA ladder.

All the PCR positive transgenic events were further tested by dot blots. Twenty-eight transgenic events, NTC plant DNA and positive plasmid DNA were analyzed in three replicates of each samples. All the transgenic events have positive dot signals except for the NTC plant. Dot blot is a fast and reliable method which can be used for testing of several transgenic events for transgene integration with minimal genomic DNA (Figure 4.4). Dot blot needs 100 to 200 ng of genomic DNA.

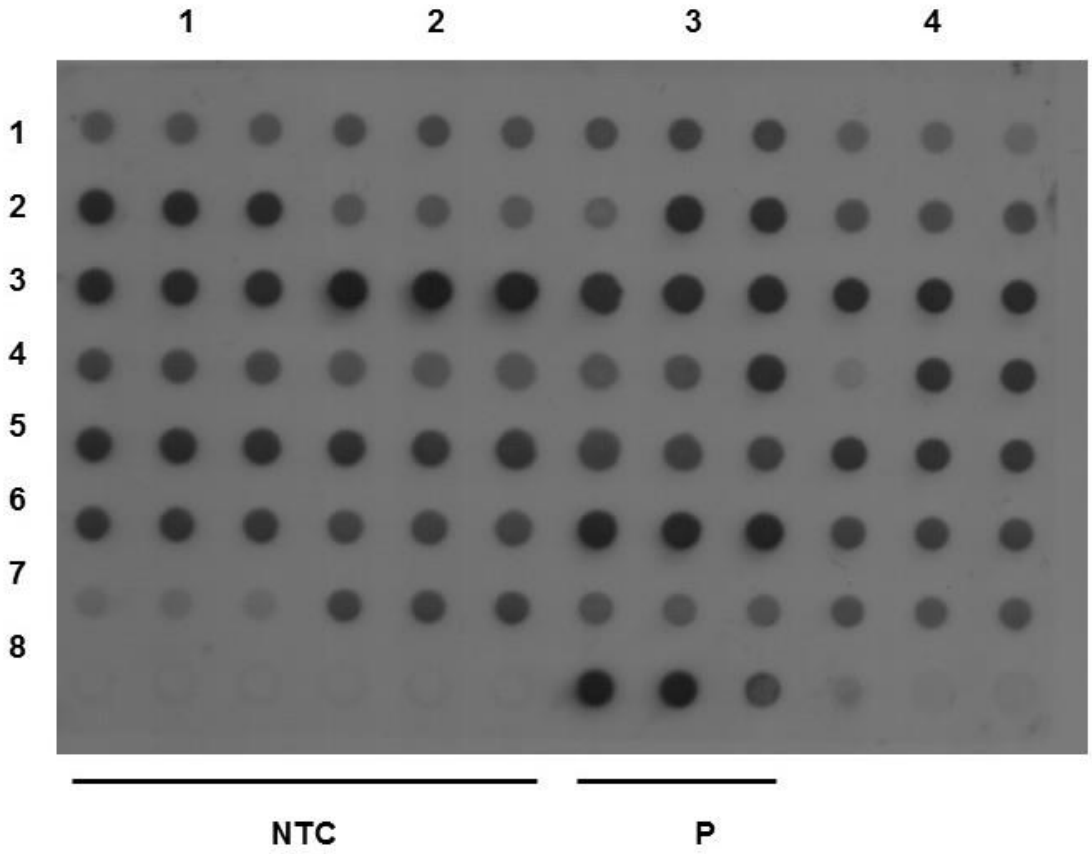


Figure 4.4: Dot blot analysis of transgenic events containing the rice *NH1* transgene. Each sample in triplets. Black signal of dot blots confirmed integration in 28 transgenic events (1 to 4 and 1 to 7 were transgenic events. NTC-non-transgenic control plant showed no signal (lower lane 8), P-Plasmid DNA as a positive control (lower lane 8).

Southern blot analysis confirmed the integration of the *NH1* gene in the PCR positive plants. The genomic DNA digested with *Hin*dIII revealed that the analyzed plants had different hybridization designs, indicating that each transgenic event resulted from an independent transgenic event with copy number (1 to 4 or multiple) of integrated transgenes (Figure 4.5).

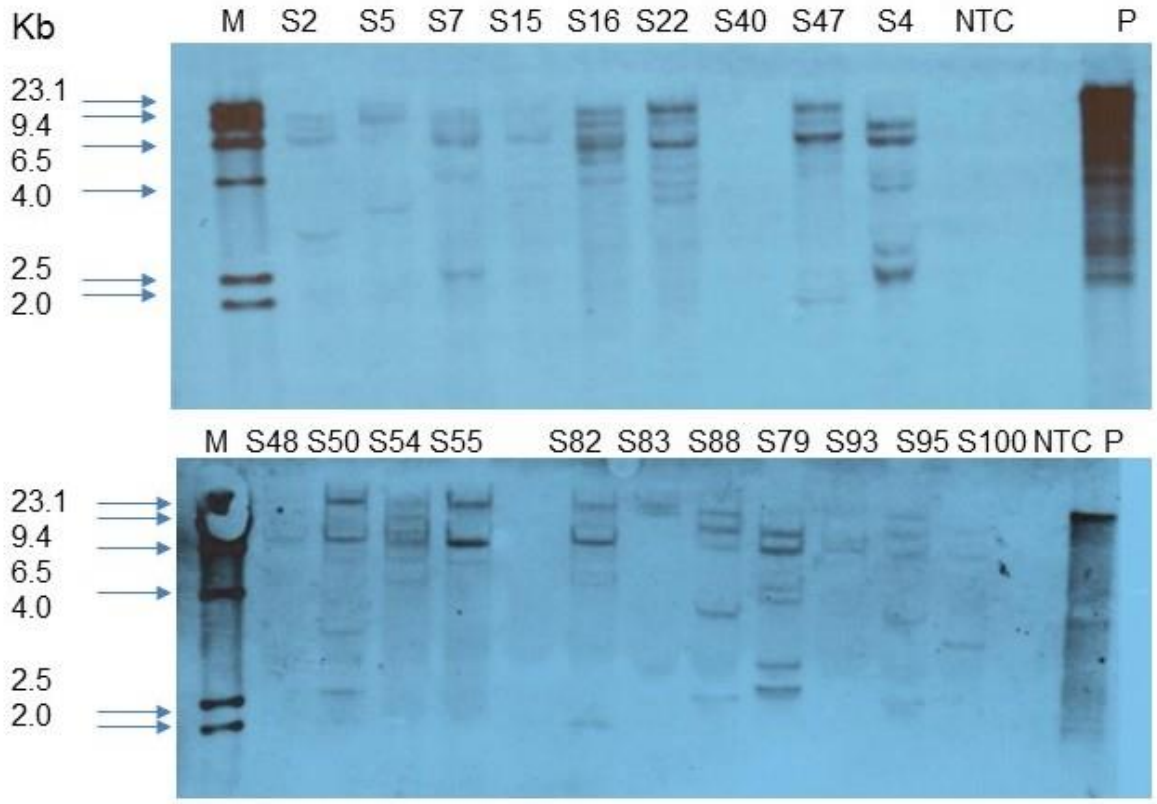


Figure 4.5: Southern analysis of transgenic events (S2 to S100) harboring *NH1* transgene. M-DIG molecular marker, NTC- non-transgenic control; P- restricted plasmid, Kb-kilobase pairs.

4.3.4 Transgenic plants growth analysis

Twenty independent (S2, S4, S5, S7, S15, S16, S40, S47, S48, S50, S54, S55, S79, S82, S83, S88, S91, S93, S95 and S100) transgenic events with three replicates were assessed in the glasshouse at 90 days after weaning in soil. All the plants developed normally with no morphological or phenotypical differences in comparison to NTC plants (Figure 4.6). In general, growth characteristics (plant height and total leaves area) were similar in all the transgenic events compared to the NTC plants except S4 and S79. These results clearly showed that there was no adverse effect of *NH1* gene in plant height, pseudostem thickness, and total number of leaves (except total leaves area of two transgenic events S4 and S79). All the transgenic plants were morphologically normal.

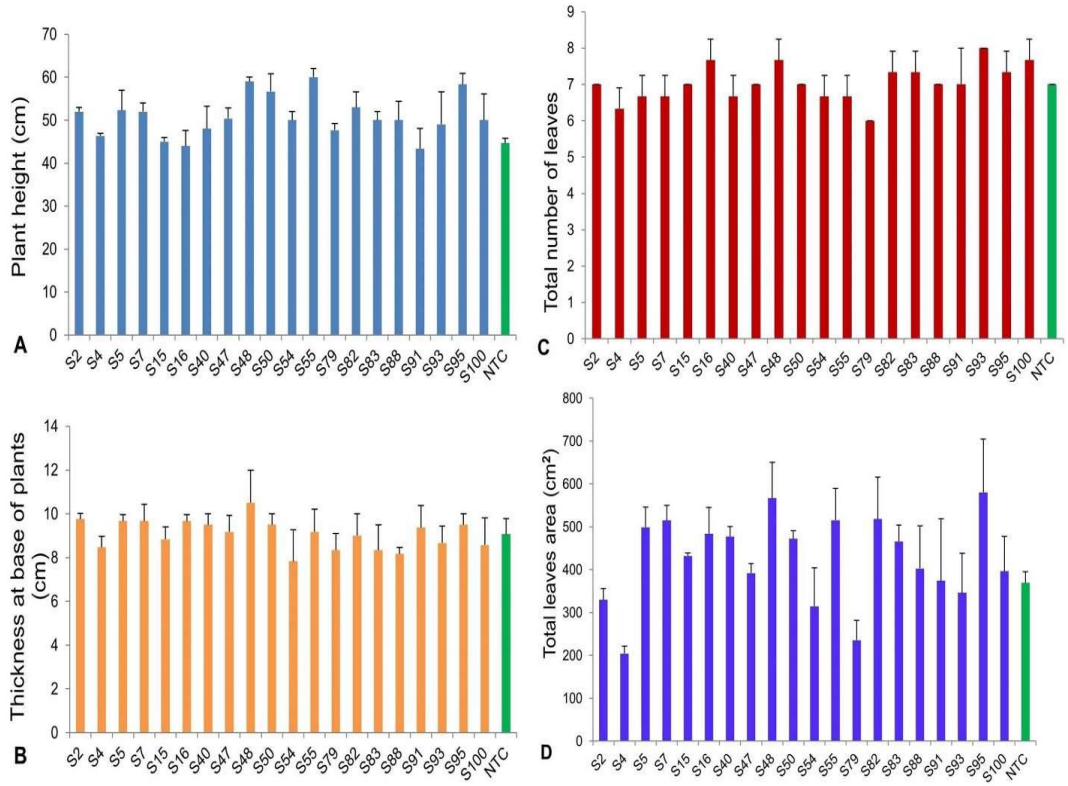


Figure 4.6: Assessment of plant growth parameters in transgenic plants (S2-S100) and non-transgenic control plants (NTC). **A)** Plant height (cm), **B)** Pseudostem thickness at base (cm), **C)** Number of total green leaves, and **D)** Totals leaves area. Plant growth parameters were recorded at 90 days after planting in pots in the glasshouse. All values were mean and standard deviation of three replicates of each transgenic events and NTC.

4.3.5 Disease evaluation against *Xanthomonas campestris* pv. *musacearum* in glasshouse

The twenty transgenic events (S2, S4, S5, S7, S15, S16, S40, S47, S48, S50, S54, S55, S79, S82, S83, S88, S91, S93, S95 and S100) were evaluated in small pots in the glasshouse. Out of the twenty tested, two events (S48 and S100) showed no symptoms at all, even at 60 dpi, demonstrated complete resistance (Figure 4.7 and Table 4.2). NTC plants developed symptoms about 13 dpi. All leaves of NTC plants were completely wilted and the plants died within 30 days (Figure 4.7 and Table 4.2). Twelve transgenic events showed reduction in disease symptoms. The six transgenic events (S4, S40, S50, S54, S55 and S79) were susceptible that showed disease symptoms and completely wilted (Table 4.2).

Figure 4.7: Disease evaluation of transgenic plants in the glasshouse against *Xanthomonas campestris* pv. *musacearum* (Xcm). **A)** Non-transgenic control plants of 'Sukali Ndiizi', **B)** Transgenic plants showed resistance (S48 and S100). The leaves with white tag were injected with Xcm. Scale bar 10 cm.

