OPTIMIZATION OF IN VITRO REGENERATION AND GENETIC TRANSFORMATION PROTOCOL FOR SELECTED BANANA AND PLANTAIN (Musa spp.) CULTIVARS AND GENERATION OF TRANSGENIC BANANA RESISTANT TO BACTERIAL XANTHOMONAS WILT

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A Thesis Submitted in Fulfillment of the Requirements for the Award of the Degree of Doctor of Philosophy (Biotechnology) in the School of Pure and Applied Sciences of Kenyatta University

APRIL 2016
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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It is my genuine gratefulness and warmest regard that I dedicate this work to my lovely husband Sammy Njoroge and our lovely kids; Vivian Muthoni, Collins Mwangi and Charles Kahariri.
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ABBREVIATIONS AND ACRONYMS

AMP  Antimicrobial peptides
ANOVA Analysis of variance
Avr  avirulence
2, 4-D 2, 4-dichlorophenoxyacetic acid
2iP  isopentenyl adenine
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
BBrMV *Banana bract mosaic virus*
BanMMV *Banana mild mosaic virus*
BCCM Bacterial co-culture medium
Bp base pair
BXW banana xanthomonas wilt
Ca²⁺ calcium ions
CaCl₂ calcium chloride
CaMV35S cauliflower mosaic virus 35s protein
CC co cultivation medium
cDNA complementary deoxyribonucleic acid
Cm centimeter
CMV *Cucumber mosaic virus*
CTAB Hexadecyltrimethylammonium bromide
Dept. Department
DIG Digoxigenin
DNA deoxyribonucleic acid
dNTPs deoxynucleotide triphosphates
Dr. Doctor
E. coli *Escherichia coli*
ECS embryogenic cell suspension
EAHBs East African Highland bananas
FAO Food and Agriculture organization
GFP Green fluorescence protein
GUS beta-glucuronidase
H⁺ Hydrogen ions
Hg mercury
HCD hypersensitive cell death
HR hypersensitive response
HRAP hypersensitive response assisting protein
IAA indole-3-acetic acid
IITA international institute of tropical agriculture
<table>
<thead>
<tr>
<th>Abbr</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>IBA</td>
<td>4-Indole-3-Butyric Acid</td>
</tr>
<tr>
<td>IM</td>
<td>intercalary meristems</td>
</tr>
<tr>
<td>K+</td>
<td>potassium ions</td>
</tr>
<tr>
<td>KARLO</td>
<td>Kenya Agricultural and Livestock Research Organization</td>
</tr>
<tr>
<td>Kg</td>
<td>kilograms</td>
</tr>
<tr>
<td>LB</td>
<td>Luria bertoni</td>
</tr>
<tr>
<td>LB</td>
<td>left T-DNA border sequence</td>
</tr>
<tr>
<td>LSD</td>
<td>least significant difference</td>
</tr>
<tr>
<td>LUC</td>
<td>Luciferase</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MA2</td>
<td>cell suspension maintenance medium</td>
</tr>
<tr>
<td>MA3</td>
<td>selection and embryo initiation medium</td>
</tr>
<tr>
<td>MA4</td>
<td>shoot germination medium</td>
</tr>
<tr>
<td>MBC</td>
<td>multiple bud clumps</td>
</tr>
<tr>
<td>Mg/L</td>
<td>miligrams per liter</td>
</tr>
<tr>
<td>Ml</td>
<td>milliliters</td>
</tr>
<tr>
<td>Mm</td>
<td>milli meters</td>
</tr>
<tr>
<td>mM</td>
<td>milli molar</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and skooog</td>
</tr>
<tr>
<td>MUG</td>
<td>methylumbelliferyl-D-galacopyranoside</td>
</tr>
<tr>
<td>N</td>
<td>North</td>
</tr>
<tr>
<td>NAA</td>
<td>naphthaleneacetic acid</td>
</tr>
<tr>
<td>Npt11</td>
<td>Neomycin phosphotransferase gene</td>
</tr>
<tr>
<td>Ng</td>
<td>nanograms</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>PC</td>
<td>Pre conditioning medium</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pflp</td>
<td>Sweet pepper ferredoxin-like protein</td>
</tr>
<tr>
<td>PR genes</td>
<td>pathogenesis related protein genes</td>
</tr>
<tr>
<td>R genes</td>
<td>resistant genes</td>
</tr>
<tr>
<td>Rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RB</td>
<td>Right T-DNA border sequence</td>
</tr>
<tr>
<td>RD1</td>
<td>Embryo development medium</td>
</tr>
<tr>
<td>RIM</td>
<td>Root induction medium</td>
</tr>
<tr>
<td>RM</td>
<td>resting medium</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT PCR</td>
<td>reverse transcriptase Polymerase chain reaction</td>
</tr>
<tr>
<td>S</td>
<td>South</td>
</tr>
<tr>
<td>SA</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>SAAT</td>
<td>Sonication assisted Agrobacterium transformation</td>
</tr>
<tr>
<td>SAR</td>
<td>systemic acquired response</td>
</tr>
</tbody>
</table>
SIM shoot induction medium
SDS sodium dodecyl sulfate
Spp Species
Taq thermos aquaticus
T-DNA Transfer DNA
TE Tris EDTA
TDZ Thidiazuron
TIFF Tagged Image File Format
U Units
USAID United States Agency for International Development
UK United Kingdom
UV ultra violet
V voltage
V/V volume by volume
Vir virulence
w/v weight by volume
µM micro molar
µFD micro farad
Xcm Xanthomonas campestris pv. musacearum
X-gluc 5-bromo-4-chloro-3-indolyl glucuronide
Xcv Xanthomonas campestris pv. Vesicatoria
YPG yeast peptone glucose
YTS yeast tryptone sucrose
Y TSA yeast tryptone sucrose agar
ZZ callus induction medium
ABSTRACT