Table 4.2: Disease evaluation of transgenic events harboring the rice *NH1* gene after of *Xanthomonas campestris* pv. *musacearum*

S. No.	Appearance of first symptoms (No. of days)	Complete wilting (No. of days)	Resistance %	Remarks
S2	24 ± 4.2 c	NCW	90.6 ± 8.1b	R
S4	17.3 ± 2.5e	29 ± 2b	0 ± 0e	S
S5	28.7 ± 2b	NCW	85.6 ±1.1c	R
S7	27.3 ± 2.5b	NCW	81 ± 5.2c	R
S15	26.3 ± 4.9c	NCW	72.3 ± 2.3c	R
S16	31.7 ± 2.5a	NCW	73.6 ±2.3c	R
S40	23.7 ± 9.0c	30.7 ± 0.5b	0 ± 0e	S
S47	26 ± 4.2c	NCW	90.8 ± 8.1b	R
S48	NW	NCW	100 ± 0a	R
S50	24.7 ± 9.3c	30.1± 1.0b	33.3 ± 28.8d	S
S54	21.7 ± 9.9d	34 ± 9.6a	0 ± 0e	S
S55	26.7 ± 3.5c	31± 3.44b	44 ± 39.1d	S
S79	19 ±7e	30.3±4.5b	0 ± 0e	S
S82	32.3 ± 2.5a	NCW	74.1 ± 20.9c	R
S83	29 ± 7.9b	NCW	78.3 ± 5.7c	R
S88	30 ± 0a	NCW	90.3 ±16.7b	R
S91	23 ± 8.0c	NCW	76.3 ± 9.2c	R
S93	23.3 ± 8.6c	NCW	63 ±10.4d	PR
S95	30.3 ± 0.5a	NCW	86.6 ± 1.4c	R
S100	NW	NCW	100 ± 0a	R
NTC	12 ± 3.4e	27.7 ± 3.1c	0 ± 0e	S

Abbreviations: NW-no wilting symptoms; NCW- no complete wilting of infected plant; R-resistance; PR- partial resistance; S-susceptible; Values were mean and standard deviation of three replicates of each transgenic events. Bacterial wilt symptoms were observed up to 60 days post inoculation (dpi). Mean values indicated with the same letter are not significantly different from each other by Tukey 's family error test ($P \leq 0.05$)

Appearance of bacterial wilt disease symptoms showed significant differences ($P < 0.05$) in resistance all the transgenic events compared to NTC plants (Table 4.2). In transgenic events delayed symptoms were observed. Transgenic events (S50, S55 and S93) were partially resistant. Two transgenic events (S48 and S100) were 100 % resistant to Xcm, with no internal symptoms observed after cutting the pseudostem at 60 dpi except some necrosis at the point of inoculation. However, yellow exudation was observed in the susceptible transgenic events and in non-transgenic control plants. The pathogenic bacteria recovered from infected and dead plants and confirmed as Xcm based on their morphology like dark yellowish, mucoid and circular colonies.

4.3.6 Expression profile of pathogenesis related genes in *NH1* transgenic events

4.3.6.1 Expression profile of *PR1* gene

Reverse transcription of the banana *PR1* gene was investigated in three transgenic events S15, S47 and S100 expressing the rice *NH1* gene and NTC plants. Expression profile of *PR1* gene varied in all the transgenic events in comparison to NTC at various time point after inoculation with Xcm indicating that *PR1* gene was induced against Xcm. At time zero, relative transcript levels for transgenic events varied from 0.5 to 0.9 which were not different from NTC (Figure 4.8 A). At 6 h and 12 h post inoculation (hpi) transcripts level increased many fold abruptly in all the transgenic events in comparison to NTC (Figure 4.8B and C). *PR1* gene had not expressed much at 24 hpi. Transcripts level of the *PR1* gene was higher in all tested transgenic events at 48 hpi than in the NTC (Figure 4.8E). Relative expression of *PR1* gene showed more than 100-fold increase in transgenic event S15, and about 700 folds increased of transcripts in S100 at 48 hpi (Figure 4.8E). Variation of *PR1* transcripts within the different transgenic events was also observed.

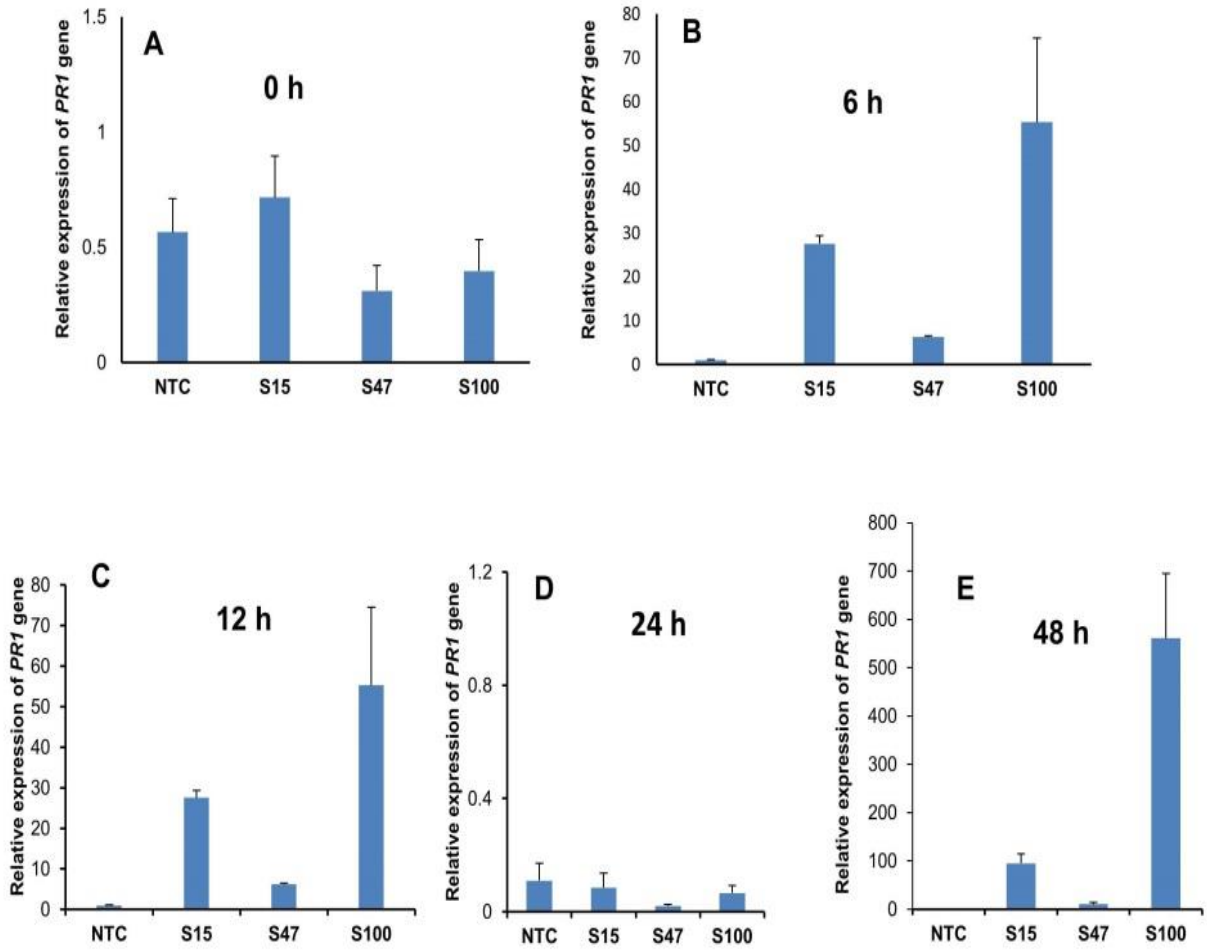


Figure 4.8: Relative expression of *PR1* gene in three transgenic plants (S15, S47 and S100) and non-transgenic control plant (NTC) after inoculation of *Xanthomonas campestris* pv. *musacearum*, **A)** at 0 hour-post inoculation (hpi), **B)** 6 hpi, C) 12 hpi, **D)** 24 hpi and **E)** 48 hpi. Values were mean ± s.e. of two biologicals and 4 technical replicates.

4.3.7.2 Expression profile of *PR2* gene

The effects of inoculation of Xcm on activation of *PR2* gene in transgenic plants with the *NH1* gene in comparison to NTC plants were also investigated at various time intervals (0, 6, 12, 24 and 48 hpi). There was a significant difference in *PR2* transcripts in all the transgenic events in comparison to NTC at 0, 6, 12 and 48 h pi (Figure 4.9A, B, C and E). The maximum induction of transcripts was in S15 and S100 at 6 hpi and 12 hpi respectively (Figure 4.9B and C). There were variations in induction of transcripts within the transgenic events as well.

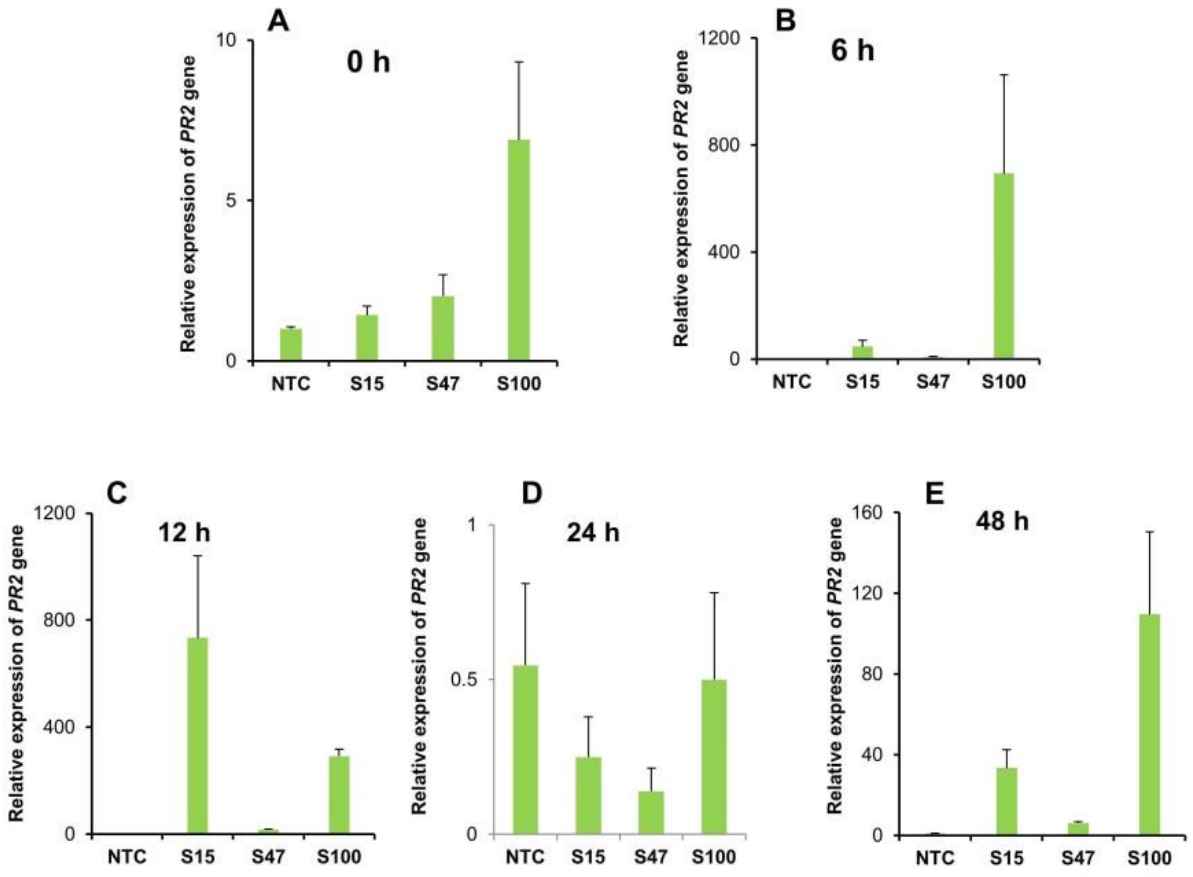


Figure 4.9: Relative expression of *PR2* gene in three transgenic plants (S15, S47 and S100) and non-transgenic control plant (NTC) after inoculation of *Xanthomonas campestris* pv. *musacearum*, **A)** at 0 hour-post inoculation (hpi), **B)** 6 hpi, **C)** 12 hpi, **D)** 24 hpi and **E)** 48 hpi. Values were mean ± s.e. of two biologicals and 4 technical replicates.

4.3.7.3 Expression profile of *PR3* gene

The expression profile of *PR3* gene in transgenic plants and NTC plants in response to Xcm was also checked at various time intervals (0, 6, 12, 24 and 48 hpi). There was no significant difference in the expression of *PR3* transcripts in the transgenic events (S15 and S47) except S100 in comparison to NTC at 0 hpi (Figure 4.10A). A significant increase in induction of transcripts was detected in the transgenic events, S15 (13 folds), S47 (3 folds) and S100 (7 folds) at 6 hpi (Figure 4.10B). The expressions of transcripts subsequently increased at 24 hpi in S15 (700 folds), S47 (50 folds) and S100 (150 folds) and expression levels remained high in events S15 (500 folds), S47 (25 folds) and S100 (700 folds) at 48 hpi (Figure 4.10 C and D).

Expression of transcripts did not significantly increase in transgenic events except S47 at 12 hpi in comparison to NTC. Even though induction of *PR3* transcripts was detected in the NTC across all time points, but it was lower than transgenic plants.

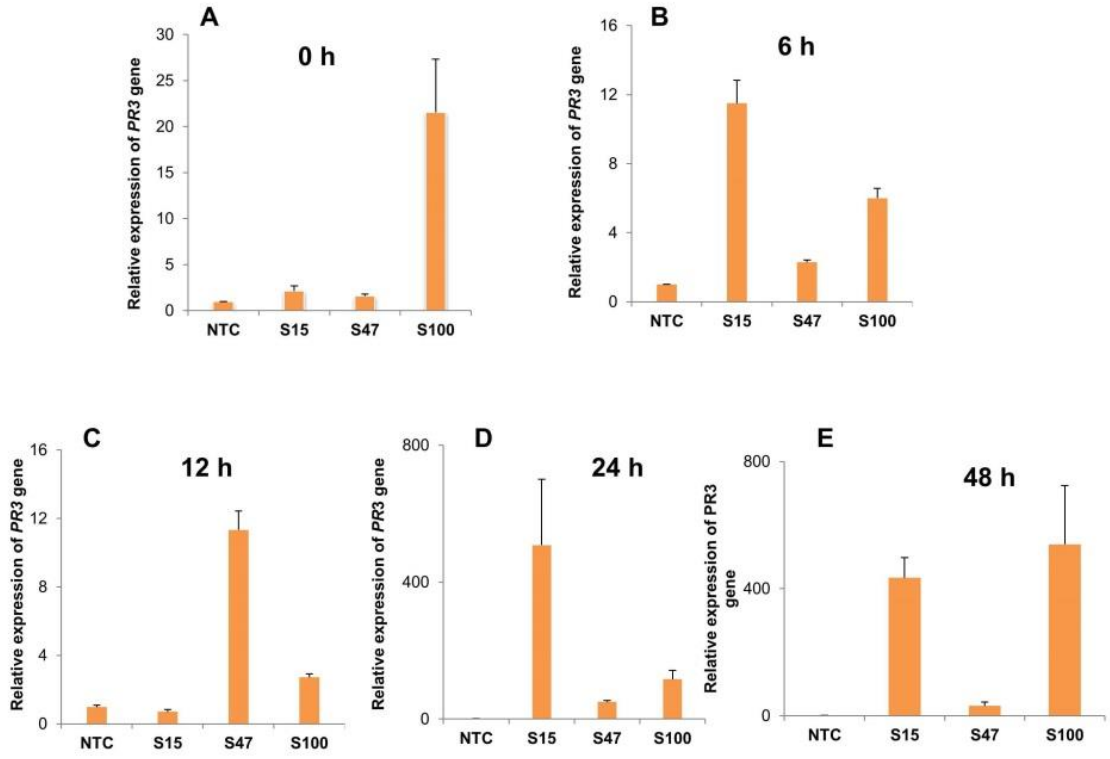
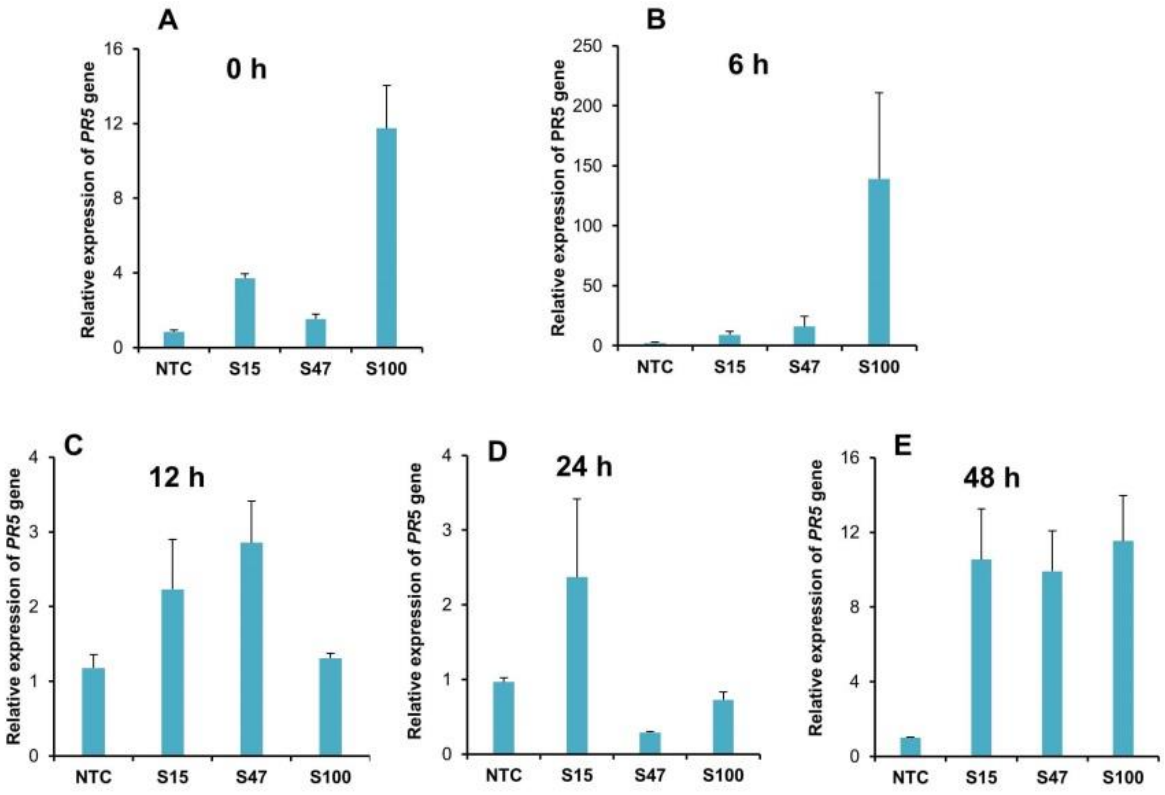


Figure 4.10: Relative expression of *PR3* gene in transgenic plants S15, S47 and S100) and non-transgenic control plant (NTC) after inoculation of *Xanthomonas campestris* pv. *musacearum*, **A)** at 0 hour-post inoculation (hpi), **B)** 6 hpi, **C)** 12 hpi, **D)** 24 hpi and **E)** 48 hpi. Values were mean ± s.e. of two biologicals and 4 technical replicates.

4.3.7.4 Expression profile of *PR5* gene

There was significant induction ($P<0.05$) of *PR5* transcripts in transgenic events S15 (4 fold), S47 (2 fold) and S100 (13 fold) in comparison to NTC at 0 hpi (Figure 4.11A). Induction of *PR5* transcripts increased at 6 hpi (Figure 4.11B). Transgenic line S100 showed maximum induction in comparison to other events and NTC at 6 hpi. At 12 hpi and 24 hpi induction was stable even though it was increased (Figure 4.11C and D). At 48 h pi *PR5* transcripts had

increased several folds in all the three transgenic events in comparison to control (Figure 4.11E).



F**igure 4.11:** Relative expression of *PR5* gene in transgenic plants (S15, S47 and S100) and non-transgenic control plant (NTC) after inoculation of *Xanthomonas campestris* pv. *musacearum*, **A)** at 0-hour post inoculation (hpi), **B)** 6 hpi, **C)** 12 hpi, **D)** 24 hpi and **E)** 48 hpi. Values were mean ± s.e. of two biologicals and 4 technical replicates.

4.4 Discussion

In this study, embryogenic cells of banana cultivar 'Sukali Ndiizi' was successfully transformed with the rice *NH1* gene. Transformed embryos were regenerated into the well-rooted plantlets on hygromycin B selective medium. These independent transgenic plants were clonally micro-propagated and multiplied in tissue culture. Well acclimatized 90 days old plants in the glasshouse were challenged with Xcm. These findings showed that over-expression of the rice *NH1* gene in transgenic banana conferred enhanced resistance to Xcm under glasshouse conditions. This is a substantial finding, considering the importance of BXW

disease in Africa (Tripathi *et al*., 2009a). It is also possible that these transgenic plants may also provide resistance to other pathogens as reported in numerous other studies using the *NPR* gene (Cao *et al*., 1998; Chern *et al.*, 2001, 2005; Lin *et al.*, 2004; Makandar *et al*., 2006; Yuan *et al*., 2007; Parkhi *et al.*, 2010). Over-expression of the *NPR1*/*NH1* gene has, however, resulted in some developmental abnormalities on the leaves of transgenic rice plants in previous studies (Fitzgerald *et al*., 2004; Chern *et al*., 2005; Quilis *et al*., 2008; Silva *et al.*, 2015). No major morphological abnormalities were seen in transgenic banana plants in the growth analysis study, although, the two transgenic events produced taller and greater total leaf area in comparison to the non-transgenic control plants (Figure 4.6). These results need to be further investigated in fully grown plants under confined field conditions.

Glasshouse assessment of transgenic plants is essential for preliminary investigation of disease resistance against pathogens. Earlier studies of artificial inoculation of the glass house plants have been demonstrated to compare well with field resistance (Amand *et al.*, 1995, Tripathi *et al.*, 2014b). However, it was reported that variability in screening tests could be challenging due to the higher pressure of inoculum of pathogens in glasshouse than the farmer`s field (Amand *et al*., 1995). In glasshouse evaluation of transgenic plants, three categories of plants were recovered. One group of transgenic plants which completely survived artificial inoculation of pathogens, called resistant, the second group called partial resistance in which the inoculated leaf as well as 2—3 other leaves wilted but the whole plant did not wilt and the third group which the whole plant completely wilted in the same way as non-transgenic control plants even though the appearance of symptoms were delayed. The reason might be the position of insertion of transgene in transgenic plants and copy number of transgene. It needs further study to the reason why some transgenic events were not show any resistance.

The results in this study of pathogenesis-related (*PR*) genes consistently show that all the *PR* genes were induced in the transgenic events at 6 hpi and 12 hpi in comparison to NTC plants. At 24 hpi, *PR1*, *PR2* and *PR5* did not show increased induction of transcripts. The reason might be that SAR spread out to the whole plant and plants were trying to neutralize the pathogens induced stresses. Non-transgenic control plants also showed induction of *PR* genes at various times after pathogen inoculation due to plant has natural resistance to survive from biotic and abiotic stresses. It needs further study to investigate *PR* genes expression in disease susceptible transgenic events. *PR* genes were again increased at 48 h to induce SAR mechanism and speed-up the healing process. The induction pattern is quite similar in all *PR* genes. PR1, the highly available PR protein, is induced up to 10,000-fold in infected tissues and accumulates to 1 to 2 % of the total leaf protein (Alexander *et al.,* 1993). Therefore, the expression of *PR1* genes can serve as display of the activation of the plant defense mechanism. It was also reported in non-transgenic *Musa* cultivar 'Cavendish' that *Musa PR1* and *PR2* (*MNPR1 and MNPR2* genes) were induced after inoculation with *Xanthomonas* (Yocgo *et al.,* 2012).

NPR1 gene is also induced by treatment with Salicylic acid, 2, 6-dichloroisonicotinic acid, or benzothiadiazole in *Arabidopsis* (Cao *et al.,* 1997; Ryals *et al.,* 1997). *PR1* gene expression is mainly responsive to salicylic acid and therefore, it is used as a marker for the salicylic acid-dependent SAR response in *Arabidopsis* and tobacco (Ryals *et al*., 1997). *PR1*, *PR2* and *PR4* genes were induced in transgenic tomato plants with *AtNPR1 gene* but no phenotypic abnormalities have been detected (Lin *et al.*, 2004). Wheat plants harboring *AtNPR1* had no constitutive increase in *PR1* gene expression (Makandar *et al.,* 2006) but tobacco plants showed increased *PR1* expression, similar to the transgenic tomato plants (Meur *et al.*, 2008). No abnormalities were seen in plant physiology in the transgenic wheat or transgenic tobacco with *NPR1* gene (Makandar *et al.,* 2006). Transgenic apple containing *NPR1* homolog *mpNPR1* had

slight increase in some *PR* genes, with no morphological changes on plant growth (Malnoy *et al.,* 2007). In rice, endogenous salicylic acid (SA) is more than other crops (Silverman *et al*., 1995). This endogenous SA is responsible for the constitutive induction of *PR* genes, and the abnormal effect on plant growth associated with constitutive SAR induction excessively to enhance defense mechanism (Clarke *et al.,* 1998; Heil *et al*., 2004). The results confirm that *NPR1* gene provides resistant due to over expression of several *PR* genes that encode various pathogenesis-related plant proteins.