Banana and plantain (Musa spp.) are important staple food crops for rural and urban consumers and provide a source of income for resource poor farmers in the humid tropics of sub-Saharan Africa. However, banana production is severely limited by both biotic and abiotic factors. Application of conventional breeding for both disease and pest resistance has resulted only in limited success due to the long generation times and the high sterility and triploidy of most cultivated bananas and plantains. Genetic transformation offers an alternative and viable means for crop improvement. However, to be successful, these applications require a rapid and efficient plant regeneration and transformation protocol for both banana and plantain. Currently, most transformation protocols for banana use cell suspension cultures. Establishing cell suspension cultures is a lengthy process, highly cultivar-dependent and most farmer-preferred banana and plantain cultivars are recalcitrant to generation of embryogenic cell suspensions. The objective of this study was to optimize a genetic transformation protocol for banana and plantain cultivars using meristematic tissues and to develop transgenic plants tolerant to BXW. Multiple bud clumps and intercalary meristematic tissues of 10 cultivars (Grande naine, Gross Michel, Gonja Manjaya, Nusu Ngombe, Ngombe, Mpologoma, Uganda green, Kayinja, Zebrina and Calcutta 4) were co-cultivated with Agrobacterium strain EHA105 harboring a binary vector pCAMBIA2301 or modified pCAMBIA2300-GFP, followed by selection and regeneration of kanamycin-resistant plantlets. Results of this study indicated 5mg/L as the optimal concentration of cytokinin for multiple bud induction. Several parameters affecting transformation efficiency were explored in this study. The optimal acetylsyringnone concentration was 200 µM and 100 µM for multiple bud clumps and intercalary meristems respectively, optimal infection time (30 minutes and 10 minutes for multiple bud clumps and intercalary meristems respectively), optimal vacuum infiltration time (2 minutes and 5 minutes for multiple bud clumps and intercalary meristems respectively), effect of explant type (multiple bud clumps had a higher transformation efficiency, 10%, compared to intercalary meristems, 7%), optimal sonication time (2 seconds for multiple bud clumps) and combined optimal sonication time and vacuum infiltration time was reported to give a higher transformation efficiency (12%) compared to sonication and vacuum infiltration alone. Uniform GFP expression was observed after the fifth sub culture. The presence and integration of the nptII and gusA genes in the progenies were confirmed by PCR and Southern blot analysis, respectively. Transformation efficiency of banana cultivar Mpologoma with hypersensitive response assisting protein (Hrap) gene was 8%. Out of the twenty lines expressing Hrap gene screened for Xanthomonas wilt resistance resulted in four resistant lines, five partial resistant and eleven susceptible lines. This study augments the ongoing genetic improvement of banana and plantain and contributes to the food security of communities living in Africa and relying on banana as a staple food.
CHAPTER ONE

INTRODUCTION

1.1 Background Information

Banana and plantain (Musa spp.) are monocotyledonous, perennial herbs, which belong to the Musa genus of the Musaceae family. They are native to South East Asia (Stover and Simmonds, 1987) and the centre of diversity is suggested to be in Malaysia or Indonesia (Daniells et al., 2001). Bananas are cultivated in more than 120 countries throughout the tropics and subtropics (Arvanitoyannis, 2008). They are distributed mainly on margins of tropical rainforests (Wong et al., 2002). Banana and plantain are ranked the fourth most important food crop in the developing world after rice, wheat and maize (Bioversity International, 2007). Total annual world production of banana is estimated at 130 million tons (Frison and Sharrock, 1999). India leads the world in banana production with an annual output of about 11 million tons (FAOSTAT, 2012). East Africa is the largest banana producing and consuming region in sub-Saharan Africa with the East and Central Africa sub-region alone producing nearly 20 million tons of bananas annually (FAOSTAT, 2011).