The variation in resistance level of transgenic plants depends on multiple factors like the position of the transgene insertion, the copy number of transgene and expression levels of the transcripts, the concentration of bacterial inoculum, conditions of the glasshouse and finally individual plant health (Tripathi *et al*., 2010; Namukwaya *et al.,* 2012). Moreover, methylation of transgenes, promoter methylation, methylation at non-coding sites can also act as mechanisms of transcriptional gene silencing (Fu *et al*., 2000). It is assumed that the high levels of *NH1* expression, as driven by the constitutive *ubiquitin* promoter causes toxicity leading to lethality due to the severe hypersensitive reaction after artificial inoculation. Similar results have been reported with the *NH3* and *OsNPR3* genes under a maize *ubiquitin* promoter (Yuan *et al*., 2007; Bai *et al*., 2010).

This is the first study to validate that insertion of the rice *NH1* gene in transgenic banana confers enhanced resistance against Xcm. Since the *NPRI*/*NH1* gene is a master regulator of SAR related activity, it may provide broad-spectrum resistance in transgenic banana against several other diseases which are caused by fungi, viruses, and nematodes. Since disease development can be affected by the age of the host plant, natural environmental conditions (rain, temperature, and humidity) and bacterial inoculum pressure and virulence, the transgenic

banana events generated in the current study need to be evaluated for agronomic performances, gene stability, and potential protection against other banana diseases in further confined field trial study in future.

CHAPTER FIVE

EXPRESSION OF THE RICE *NH1* GENE IN BANANA PROVIDES RESISTANCE TO *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* RACE 1

5.1 Introduction

Fusarium wilt (Panama disease) is known as one of the deadliest soil born fungal disease of banana and is caused by *Fusarium oxysporum* f. sp. *cubense* (E. F. Smith; Snyder and Hansen (Foc) (Ploetz, 2015). There are three races (1, 2, and 4) of Foc which severely infect banana cultivars, and race 3 shows infection in Heliconia species (Waite, 1963; Wu *et al*., 2010). Foc race 1 attacks 'Gros Michel' (AAA genome), 'Sukali Ndiizi' and 'Silk' (AAB genome) banana cultivars. Bancroft (1876) reported first time about Fusarium wilt disease in banana in Australia. Fusarium wilt was originated in South-East according to phylogenetic research by various researchers (O' Donnell *et al*., 1998; Ploetz and Pegg, 2000). Banana production is seriously threatened by the Fusarium wilt globally (Ploetz, 2015). The banana growing companies in Central American countries and the Caribbean islands was completely devastated by Foc race 1 in the mid-twentieth century causing a shift to the resistant cultivar 'Cavendish Williams', which now constitutes of 99 % of export banana (Ploetz and Pegg, 2000).

Later, a new fungal strain of Foc was discovered known as Tropical race 4 (TR4) has overawed Foc resistance in 'Cavendish Williams' and other banana varieties (cooking, roasting, juice producing and plantain) which are grown mainly by small holder farmers. Foc race 4 was identified in banana growing region of Taiwan in the 1960 (Hwang and Ko, 2004). It infects mainly dessert cultivars especially 'Cavendish Williams' which is highly prone to race 1 and 2. Foc race 4 is further divided into subtropical and tropical race 4. Subtropical race 4 infects banana plants growing in the region with cold weather, whereas tropical race 4 infects plants

in any hot and humid environment or other predisposing factors (Ploetz, 2006; Wu *et al*., 2013). The most of global banana production (approximately 80 %) is depend on TR4 susceptible germplasm mainly 'Cavendish' and 'Gros Michel' cultivars (Ploetz, 2015). The history of Foc subspecies has been reviewed (Stover, 1962; Ploetz and Pegg, 2000; Pérez-Vicente, 2004; Ploetz, 2015). Fusarium wilt has infected banana plantation rapidly over the last ten years in East and Central African region and has now become a major production limitation of banana causing severe losses in farmer fields (Tushemereirwe *et al*., 2004b). The 'Sukali Ndiizi' is important dessert cultivar in East and Central Africa and highly prone for fungal diseases (Tushemereirwe *et al*., 2004b).

Infected planting material, rain water, infected soil, cutting tools, footwear, grazing animals, and tractors can efficiently disseminate the pathogen from one localized place to the entire field. The fungus can stay alive in soil for more than 20 years, and has a long survival period (Stover, 1972; Vicente *et al*., 2004). Banana infection occurs through primary and secondary roots (Li *et al.*, 2011). Early detection of disease symptoms in the banana plants and speedy laboratory diagnostic and strict quarantine regulation are the important steps to containment of any eventual outbreak (Vicente *et al.,* 2004; Ploetz, 2015).

Currently, there are no effective technology available for prevention and spread of Foc (Ploetz, 2015). Chemical control or biocontrol methods are ineffective or partly effective, and Fusarium spores mainly chlamydospores remain viable in the soil for several years making infested ground unsuitable for cultivation of disease prone banana varieties (Ploetz, 2015). The only option for controlling the disease is use of Foc resistant banana varieties generated by either banana breeding or genetic engineering (Paul *et al.,* 2011; Dale *et al*., 2017). Few banana varieties are resistance to Foc in wild banana varieties but most of banana cultivars are prone

with this deadly fungal disease (Ploetz, 2006). Conventional banana breeding is not very promising to provide a solution due to the long generation cycle, polyploidy and seedless nature of crop (Pillay and Tripathi, 2007).

Expression of the *AtNPR1* gene or its homologs has been shown to enhance resistance to fungal, viral and bacterial pathogens in several economically important crops including rice, wheat, grape, carrot, tomato, apple, citrus, tobacco and strawberry (Chern *et al*., 2005; Le Henanff *et al*., 2009; Lin *et al.*, 2004; Makandar *et al.*, 2006; Malnoy *et al.*, 2007; Meur *et al.*, 2008; Wally *et al*., 2009; Yuan *et al*., 2007; Zhang *et al*., 2010; Silva *et al*., 2015). These reports indicate that *AtNPR1* and its homologs are useful genes for genetic modification to develop disease resistance crops (Cao *et al*., 1998; Chern *et al.*, 2001; Friedrich *et al*., 2001; Lin *et al.*, 2004; Silva *et al*., 2015).

Genetic engineering has the potential to provide disease resistance resistant banana. Various published reports (Paul *et al.*, 2011; Mahdavi *et al.*, 2012; Magambo *et al*., 2016; Dale *et al*., 2017) confirm that genetic modification of banana can provide significant resistance against Foc subspecies. An endogenous banana *Musabag1* gene have shown resistance against Foc race 1 in transgenic banana cultivar 'Rasthali' (Ghag *et al*., 2014) and a nematode anti-apoptosis gene *Ced 9* has conferred resistance in 'Lady Finger' banana (Paul *et al*., 2011). Till now no genetically modified disease resistant bananas are commercially available. Here it was investigated whether expression of the rice *NH1* gene in banana would confers resistance to Foc race 1.

5.2 Materials and methods

5.2.1 Multiplication of *NH1* transgenic banana plants

Fifteen transgenic events with *NH1* gene were used for disease evaluation against Foc race 1. These 15 transgenic events were multiplied by monthly subculture in tissue culture with 16/8 light dark cycle at 26 ± 2 °C in 3 months. Five to eight replicates of each transgenic event were weaned and acclimatized in the glasshouse in sterile soil under natural conditions. Ten non-transgenic (positive) control plants of 'Sukali Ndiizi' cultivar (5 replicates for inoculation and 5 replicates for non-inoculation) were also included. Additionally, ten non-transgenic (negative) control plants of the East African highland cooking banana cultivar 'Kisansa' which is supposed to be resistant cultivar to Foc race 1 (5 replicates for inoculation and 5 replicates for non-inoculation) were also included.

5.2.2 Fungal culture maintenance

Foc race 1 (VCG 0124) culture was acquired from National Agriculture Research Laboratory (NARL), Uganda. Cultures were maintained in potato dextrose agar medium (PDA) at 25 ± 3 °C by regular two monthly subcultures into fresh medium supplemented with Ampicillin (200 mg/L).

5.2.3 Preparation of fungal spores

Foc race 1 (VCG 0124) isolate was grown on 90 mm petri dishes containing potato dextrose agar medium (PDA) supplemented with ampicillin (200 mg/L) at 25 ± 3 °C for 10 days. Red millet (*Echinochloa esculenta*) grains were used to grow fungal pathogens on its surface to inoculate 90 days fully grown potted transgenic plants in glasshouse (Smith *et al*., 2008). Millet grains were washed 3—4 times with running tap water in a bucket to remove all the dust

particles and finally washed 1 time with distilled water. It was autoclaved for 60 min at 121 °C then cool down up to room temperature before adding fungal spores. Aliquots of 50 gm millet grains in 50 ml sterile falcon tubes dispensed to polythene bag with 5 pieces (1 cm^2) of fungi were added in each bag and mixed properly. All the packets were incubated at 25 ± 3 °C for 8 to 10 days to grow on the surface of red millets. Millet grains were mixed properly for uniform growth of fungal spores every alternate day. Before adding spores to each plant, all the packets were opened in a 15 L plastic bucket and mix properly to make a uniform spore culture.

5.2.4 Infection of plants

Fully acclimatized well rooted 90-day old plants bearing six to eight leaves were used for fungal infections. Each plant was up rooted and carefully replanted in a new pot containing fungal spores. Plastic pots were initially (30 cm diameter) one quarter filled with sterile soil and with gravel on top then 50 gm millets with fungal spores was added after which plant was planted and pot was filled with sterile soil and gravel. For consistency of equal inoculum pressure 50 gm of fungal spore coated pearl millets were used for each transgenic as well as non-transgenic control plant. Plants were irrigated every alternate day. Leaf symptoms like yellowing, browning and wilting were recorded at 2^{th}, 4^{th}, 6^{th} and 8^{th} week interval. Final data were recorded on 9^{th} week and the experiments were terminated. Foc race 1 was re-isolated from symptomatic plants to confirm presence of pathogens in infected plants. Photographs were captured using Nikon D7100 camera.

5.2.5 Disease evaluation scale

For Fusarium wilt disease evaluation yellowing and wilting of leaves, splitting or tearing of pseudostem, reddish brown colouration of internal corm and pseudostem were recorded using method reported by Mak *et al.,* (2001) with some modifications. Disease evaluation scale

(Table 5.1) was divided into 1—5 for yellowing and wilting of leaves (mild, moderate, severe and dead). Plant pseudostem splitting was divided into 1—4 scale and corm discoloration were categorized into a 1—5 scale (Table 5.1). External symptoms were recorded first by counting number of yellow and wilted leaves in each plant. For assessment of the internal symptoms, each plant was removed from the pot and upper half of the pseudostem and leaves were discarded. Corm and the root section and 5 cm of pseudostem was retained (Magambo *et al*., 2016). The roots of each plant were washed in a bucket full of water to remove all the soil from the roots and pseudostem after that primary and secondary roots were also cut with clean knife and discarded then pseudostem was cut longitudinally through the corm in to two halves. Five-point scales were used to compare the level of discolorations due to infection in corm tissue and to calculate the rhizome discoloration index (RDI). To determine resistance levels of the transgenic plants in comparison to control non-transgenic plants, DSI (%) was calculated through LSI and RDI of each replicate (Magambo *et al*., 2016).

5.2.6 Disease severity index

Disease severity index was calculated using the formula as mentioned below:

Disease severity index for leaf and rhizome (DSI) (%) =

$$\frac{\Sigma\ (\text{No.of scale x No.of plantlets in the scale})}{\Sigma\ (\text{No of treated plantlets})\ Maximum\ scale}\ x100$$

Disease scale category is based 1-5 on leaves and rhizome symptoms grading.

The DSI is further classified into four groups mainly (0—20 %) resistant, (21—40 %) tolerant, (41—70 %) susceptible and (71-100 %) highly susceptible (Mak *et al*., 2001). If the fungal spores' inoculated plant is resistant for leaf symptoms (LS) and tolerant in rhizome symptoms (RS), the cultivar is considered as disease tolerant.

Table 5.1: Description of disease evaluation scales for yellowing and wilting of leaves, pseudostem splitting or tearing and rhizome or corm reddish brown discoloration

Disease severity scale	**Leaf yellowing**	**Wilting**	**Pseudostem splitting**	**Corm discolorations**
1	No yellowing of leaves	No wilting	No splitting	No discoloration of corm
2	Yellowing of lower leaves (Mild)	Mild wilting <30 %	Mild splitting	Discolorations of root and corm intersection
3	Yellowing of the most of lower leaves (Moderate)	Moderate > 50 %	Moderate	Discoloration of 30 % of corm
4	Yellowing of the all the leaves (Severe)	Severe > 90 %	Severe	Discoloration of 30 to 50% corn
5	All the leaves turned brown (Dead plant)	Dead plant		Discoloration of 50 to 100 % of corm

If RS is tolerant and LS is susceptible, the cultivar is known susceptible. The rank of the cultivar is measured as resistant when both LS and RS show no symptoms either in leaves or rhizome.

If the both responses are tolerant, then cultivar is considered as tolerant (Mak *et al*., 2001; Magambo *et al.*, 2016).

5.2.7 Plant RNA extraction and cDNA synthesis

Total plant RNA was isolated using Qiagen RNeasy mini-kits (UK) with slight modifications from freshly collected leaf sample (200 mg) from transgenic and NTC plant samples. The RNase Inhibitor, murine (NEB, UK) was used during RNA extraction to prevent RNA degradation. Total RNA was treated with DNase I (NEB, UK) at 37 °C for 1 h. The cDNA was synthesized with 2 µg of DNase treated RNA using the Maxima First Strand cDNA Synthesis kit (Fermentas, UK) and oligo-dT primers according to manufacturer's instruction with slight modifications. Program used for cDNA synthesis was 25 °C for 10 min, 55 °C for 30 min and reaction was terminated at 85 °C for 5 min. ABI 7500 (Applied Biosystems, Foster City, CA, USA) thermocycler was used for cDNA synthesis.

5.2.8 Reverse Transcription (RT-PCR) analysis

Reverse Transcription-PCR (RT-PCR) was performed using 1 µL cDNA and primer pairs specific to the *NH1* gene: forward *NH1* primer 5`ACTTAGCTCGGATGACGGCAC 3` and reverse *NH1* primer 5` AGCAATGGTGTTCATCTCCTTGGT 3`. Amplification of the *Musa 25* S ribosomal gene, used as an internal control to determine the quality of cDNA, was performed using the forward primer *Musa F*: 5'- ACATTGTCAGGTGGGGAGTT -3' and the reverse primer *Musa R*: 5'- CCTTTTGTTCCACACGAGATT -3'. For the *NH1* gene-specific primers, PCR conditions were set as follows: 95 ^{0}C for 10 min for denaturation and 35 cycles of amplification of cDNA at 94 ^{0}C for 30 s, 55 ^{0}C for 90 s and 72 ^{0}C for 60 s, and 72 ^{0}C for 10 min for extension of amplified product. For the *Musa* 25 ribosomal gene PCR conditions were set as follows: 95 ^{0}C for 10 min for denaturation and 35 cycles of amplification of cDNA at 94

^{0}C for 30 s, 60 ^{0}C for 40 s, and 72 ^{0}C for 50 s, and 72 ^{0}C for 7 min for extension of amplified product. The amplified PCR products were separated by electrophoresis on 1.2 % agarose gel, visualized under a UV transilluminator. Gel documentation system with attached camera and computer was used to capture photograph. A negative control (non-transgenic control plant cDNA) was included in each experiment.

5.2.9 Quantitative real time PCR (qRT-PCR)

For quantitative real-time (qRT-PCR), synthesized cDNA was diluted 10-fold with nuclease free distilled water in PCR workstation. Primers used for qRT-PCR were *NH1* gene specific forward primer F: 5`TGTCCTTGATAAGGTTGAAGTAGA 3` and reverse primer R: 5`CAAGGTTTGACCGGACTACCA 3`. The *Musa* 25S ribosomal gene specific primes as endogenous reference gene was used as mentioned previously.

qRT-PCR was achieved with the 7500HT Real-Time PCR machine supplied by Applied Biosystems, Foster City, CA, USA using a Maxima SYBR Green/ROX qPCR master mix kits for detection (Thermo Scientific, UK). The reaction mixture contained 2 µl of 10-fold diluted cDNA, 0.3 µl of 10 µM of forward and reverse primers, 10 µl Maxima SYBR-Green/ROX master mix, and remaining nuclease-free water to make reaction volume 20 µl was added into respective wells of a 96 well qRT-PCR micro-titer plate and reaction was performed. Each reaction was set up in triplet. Non-template control reactions, containing water were also included into the analysis. qRT-PCR conditions were as follows: pre-incubation for 10 min at 95 °C, followed by 40 cycles consisting of a denaturation step at 94 °C for 10 s, primer annealing at 60 °C for 30 s and primer extension at 72 °C for 45 s. Amplified product specificity was determined by melting curve analysis (60 °C to 95 °C). PCR completion was followed by melt curve analysis (60–95 °C every 0.5 °C for 1 s) to remove non-specific amplified products.

Relative gene expression was determined with comparative CT method (Livak and Schmittgen, 2001): $2^{-\Delta\Delta CT}$; with $\Delta\Delta CT = \Delta CT_{TG}$ (CT target gene – CT reference gene) – ΔCT_{CG} (Non-transgenic control plant – CT reference gene). The *Musa* 25S ribosomal gene was used as an endogenous reference.

5.2.10 Statistical analysis

The data generated for the transgenic events and non-transgenic control plants (NTC) 'Sukali Ndiizi' and 'Kisansa' were scrutinized using disease severity index (DSI) values for leaves and rhizomes. The one-way analysis of variance (ANOVA) was achieved using Minitab 16 statistical software program. Dunnett's test was performed to examine the significance value ($p < 0.05$).

5.3 Results

Fifteen independent transgenic events were multiplied and assessed for resistance against Foc race 1 under glasshouse conditions.

5.3.1 Disease evaluation of transgenic events in glasshouse

Fifteen independent transgenic events (S13, S15, S16, S22, S47, S48, S50, S55, S62, S81, S83, S91, S93, S95 and S100) with (5–7 replicates each) were inoculated with Foc race 1 in the glasshouse (Figure 5.1). The 'Sukali Ndiizi' cultivar (AAB genome) as positive controls and East African Highland banana cultivar (EAHB) 'Kisansa' (AAA genome) as negative control were evaluated as non-transgenic controls. Disease symptoms were recorded every two weeks. Final recording of symptoms was performed at nine weeks post inoculation (wpi), the external symptoms (yellowing of leaves, wilting of complete plant, pseudostem splitting) and internal disease symptoms like rhizome discoloration were assessed after uprooting of the plant.

Figure 5.1: Transgenic banana plants with *NH1* gene inoculated with *Fusarium oxysporum* f. sp. *cubense race* 1 Photograph was taken at starting point of experiment. Scale bar 10 cm.

All inoculated plants of susceptible cultivar ‘Sukali Ndiizi’ (non-transgenic control) expressed disease symptoms like and yellowing of lower leaf and pseudostem tearing after 13—20 days post inoculation (dpi). Yellowing of leaves appeared first in the older leaves and then progressed upwards to the new leaves. The first symptom that appeared was swelling at the base of non-transgenic inoculated control plants ‘Sukali Ndiizi’ at 2 wpi and slight tearing of outer bract leaf on 2 to 3 wpi after inoculation (Figure 5.2A). That indicated pathogens successfully entered in the primary and secondary roots and blocked the vascular system of plants in the pseudostem. Gradually, pseudostem splitting became larger in size in 6 to 8 wpi (Figure 5.2B). Secondary symptoms such as the yellowing and browning of the lower leaves of symptomatic plants appeared at 3 to 4 wpi due to the blockage of the vascular systems. Gradually, this increased to other leaves until eventually most of the leaves turned yellowish

and brown and finally wilted. It was observed that sometimes, whole plant completely wilted with the bigger size of pseudostem splitting lesion.

Figure 5.2: Non-transgenic control plant of 'Sukali Ndiizi' showing early and delayed symptoms of pseudostem tearing after infection of *Fusarium oxysporum* f. sp. *cubense* race 1, **A**) early symptom appeared on 2rd to 3rd week post-inoculation and **B**) late symptoms profuse tearing of pseudostem at 6th week post-inoculation. Scale bar 10 cm.

The susceptible cultivar 'Sukali Ndiizi, showed both external symptoms like yellowing and wilting of most of the leaves and internal disease symptoms like rhizome and pseudostem discolouration in all replicates and most of the plants had pseudostem tearing (Figure 5.3A and 5.4). According to observations the resistant cultivar 'Kisansa' showed no external or internal symptoms of Fusarium wilt. This confirmed that Kisansa is resistant against Foc race 1 (Figure 5.3B and 5.4). This proved Foc race 1 culture was highly virulent and the technique of infection was uniform and robust. Rhizome and pseudostem reddish brown colouration were the most suitable indicator for assessment of susceptible, partial susceptible, tolerant and resistance plants in my observation because yellowing and wilting of banana plants leaves gave variable symptoms and sign.

Fifteen transgenic events (S13, S15, S16, S22, S47, S48, S50, S55, S62, S81, S83, S91, S93, S95 and S100) were challenged for Foc race 1 resistance up to 9 wpi in the glasshouse. Most of the transgenic events showed a significant resistance ($P<0.05$) compared to NTC plants of the susceptible cultivar ‘Sukali Ndiizi’ (Figure 5.3 C and D).

Out of 15 transgenic events evaluated, three transgenic events (S15, S22 and S62) fall in the category of resistant (with 0-20 % of DSI) and seven transgenic events (S47, S48, S50, S55, S82, S95 and S100) were tolerant with reduced external visual symptoms like yellowing of leaves and internal symptoms like discolorations of rhizome (Figure 5.4 and 5.5). The remaining five transgenic events (S13, S16, S83, S91 and S93) were found to be susceptible. These plants showed external as well as internal disease symptoms. Disease severity index for leaves and rhizome are shown in Figure 5.5. Some variations in disease symptoms appearance were also observed within replicates of independent transgenic events. No pseudostem splitting was seen in any transgenic events.

Figure 5.3: Evaluation of transgenic events harboring the rice *NH1* transgene against *Fusarium Oxysporun* f. sp. *cubense* race 1 in the glasshouse. **A**) Non-transgenic 'Sukali Ndiizi' inoculated plants showing severe disease symptoms **B**) Non-transgenic Kisansa inoculated plants showing no disease symptoms, **C and D**) Inoculated transgenic events (S15, S22, S48, S62, S47, S55, S82, S91, S95 and S100) showing no symptoms or minimal yellowing of lower leaves. Photographs were taken on 9th week of post-inoculation. Scale bar 10 cm.

Figure 5.4: Internal disease symptoms in vertical section of rhizomes and pseudostem of non-transgenic control and transgenic plants**.** PC- positive non-transgenic control inoculated 'Sukali Ndiizi' showing rhizome discoloration and pseudostem tearing, NC-negative non-transgenic control inoculated 'Kisansa' showing no rhizome discoloration, transgenic events (S15-S100) showing no symptoms or reduced symptoms of rhizome discoloration. All the photographs have been taken on 9th weeks of post-infection. Scale bar 4 cm.

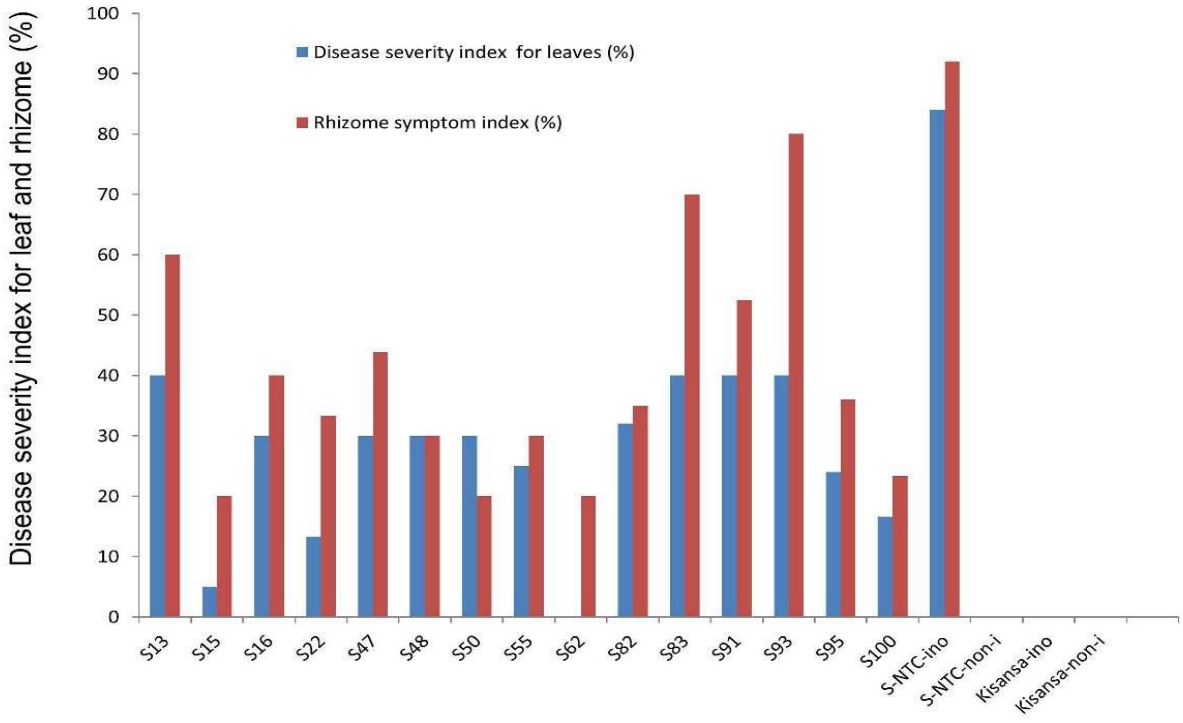


Figure 5.5: Disease severity index (DSI) assessment of transgenic plants in the glasshouse after infection of *Fusarium oxysporum* f. sp. *cubense* race 1. Percentages of leaves and rhizome symptoms were shown at nine weeks. Transgenic plants: S13 to S100; S-NTC-ino- 'Sukali Ndiizi' non-transgenic control inoculated plants; S-NTC-non-ino- 'Sukali Ndiizi' non-transgenic control non-inoculated plants; Kisansa-ino- 'Kisansa' inoculated plants; Kisansa-non-i- Kisansa non-inoculated plants. Values were based on 5 replicates of each transgenic events and non-transgenic control plants.