Bananas and plantains provide food security and a valuable source of income through local and international trade (Frison et al., 1997). Bananas provide a starch staple across some of the poorest parts of the world in Africa (with
consumption up to 400 kg per person per year) and Asia, while dessert bananas are a major cash crop in many countries (FAO, 2007).

Banana and plantain production is severely limited by various diseases and pests, such as Banana Xanthomonas wilt (BXW) disease caused by the bacterium *Xanthomonas campestris* pv. *Musacearum* (*Xcm*), Banana bunchy-top virus (BBTV), Banana streak virus (BSV), black sigatoka or black leaf streak (*Mycosphaarella fijiensis*), Fusarium wilt (*Fusarium oxysporum* f. sp. *cubense*), burrowing nematodes (*Radopholus similis*) and weevil, (Jones, 2000; Tushemereirwe *et al.*, 2004; Tripathi *et al.*, 2008). The most devastating disease that significantly causes banana and plantain losses in the Great Lakes region of East and Central Africa is BXW. The disease was first reported in Ethiopia on *Ensete* species, closely related to banana (Yirgou and Bradbury, 1974), but over the years, it has slowly spread to other parts of Eastern Africa region such as Uganda, Rwanda, Tanzania, Democratic Republic of Congo, Burundi and Kenya (Tushemereirwe *et al.*, 2003; 2004; Mbaka *et al.*, 2009; Tripathi *et al.*, 2009). The impacts of BXW are both extreme and rapid, unlike those of other diseases that cause gradually increasing losses over years. The disease leads to absolute yield losses and death of the mother plants that would otherwise contribute to the ratoon plant production cycles (Tripathi *et al.*, 2009).
Conventional breeding programs for banana and plantain improvement are hampered by high sterility of most of the cultivars, variable ploidy levels, and long generation times of the crops. This underscores the need for integration of biotechnological tools into the breeding programs (Rout et al., 2000). Genetic transformation offers an alternative and viable option for banana improvement and complements existing conventional breeding strategies. In addition, gene transfer offers the possibility to add novel traits without altering the qualities of the preferred cultivar.

Genetic transformation of banana has been performed by different techniques such as particle bombardment (Sagi et al., 1995), Agrobacterium-mediated transformation (May et al., 1995; Ganapathi et al., 2001) and electroporation (Rout et al., 2000). Gene transfer by Agrobacterium is the method of choice for genetic transformation of most plant species due to its ability to transfer large segments of DNA with minimal rearrangement and with fewer copies of inserted genes at higher efficiencies and with lower cost (Raineri et al., 1990; Lindsey, 1992). Currently, most of the transformation protocols for banana are based on embryogenic cell suspensions (ECSs) (Becker et al., 2000; Ganapathi et al., 2001; Khanna et al., 2004; Tripathi et al., 2012). Initiation and maintenance of ECS cultures is difficult, highly cultivar-dependent and require more time to obtain transgenic banana plants. Hence, it is necessary to develop alternative regeneration and transformation protocols to obtain transgenic banana plants within a short time.
particularly for cultivars that are recalcitrant to formation of ECSs. Transformation systems using apical meristems, corm slices, intercalary meristems and multiple buds have been reported (May et al., 1995; Tripathi et al., 2005; 2008; Yip et al., 2011) but the transformation efficiencies are low and often led to generation of chimeric plants. Tissue culture techniques can be optimized to dilute chimeras and obtain uniformly transformed plant. This study aimed to optimize a cultivar-independent genetic transformation protocol for a diverse range of banana and plantain cultivars using multiple bud clumps (MBCs) and intercalary meristematic tissues. The use of these explants will ensure rapid production of transgenic plants within a short duration as compared to ECSs. The optimized protocol will provide a rapid and feasible means of introducing genes of agronomic importance to this valuable crop independent of cultivar differences.