5.3.2 Gene expression analysis

To determine whether there was any relationship between *NH1* transgene expression and disease resistance in transgenic and nontransgenic control plants (NTC), ten transgenic events (S15, S16, S22, S47, S55, S62, S82, S91, S93 and S100) and NTC were further selected for qRT-PCR. Initial study was performed by RT-PCR of selected transgenic events and NTC. An amplified *NH1* transcript of 280 bp was present in the tested transgenic events (Figure 5.6). NTC plant has shown no amplified product. The amplification of the internal control *Musa 25S* ribosomal gene amplified product was seen in all the transgenic events and NTC. The size of

amplified product from *Musa 25S* primers was 106 bp. This was achieved to determine the quality of cDNA.

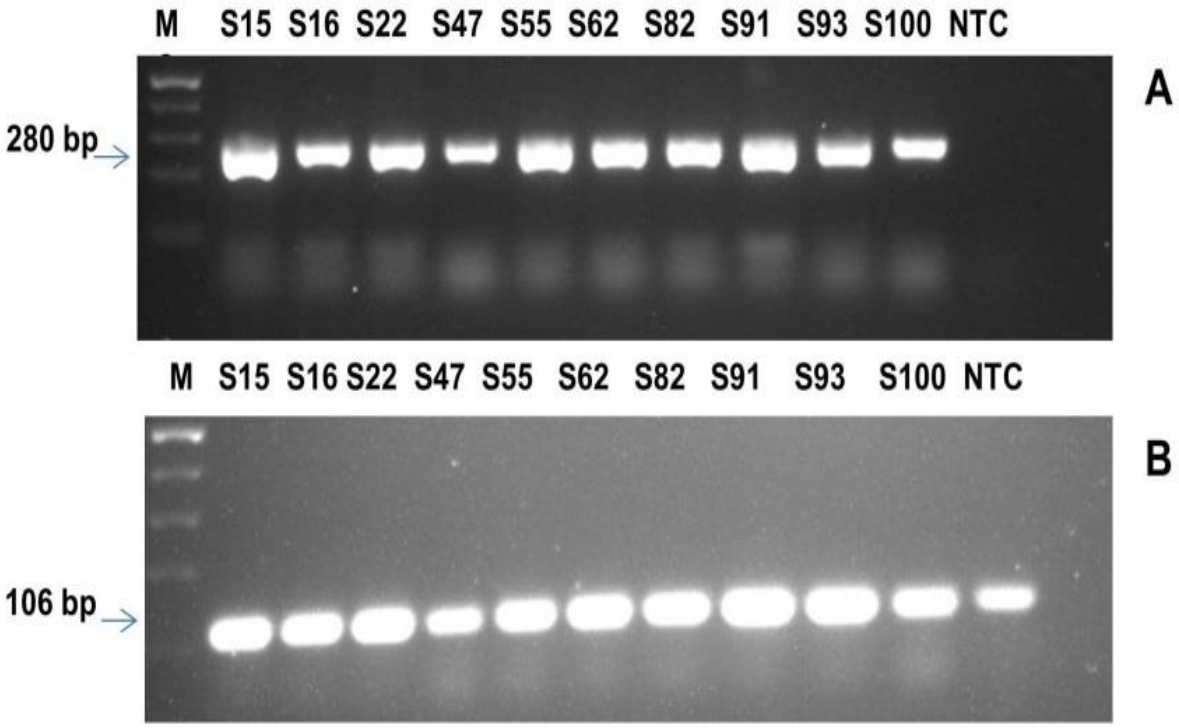


Figure 5.6: Gene expression analysis by Reverse Transcription-PCR (RT-PCR)**. A)** RT-PCR was performed from 10 transgenic plants (S15, S16, S22, S47, S55, S62, S82, S91, S93 and S100) and non-transgenic control plant using *NH1* gene-specific primer pairs and **B)** *Musa 25S* ribosomal gene specific primers, respectively. Abbreviations: M – 1kb plus molecular weight marker, NTC - non-transgenic control. 1.2 % agarose gel was used.

To understand the variation in resistant level of transgenic plants, qRT-PCR was performed with all the RT-PCR tested transgenic events. Variable amounts of transcripts were seen in transgenic events and NTC plants (Figure 5.7). This was well correlated with resistant transgenic events. Transgenic events (S15, S22, S47, S55, S62, S82 and S91), which showed resistance had higher expression in comparison to NTC plants. These transgenic events showed reduced external symptoms like yellowing and wilting of leaves and internal symptoms like rhizome discoloration.

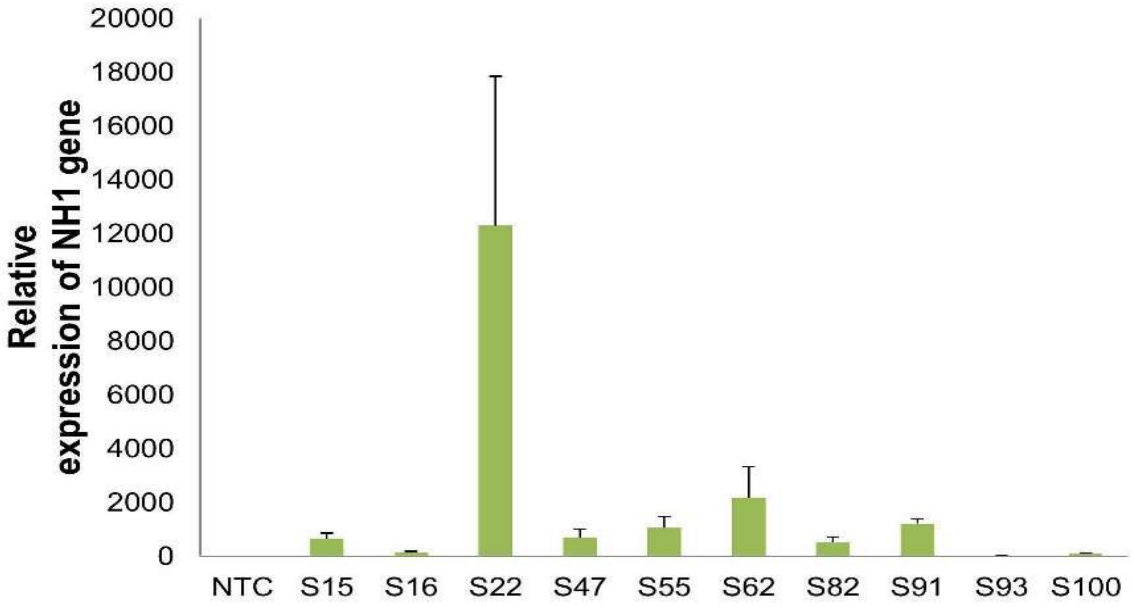


Figure 5.7: Relative expression of *NH1* transcripts in transgenic events (S15, S16, S22, S47, S55, S62, S82, S91, S93 and S100) and non-transgenic control plants (NTC). Expression of *NH1* gene was normalized with *Musa* 25S ribosomal gene (internal control) and non-transgenic control plant as a calibrator. Relative expression was determined from two biological replicates and six technical replicates. Values were mean ± s.e.

5.4 Discussion

In this study, the constitutive expression of the rice *NH1* gene, homolog of *Arabidopsis thaliana NPR1* gene, in genetically modified banana showed resistance to Foc race 1 under glasshouse assessment. Fusarium wilt susceptible cultivar 'Sukali Ndiizi' was successfully transformed with *NH1* gene using embryogenic cells. Fifteen transgenic events were clonally multiplied by regular subculturing in the plant transformation growth room facility. Five to seven replicates of each transgenic and NTC plants of 'Sukali Ndiizi' and 'Kisansa' were weaned in soil and acclimatized for the glasshouse evaluation against Foc race 1. Most of the tested transgenic events showed reduced external and internal symptoms of disease severity and increased level of resistance to Foc race 1 in comparison to NTC inoculated plants. Variations in resistance levels is well corelated with the presence of varied quantity of transcripts (Figure 5.7) in tested transgenic plants. Transcripts were more in S15, S22, S47,

S55, S62, S82 and S91 transgenic events in comparison to other transgenic events and NTC plants and performance of these events was also better against Foc race 1. Some of the transgenic events (S16 and S93) did not show enhanced resistance which might be due to post-transcriptional gene silencing, and positional effects of integration of gene sequences into the banana genome (Matzke and Matzke 1998; Kooter *et al.,* 1999).

The generation of transgenic 'Sukali Ndiizi' plants with enhanced resistance to deadly fungal pathogens is an important result toward development of disease resistance banana to reduce the impact of economic losses caused by this fungal disease. There are previous reports demonstrating resistance to Foc race 1 and TR4 using genetic engineering (Ghag *et al*., 2014; Paul *et al*., 2011; Dale *et al.,* 2017). However, to my knowledge this is the first report to demonstrate that over expression of the rice *NH1* gene in genetically modified banana confers significant resistance to Foc race 1. Fusarium wilt resistant transgenic plants were morphologically like non-transgenic plants in the glasshouse study, showing that the constitutive expression of the *NH1* gene is not affecting adversely on plant morphology of the genetically modified banana plants. This study has generated promising transgenic events for a future field trial to confirm the status of disease resistance with aim of enhancing food security for African farmers. Since the *NPRI*/*NH1* gene is SAR related, it may potentially provide broad-spectrum resistance to transgenic banana against diseases caused by fungi, viruses and even nematodes. This study is supported by similar other studies related to application of *NPR1* gene to generate disease resistance transgenic plants. As reported by Cao *et al.,* (1998) that expression of *Arabidopsis NPR1* leads to enhanced resistance with no unfavorable effect on the plant growth and morphology. Thus, for the first time, a single gene was demonstrated to confer resistance to bacterial and fungal pathogens in banana. Further, Chern *et al.,* (2001) reported that over-expression of the *Arabidopsis NPR1* in rice conferred enhanced resistance

to *Xanthomonas oryzae* pv. *oryzae* (Xoo) a severe bacterial blight pathogen. In a separate study, *Arabidopsis NPR1* gene was inserted into a tomato which showed drought tolerance and resistance to various pathogens (Lin *et al*., 2004). An apple *NPR1* gene homologue, *MpNPR1*, showed disease resistance when overexpressed in apple cultivars (Malnoy *et al*., 2007). Both monocot and dicot species share a conserved signal transduction pathway controlling *NPR1*-mediated defense mechanism to fight against pathogens (Chern *et al*., 2001). Transgenic rice plants with the rice *NH1* gene showed resistance to Xoo. The resistance was also shown to next generation plants and it was confirmed with the presence of the transgene and less inoculum of Xoo bacteria in the rice plants by bacterial growth study (Chern *et al*., 2005). Overexpression of the rice *OsNPR1 gene* in the both rice cultivars, Gui99 or TP309 led to enhanced resistance to blast fungus *Magnaporthe oryzae* and bacterial infection of *Xanthomonas strain* (Feng *et al*., 2011). According to Dutt *et al*. (2016) genetically modified orange plants with *Arabidopsis NPR1* gene showed resistance in confined field study up to 36 months of planting. The *NPR1* gene affects the SA, JA and ET pathways and is thus it is master regulator to pathways for plant defense (Dong, 2004). NPR1 protein is basically acts on nucleus to initiate defense responses when pathogens enter in plants (Kinkema *et al.*, 2000). The NPR1 protein makes an oligomer protein and is present in cytoplasm under nonstress conditions. SAR induction occurs due to entry of pathogens in plant cell, monomeric form of NPR1 proteins appears in cytoplasm through redox changes, moves to the nucleus of the plant cells, and activates several *PR* genes for plant defense induction (Mou *et al.*, 2003).

Since resistance in plants against pathogens can be affected by the age and health of the infected plant, natural environmental conditions (rain, temperature and humidity) and inoculum quantity and virulence of the pathogen, the transgenic events generated in this study need to be

further evaluated for gene stability and potential protection against both Xcm and Foc race 1 during confined field trials.

This study suggests that *NH1* gene is a valuable gene for the improvement of banana through gene technology which provide resistance to both pathogens Foc race 1 and Xcm. Transgenic banana expressing the *NH1* gene appears to have potential to control bacterial as well as fungal diseases in banana and thus improve the food security and livelihoods in banana farmers who grow banana as a main food crop.

CHAPTER SIX

GENERAL DISCUSSIONS, CONCLUSIONS RECOMMENDATIONS AND FUTURE RESEARCH

6.1 General discussions

East and Central African countries grow and consume the maximum banana in Africa. In these countries banana is the real food security crop. Uganda is the world's third-largest banana producer after India and China (FAOSTAT, 2015). All the banana which has been grown worldwide are produced by farmers which have small land area. They grow banana for their own consumptions as a staple food and local nearby markets rather than for international export. In Africa, banana mainly cooking type is a staple food. However, their production is vulnerable to many serious diseases caused by pests and pathogens including nematodes, weevils, bacteria, fungi, and viruses. The Banana Xanthomonas wilt (BXW) and Fusarium wilt (FW) are two major production constraints in the cultivation of banana, especially in African countries. The economic impacts of BXW and FW are severe and fast than other pest and diseases which cause gradual losses in few years, since it causes the death of mother plant which produces several suckers (daughter plants) in her life time.

Classical breeding for the improvement of banana is time-consuming and challenging due to polyploidy of banana cultivars, long generation cycle and seedless nature of the cultivated banana. As such, genetic engineering of banana is likely the best option for improving disease susceptible cultivars by inserting a gene of interest to develop disease and pest resistance plants which have more production and good fruit quality. Recently, transgenic banana with either *Hrap* or *Pflp* gene, grown in a confined field showed that genetic engineering could provide a solution to farmers in Uganda for bacterial disease (Tripathi *et al.,* 2014a). These transgenic

events also demonstrated increased and better yield. Currently, these transgenic bananas have been planted on multi-locational trials to collect more on disease resistance and agronomic performance. Recently, Dale *et al.,* (2017) demonstrated that transgenic technology can provide lasting solution against fungal pathogen Foc tropical race 4 in three-year field trial of transgenic 'Cavendish' banana in Northern part of Australia. These are the potential examples that transgenic technology can provide disease resistant banana.

In this study, the sole aim was to established banana transformation system by using embryogenic cells (EC) of banana cultivars. It is essential for genetic engineering of banana to have EC of the farmer`s preferred cultivars of banana. Even though developing EC and optimizing their regeneration and transformation efficiency, are time consuming and laborious tasks but EC are preferred material for gene insertion due to high regeneration and transformation efficiencies. It takes about 15 to 18 months depending on banana cultivars (Tripathi *et al.*, 2015). EC is an essential part of the success of *Agrobacterium*-mediated transformation system even though there are other methods also available for banana transformation like apical meristematic tissue. Those methods are not reliable due to development of chimeric transgenic plants and very low transformation efficiency. Although, developing EC is highly cultivar dependent, EC of banana cultivars like 'Gros Michel', 'Cavendish Williams' and 'Sukali Ndiizi' have been developed in this study and regeneration and transformation systems have been established and optimized using *gus*A reporter gene (Tripathi *et al.,* 2015). This is robust system and easily reproducible. Developed EC can be used with any gene of interest to develop transgenic banana plants easily in 7 to 9 months. These EC can be used up to 2 years with replenishment of new culture media every 10-14 days and discarding old media. Even though, this is a laborious process, but this is the best way to develop transgenic banana (Tripathi *et al.,* 2015).

EC of ‘Sukali Ndiizi’ was used for successful insertion of the rice *NH1* gene through *Agrobacterium*-mediated transformation. ‘Sukali Ndiizi’ is the one of the highly susceptible cultivars for fungal and bacterial diseases. The transgenic nature of generated events was tested by molecular methods i.e. PCR, Dot blots, and Southern blot analysis. Evaluated transgenic events showed the considerable levels of resistance against BXW and Fusarium wilt in a much-regulated glasshouse environment, using higher inoculum pressure of pathogens than the natural environment.

6.2 Conclusions

This study has shown that the rice *NH1* gene is an effective strategy for controlling banana Xanthomonas wilt and Fusarium wilt especially in the ‘Sukali Ndiizi’ cultivar of East Africa. It will be of interest to determine whether this resistance is maintained in farmers` fields and over several generations of the crop. To this end, transgenic plants established during this research will be part of the confined field trials to be conducted over the coming years in Africa after getting regulatory approval from the host country. Ultimately, this available strategy will be adapted to other banana cultivars to engineer resistance in farmer-preferred banana cultivars and control the diseases through the application of genetic engineering.

There are various plant disease control methods were invented and applied, even though over 20—40 % of food production losses due to plant pathogens and pests (Savary *et al*., 2012). Banana production is equally affected by several plant diseases in Africa primarily due to two major deadly diseases of banana especially BXW and FW. The means of small growers, who are the 80 % of food cultivators in Africa, to control important banana diseases and pests are very limited. It is, therefore, important to plant science researchers to explore new disease management strategies to enhance banana production for future generation through application

of modern crop biotechnology. Banana are micro-propagated either by suckers from mother plants or *in-vitro* plantlets developed in plant tissue culture laboratories through clonal mode of multiplication in absence of proper seed production, there are highly vulnerable for several pathogens and pests than other cultivated food and fruit crops. Therefore, additional efforts need to be taken to ensure sustainable production. The application of genetic engineering offers remarkable opportunities to develop a new disease and pest resistant crop for the African continent and other parts of the world. The recent sequencing of the banana genome will provide a fundamental insight into genetic engineering and new gene discovery (D'Hont *et al*., 2012) as it would now be promising to use genes isolated from resistant banana species and insert them into susceptible banana cultivars to make them diseases and pest resistance. Overall plant biotechnology has enormous potential to feed the growing population on the planet.

6.3 Recommendations

i. The optimized protocol for production of embryogenic cells, generation of transgenic events of various banana cultivars described in this study can be used to introduce transgenes for important traits in banana.

ii. The embryogenic cells developed from three banana cultivars especially 'Cavendish Williams' 'Gros Michel' and 'Sukali Ndiizi' can be used for mass propagation and transformation of banana.

iii. The embryogenic cells developed in this study can be used to test new biotechnological technology like CRISPR/Cas9 for genome editing.

iv. Overexpression of rice *NH1* gene in transgenic banana can provide resistance to both Xcm and Foc race 1.

6.4 Future research

i. An extra effort is required to develop embryogenic cells of other important banana and plantain cultivars, which are economically important for banana farmers.

ii. 'Sukali Ndiizi' transgenic events with *NH1* gene need to be evaluated in confined field trials to reach more conclusive results regarding resistance against bacterial and fungal diseases.

iii. Agronomic performance of transgenic plants needs to be monitored under field conditions.

iv. Transgenic plants should be tested for the off-target effects and negative impact of NH1 protein on soil microbe community and soil properties.

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APPENDICES

APPENDIX I

STATISTICAL ANALYSIS

All the data analysis was performed with Minitab statistical program www.minitab.com

1. One-way ANOVA: Number of plantlets generated per 1 ml of settle cell volume of embryogenic cells of each cultivar

Source	DF	Sum of Square	Mean Square	F-Value	P-value
Exp.	2	1453902222	726951111	265.85	≤0.001
Error	6	16406667	2734444		
Total	8	1470308889			

S = 1654 R-Sq = 98.88% R-Sq(adj) = 98.51%

2. One-way ANOVA: Number of transgenic plantlets generated per 1 ml of settle cell volume of embryogenic cells of each cultivar

Source	DF	Sum of square	Mean Square	F-value	P- value
C2	2	2844.2	1422.1	75.73	≤0.001
Error	6	112.7	18.8		
Total	8	2956.9			

S = 4.333 R-Sq = 96.19% R-Sq(adj) = 94.92%

3. One-way ANOVA: Sukali Ndiizi (*NH1)* transgenic events growth analysis

i. Plant height(cm) of banana plants versus events

Source	DF	Sum of square	Mean-Square	F-value	P-value
Events	20	1406.2	70.3	5.60	≤0.001
Error	42	527.3	12.6		
Total	62	1933.6			

S = 3.543 R-Sq = 72.73% R-Sq(adj) = 59.74%

ii. One-way ANOVA: Thickness of pseudostem of banana plant at base (cm) versus events

Source	DF	Sum of square	Mean Square	F-value	P-value
Events	20	25.584	1.279	1.82	≤0.052
Error	42	29.587	0.704		
Total	62	55.171			

S = 0.8393 R-Sq = 46.37% R-Sq(adj) = 20.84%

iii. One-way ANOVA: Number of total leaves in each banana plants versus events

Source	DF	Sum of square	Mean Square	F-value	P-value
Events	20	13.937	0.697	2.93	≤0.002
Error	42	10.000	0.238		
Total	62	23.937			

S = 0.4880 R-Sq = 58.22% R-Sq(adj) = 38.33%

iv. One-way ANOVA: Total leaves area cm^2 of each banana plant versus events

Source	DF	Sum of square	Mean Square	F-value	P-value
Events	20	95649019	4782451	4.86	≤ 0.001
Error	42	41289948	983094		
Total	62	136938967			

S = 991.5 R-Sq = 69.85% R-Sq(adj) = 55.49%

4. One-way ANOVA: Glass house screening of Sukali Ndiizi (*NH1*) transgenic events against *Xanthomonas campestris* pv. *musacearum*

i. One-way ANOVA: Number of days for appearance of first BXW symptoms versus events

Source	DF	Sum of square	Mean -square	F-value	P-value
Events	20	1413.9	74.4	2.10	≤ 0.029
Error	34	1202.0	35.4		
Total	53	2615.9			

S = 5.946 R-Sq = 54.05% R-Sq(adj) = 28.37%

ii. One-way ANOVA: No. of days for complete wilting of susceptible transgenic events versus events

Source	DF	Sum of square	Mean-Square	F-value	P-value
events	6	67.9	11.3	0.45	≤0.833
Error	10	254.0	25.4		
Total	16	321.9			

S = 5.040 R-Sq = 21.09% R-Sq(adj) = 0.00%

5. One-way ANOVA: Glass house screening of Sukali Ndiizi (*NH1*) transgenic events against *Fusarium oxysporum* f. sp. *cubense* race 1

i. One-way ANOVA: No of yellow leaves (grade 1-5) versus number of Replicate

Source	DF	Sum of Square	Mean-Square	F-value	P-value
No of Rep	18	47.771	2.654	4.15	0.001
Error	82	52.467	0.640		
Total	100	100.238			

S = 0.7999 R-Sq = 47.66% R-Sq(adj) = 36.17%

ii. One-way ANOVA: Number of wilted leaves (grade 1-5) in each plant due to Fusarium wilts versus No of Replicate

Source	DF	Sum of Square	Mean Square	F- value	P- value
No of Rep.	18	241.36	13.41	6.78	0.001
Error	82	162.07	1.98		
Total	100	403.43			

S = 1.406 R-Sq = 59.83% R-Sq(adj) = 51.01%

iii. One-way ANOVA: Fusarium wilt leaves symptoms (grade 1-5) versus number of replicates

Source	DF	Sum of Square	Mean Square	F-value	P-value
No of Rep.	18	138.62	7.70	2.59	0.001
Error	82	243.58	2.97		
Total	100	382.20			

S = 1.723 R-Sq = 36.27% R-Sq(adj) = 22.28%

iv. One-way ANOVA: Wilting (grade1-5) in each plant from Fusarium wilt versus Wilting (1-5)

Source	DF	Sum of Square	Mean Square	F-value	P-value
Wilting (1-5)	4	87.53488	21.88372	.000	0.001
Error	81	0.00000	0.00000		
Total	85	87.53488			

S = 0 R-Sq = 100.00% R-Sq(adj) = 100.00%

v. One-way ANOVA: Pseudostem splitting (grade1-3) from Fusarium wilts versus No of Replicate

Source	DF	Sum of Square	Mean Square	F-value	P-value
No of Rep.	18	20.760	1.153	9.52	0.000
Error	82	9.933	0.121		
Total	100	3 0.693			

S = 0.3480 R-Sq = 67.64% R-Sq(adj) = 60.53%

vi. One-way ANOVA: Corm symptoms from Fusarium wilt in each plant (grade 1-5) versus Number of Replicate

Source	DF	Sum of Square	Mean Square	F-value	P-value
No of Rep.	18	134.284	7.460	22.02	0.001
Error	82	27.775	0.339		
Total	100	162.059			

S = 0.5820 R-Sq = 82.86% R-Sq(adj) = 79.10%

APPENDIX II

GENOMIC DNA EXTRACTION FROM BANANA LEAF TISSUES

Hexadecyltrimethylammonium bromide (CTAB) buffer

Component	**Final Concentration**	**Stock Concentration**	**Amount added for 200 ml**	**Amount added for 1000 ml**
CTAB	2%		4 g	20 g
Tris-Hcl, pH 8	100 mM	1 M	20 ml	100 ml
EDTA	20 mM	500 mM	8 ml	40 ml
NaCl	1.4 M	5M	56 ml	280 ml
Dissolve CTAB and warm at 60 ℃, bring to volume			200 ml	1000 ml

Before use, add 7 µl of beta- mercaptoethanol to per ml of CTAB and warm at 65 ℃

Procedure

1. Grind 1.5 g leaf tissue in a chilled mortar with liquid nitrogen and transfer the powder to a 15 ml falcon centrifuge tube. Use clean and autoclave mortar and pestle for each sample. Put pestles and mortars in -80 ℃ for 2 hours so that grounded tissues do not stick on the wall of mortar. Use up to 1.5 g tissue in 15 ml falcon tube.
2. Keep samples in liquid nitrogen until used.
3. Warm CTAB extraction buffer to 60 ℃ and add beta-mercaptoethanol.
4. Add 3 ml of pre-warmed CTAB buffer for each gram of tissue. Mix it properly and place in water bath at 60 ℃ for 90 min. During this period, mix several times by inverting the tubes.
5. Add chloroform (2/3 of the volume of the CTAB buffer) to each tube and mix gently by inversion until an emulsion is formed (about 20 to 25 times). Perform this step under fume hood.