1.2 Problem statement

Banana and plantain play an important role in the food security of developing countries, especially in sub-Saharan Africa (FAOSTAT, 2010). They are cultivated in over 120 countries covering about 10 million ha, with an annual production of 130 million tons (FAO, 2009). In Africa, banana and plantain provide more than 25% of the food energy requirements for more than 100 million people, of whom 20 million are from East Africa alone (FAOSTAT, 2004). The crop is threatened by many pests and diseases such as BXW disease caused by the bacterium *Xcm*, viral disease such as BBTV and BSV, fungal disease like black
sigatoka or black leaf streak (*Mycosphaerella fijiensis*) and Fusarium wilt (*Fusarium oxysporum* f. sp. *cubense*), burrowing nematodes (*Radopholus similis*) and weevils, (Jones, 2000; Tushemereirwe *et al.*, 2004; Tripathi *et al.*, 2008). It is therefore anticipated that these constraints could be solved by use of modern biotechnology provided a relatively rapid and cultivar-independent genetic transformation system is available. Therefore, development of a reliable and efficient regeneration and transformation protocol of farmer-preferred cultivars using MBCs and meristematic tissues as explants is a prerequisite for genetic improvement of banana and plantain by genetic engineering.

1.3 Null hypotheses

i. Multiple bud clumps and intercalary meristematic tissues of banana are not viable explants for generating uniformly transgenic plants by *Agrobacterium* mediated transformation of bananas and plantain cultivars preferred by farmers.

ii. *Hrap* gene does not confer resistance against BXW transgenic Kenyan banana cultivars expressing the gene.

1.4 Objectives of the study

1.4.1 General objective

To optimize a genetic transformation protocol for selected banana and plantain cultivars preferred in by farmers using multiple bud clumps (MBCs) and
meristems and to generate transgenic lines expressing \textit{Hrap} gene with potential to create resistance against \textit{Xanthomonas campestris pv. Musacearum}.

1.4.2 Specific objectives

i. To optimize transformation protocols and selection steps for a broad range of banana and plantain cultivars using (intercalary meristems) IM and MBCs as explants and \textit{gusA} and \textit{gfp} as reporter genes

ii. To develop transgenic plants of Kenyan banana cultivar expressing \textit{Hrap} gene cassette for resistance against \textit{Xcm} and evaluate the level of disease resistance in transgenic banana lines

1.5 Justification

Genetic improvement of bananas and plantains through conventional breeding is severely limited by factors such as lack of genetic variability, ploidy variations and low female and male fertility (Vuylsteke and Swennen, 1992; Pillay \textit{et al.}, 2012). Genetic engineering has been considered as an attractive tool to complement traditional breeding methods in banana improvement. However, an essential prerequisite for banana and plantain genetic engineering is the availability of a reliable, efficient and cultivar-independent regeneration and genetic transformation system for farmer-preferred cultivars.
Genetic transformation of banana was reported as early as 1995 (Sági et al., 1995; May et al., 1995), though the development was hampered by limitation of starting material for transformation. Currently, most transformation protocols for banana and plantain use ECSs (Tripathi et al., 2012). The use of ECSs as opposed to the use of meristems in the genetic transformation of banana and plantain is ideal because a single cell origin of somatic embryogenic cultures would lead to the development of uniformly transformed plants devoid of chimerism in regenerated plants (Ganapathi et al., 2001; Tripathi et al., 2012). However, establishing cell suspensions is a lengthy process, highly cultivar dependent and most banana and plantain cultivars are recalcitrant to generation of ECSs. The use of MBCs unlike other explants limits on the frequent field-trip required for other methods like in the collection of immature flowers and is not influenced by time or seasons (Strosse et al., 2006).

Transformation systems using apical meristem, corm slices, intercalary meristem and multiple buds have been reported (May et al., 1995; Tripathi et al., 2005; 2008; Yip et al., 2011) but the transformation efficiencies are low and often led to chimeras. Optimization of tissue culture techniques can be used to avoid generation of chimeras. The meristematic tissues as a target for genetic transformation have the potential to regenerate plants from many different cultivars, unlike somatic embryogenesis, which is restricted to only a few cultivars (Tripathi et al., 2005; 2008). However, use of meristematic tissues results in the
development of chimeric plants since multiple cells are involved in shoot development and not all of them may be transformed. The dilution of chimerism and subsequent recovery of uniformly transformed plants can be achieved through tissue culture manipulation and optimization of selection steps. Therefore, there is need to optimize a genetic transformation protocol that will be applicable to a broad range of bananas and plantains using meristematic tissues.