6. Centrifuge the mixture at 4000 rpm for 10 minutes at room temperature.
7. Label set of new falcon tubes and place 10 µl of RNAse (32 mg/ml) into each tube. Pipette the upper aqueous layers from the last step into new tube without disturbing the interface. Invert each tube several times to mix.
8. Incubate for 30 minutes at 37 ℃. Repeat chloroform step 5 and centrifugation step 6.
10. Remove the upper, aqueous layer (should be clear) into a new tube and add equal volume of Isopropanol.
11. Invert the tube gently about 20 to 25 times to mix and keep at -20 °c for overnight for precipitation.
12. Centrifuge the solution for 15- 20 minutes at 4000 rpm.
13. Discard the isopropanol and dry pellets in Laminar Hood.
14. Dissolve the DNA pellet in 400 µl of TE buffer (1X) and transfer to 1.5 ml microfuge tube.
15. Add 800 µl of absolute ethanol and 40 µl of 3M sodium acetate for re-precipitation of DNA.
16. Keep the tube at -20 °c for 4-6 hours and spin at 14000 rpm.
17. Discard the supernatant and dry the pellets in laminar hood for 1 hour. There should be no droplets at wall of microfuge tube.
18. Suspend the pellet in 100 µl of double distilled sterile water or TE buffer (1X).
19. Load 5 µl of DNA with blue dye and test on agarose gel.
20. Check DNA concentration on Nanodrop.
21. This DNA is suitable for Southern analysis and PCR.

APPENDIX III

VARIOUS MEDIA FOR BANANA TISSUE CULTURE AND TRANSFORMATION

A. Composition of MS medium (Murashige and Skoog, 1962)

Constituents	**MS Medium (mg/l)**	
Stock I (Macronutrients)	MW	**10X (gm\l)**
NH_4NO_3	1650	16.5
KNO_3	1900	19.0
$CaCl_2.2H_2O$	440	4.4
$MgSO_4.7H_2O$	370	3.7
KH_2PO_4	170	1.7
Stock II (Micronutrients)		**100X (gm/l)**
KI	0.83	0.083
H_3BO_3	6.2	0.62
$MnSO_4.4H_2O$	22.3	2.23
$ZnSO_4.7H_2O$	8.6	0.86
$Na_2MoO_4.2H_2O$	0.25	0.025
$CuSO_4.5H_2O$	0.025	0.0025
$CoCl_2.6H_2O$	0.025	0.0025
Stock III (Iron)		**100X (gm/l)**
$Na_2EDTA.2H_2O$	37.3	3.73
$FeSO_4.7H_2O$	27.8	2.78
Stock IV (Vitamins + Inositol)		**200X (gm/l)**
Nicotinic Acid	0.5	0.1
Pyridoxine. HCl	0.5	0.1

Thiamine. HCl	1	0.2
Glycine	2.0	0.4
Myo-Inositol	100	20
Stock V (Antioxidant)		**gm/10 ml**
Ascorbic Acid	10 mg/ml	0.1

B. Composition of Morel Vitamins (Morel and Wetmore, 1951)

Components	**Final concentration (mg/l)**	**200X (500 ml)**
Biotine (1mg/ml)	0.01	1 ml
Calcium panthotenate	1	100 mg
Myo-inositol	100	10 g
Nicotinic Acid	1	100 mg
Pyridoxine HCl	1	100 mg
Thiamine HCl	1	100 mg

C. Preparation and storage of growth regulator solution

Growth regulators	**Stock Solution**	**Solvent**	**Solution storage**	**Sterilization**
Auxins				
2,4-D	1 mg/l	EtoH/1N NaOH	0-5^{o}C	CA/F
2,4,5-T	1 mg/l	EtoH/1N NaOH	0^{o}C	CA/F
IBA	1 mg/l	EtoH/1N NaOH	0^{o}C	CA/F

NAA	1 mg/l	1N NaOH	0-5^{o}C	CA
IAA	1 mg/l	1N NaOH	0^{o}C	F
Cytokinins				
BAP	1 mg/l	1N NaOH	0-5^{o}C	CA
KIN	1 mg/l	1N NaOH	0^{o}C	CA/F
TDZ	1 mg/l	1N NaOH	0^{o}C	CA/F
ZET	1 mg/l	1N NaOH	0^{o}C	CA/F

CA- coautoclavable with other media components; CA/F- coautoclavable with other media components, however, some loss of activity may occur. Therefore, filter sterilization was performed in critical experiments; F- filter sterilization (0.22 μm).

D. Composition of proliferation medium (PM) using stock solutions

Proliferation media	**1 L**	**500 ml**
Macros (10X)	100 ml	50 ml
Micros (100X)	10 ml	5 ml
Fe^{3+} (100X)	10 ml	5 ml
Vitamins (200X)	5 ml	2.5 ml
Sucrose	30 g	15 g
Ascorbic acid (10mg/ml)	1 ml	0.5 ml
BAP (1mg/ml)	5 ml	2.5 ml
Gelrite	2.4 g	1.2 g
pH 5.8		

E. Composition of proliferation medium (PM) using MS premix

Proliferation media	**1 L**	**500 ml**
MS salts + Vitamins Premix	4.4 g	2.2 g
Sucrose	30 g	15 g
Ascorbic acid (10 mg/ml)	1 ml	0.5 ml
BAP (1 mg/ml)	5 ml	2.5 ml
Gelrite	2.4 g	1.2 g
pH 5.8		

Note: MS salt premix (Duchefa, M0222) contains MS salts, vitamins, iron and myo-inositol.

F. Composition of rooting medium (RM)

Proliferation media	**1 L**	**500 ml**
MS salts + Vitamins Premix	4.4 g	2.2 g
Sucrose	30 g	15 g
Ascorbic acid (10 mg/ml)	1 ml	0.5 ml
IBA (1 mg/ml)	1 ml	0.5 ml
Gelrite	2.4 g	1.2 g
pH 5.8		

G. Multiple bud induction medium (MBI or modified P4)

Stock	**Final concentration**	**500 ml**	**1 L**
MS salts + Vitamins + myo-inositol Premix		2.2 g	4.4 g
Ascorbic acid (10 mg/ml)	10 mg/l	0.5 ml	1 ml
IAA (1 mg/ml)	0.175 mg/l	88 µl	175 µl
BAP (1 mg/ml)	22 mg/l	11.3 ml	22.7 ml
Sucrose	30 g/l	15 g	30 g
Gelrite	3 g/l	1.5 g	3 g
pH 5.8			

H. Callus induction medium (CIM or ZZs)

Composition	**Working concentration**	**1 L**	**500 ml**
Macros (10X)	½	50 ml	25 ml
Micros (100X)		10 ml	5 ml
Iron (100X)		10 ml	5 ml
Vitamins + myo-inositol (200X)		5 ml	2.5 ml
Ascorbic acid (10 mg/ml)		1 ml	500 µl
2,4-D (1 mg/ml)		1 ml	500 µl

Zeatin (1 mg/ml)	0.219 mg/l	219 µl	210 µl
Sucrose	30 g/l	30 g	15 g
Gelrite	3 g/l	3 g	1.5 g
pH 5.8			

Note: For preparing ZZ liquid medium, do not add gelrite

I. Embryo Development Medium

Components	**Working concentration**	**1 L**
SH salts premix	3.2 g	3.2 g
MS Vitamins (200X)	1X	5 ml
Glutamine	100 mg/l	100 mg
Malt extract	100 mg/l	100 mg
Biotin (1 mg/ml)	1 mg/l	1 ml
Proline	230 mg/l	230 mg
Citric acid	60 mg/l	60 mg
Ascorbic acid (10 mg/ml)	60 mg/l	6 ml
Cysteine	400 mg/l	400 mg
NAA (1 mg/ml)	0.2 mg/l	200 µl
2 iP (1 mg/ml)	0.2 mg/l	200 µl
Kinetin (1 mg/ml)	0.2 mg/I	200 µl
Zeatin (1 mg/ml)*	0.1 mg/l	100 µl
Lactose	10 g/l	10 g
Sucrose	45 g/l	45 g
Gelrite	3 g/l	3 g

pH 5.8

*Zeatin should be filter sterilized and add after autoclaving, when the temperature of the medium has come down to 50^0c.

Schenk & Hildebrandt (SH) Basal salt medium-Premix (Duchefa, S0225)

J. Embryo maturation medium

Composition	**Working concentration**	**1 L**	**500 ml**
Macros (10X)	½	50 ml	25 ml
Micros (100X)		10 ml	5 ml
Iron (100X)		10 ml	5 ml
Vitamins (200X)		5 ml	2.5 ml
Ascorbic acid (10 mg/ml)		1 ml	500 µl
Sucrose	30 g/l	30 g	15 g
Gelrite	3 g/l	3 g	1.5 g
pH 5.8			

K. Germination medium

Composition	**Working concentration**	**1 L**
Macros (10X)	1X	100 ml
Micros (100X)	1X	10 ml
Iron (100X)	1X	10 ml
Moral Vitamins (200X)	1X	5 ml

IAA (1 mg/ml)	2 mg/l	2 ml
BAP (1 mg/ml)	0.05 mg/l	500 µl
Sucrose	30 g/l	30 g
Gelrite	3 g/l	3 g
pH 5.8		

M. Callus induction using flower

Components	**Working concentration**	**1 L**
MS salts + vitamins premix (Duchefa, M0222)	4.4 g	4.4 g
Biotin (1 mg/ml)	1 mg/l	1 ml
IAA (1 mg/ml)	1 mg/l	1 ml
2,4-D (1 mg/ml)	4 mg/l	4 ml
NAA (1 mg/ml)	1 mg/l	1 ml
Sucrose	30 g/l	30 g
Gelrite	3 g/l	3 g
pH 5.7		

N. Callus Induction medium for multiple buds

Components	**Working concentration**	**1 L**
MS salts + vitamins premix (Duchefa, M0222)	4.4 g	4.4 g
Glutamine	100 mg/l	100 mg
Malt extract	100 mg/l	100 mg

Biotin (1mg/ml)	1 mg/l	1 ml
2,4-D (1 mg/ml)	1 mg/l	1 ml
Sucrose	45 g/l	45 g
pH 5.3		

O. Bacterial co-culture medium (BCCM- A+B): 500 ml

BCCM (A): 300 ml – filter sterilize

Components	**Stock**	**Amount for 300 ml**
Sucrose		15 g
Maltose		15 g
Glucose		5 g
L-Glutamine		50 mg
Malt extract		50 mg
Proline		150 mg
L-Cystein		200 mg
MS vitamins + myo-inositol	200X	5 ml
Ascorbic acid	10 mg/ml	500 µl
Biotin	1 mg/ ml	500 µl
Acetosyringone	400 mM	400 µl
Bring volume to 300 ml, pH 5.3, filter sterilize & dispense 30 ml aliquots in falcon tubes & store at -20^{0}C		

Note: For preparing acetosyringone (400mM) stock, dissolve 78.4 mg per ml DMSO and filter sterilize. Store at -20^{0}C.