Use of genetic transformation technologies for *Musa* spp., may provide an alternative approach in managing and controlling BXW disease. Therefore, the development of cultivar-independent banana and plantain transformation protocols and generation of BXW disease resistant banana and plantain cultivars would complement the crop improvement efforts aimed at enhancing food security.
CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of banana and plantain

Banana and plantain belong to the genus *Musa* of the family *Musaceae* (includes the genera *Ensete* and *Musella*), in the monocot order *Zingiberales* (Kress *et al*., 2001; Kress and Specht, 2006). The genus has over 60 known wild species with known diploid (2n) chromosome numbers of 22, 20, or rarely 18 or 14 (Simmonds, 1962; Shepherd, 1999). The taxonomy of the species within the genus *Musa* remains poorly resolved because of the widespread vegetative reproduction and natural occurrence of many hybrids. The genus is grouped into 5 sections (*Callimusa, Australimusa, Eumusa, Rhodochlamy* and *Incertae sedis*) on the basis of chromosome numbers, orientation and arrangement of flowers in the inflorescence. The species in the sections *Callimusa* and *Rhodochlamy* do not produce fruits and are used as ornamentals. The species in section *Australimusa* are seedless edible bananas.

The largest and most diversified section *Eumusa* also called *Musa* (true bananas) has 13-15 species (Karamura, 1998). The vast majority of the cultivated banana (Pollefeys *et al*., 2004) are derived from two wild species; *Musa acuminata* Colla (A genome) and *Musa balbisiana* Colla (B genome), both of which belong to section *Eumusa* (Simmonds and Shepherd, 1955). Some diploid banana are still grown today but triploids are the most widespread and economically important
bananas. Tetraploids occur very rarely or are bred artificially. Sterile hybrid progenies with the genome AAA have been formed through crossing of the seeded edible diploids (AA) over time with several of the many subspecies of *Musa acuminata*. Diverse hybridisations with *Musa balbisiana* resulted in mostly sterile hybrid progeny mainly with the genomes AB (dessert bananas), AAB (plantains) and ABB (cooking bananas) (Kagy and Carreel, 2004). Edible banana are classified into; groups, subgroup and clones. *Musa* AAA group has three subgroups; Cavendish, Gros Michel and Green-Red (Cheesman *et al.*, 1933). Cavendish subgroup is divided into clones (Daniells, 1990), though other taxonomists regard Dwarf Cavendish, Giant Cavendish, Grand Naine and Lacatan as morphotypes (Stover and Simmonds, 1987; Lebot *et al.*, 1994). The subgroups in *Musa* AAB group include; Plantains, Popoulu and Maia Maoli (pacific plantains), Mysore, Silk, Pome and Pisang Raja (De Langhe and Valmayor, 1980; Swennen and Vuylsteke, 1987; Lebot *et al.* 1994).

Majority of plantain, cooking banana and dessert banana are sterile seedless triploids (INIPAB, 1995; Zeller, 2005). Plantain and cooking banana usually are bi-specific triploid hybrids, AAB and ABB, respectively; with the exception of the East African Highland Bananas (EAHBs) which are starchy AAA triploids used for beer production or as a cooked vegetable (Karamura, 1999; Karamura and Pickersgill, 1999; Carreel *et al.*, 2002).
2.2 Importance of bananas and plantains

Banana and plantain (*Musa* spp. L.) are important staple food for nearly 400 million people in many developing countries, especially in Africa. Total global production ranks fourth after maize, rice and wheat. They are a major staple in Africa, Latin America, and Asia. In the East African highlands, consumption may be as high as 1 kilogram per person per day.

Banana is used as a staple food, processed into flour or fermented to produce vinegar, banana juice beer and wine (Pillay and Tripathi, 2007). Commercial beer production from banana is a dominant activity constituting 64% of annual beer produced in some parts of the region such as Rwanda, Burundi and Uganda. Banana flowers, trunk and leaf buds are used as a source of vegetables and banana sap is used as a dye and ink (Nelson *et al.*, 2006).

The leaves of *Musa* are used for their fibre content, when fresh as plates for eating or wrapping food parcels for steaming, and when dry as strips for weaving into various articles and for roofing shelters. Manufacture of banana fiber based handicrafts such as baskets, ropes, mats and lamp shades has become an important economic activity in banana growing regions. Different parts of banana have been used as a source of fodder for animals, which in turn provide manure for farming. Banana and plantain combat soil erosion on steep slopes and may be used as mulch for maintaining and improving soil fertility (Gold *et al.*, 1999). The crop is
an important source of income for the resource poor farmers upon selling the excess production in local markets. Dessert bananas are considered a major cash crop in many countries (FAO, 2007).

Other uses of banana are cultural and medicinal. Specific banana cultivars mark specific cultural values such as birth, marriages, deaths and other special ceremonies and rituals. Different parts of banana have been used to treat abdominal ailments such as ulcers, worm infections among others (Karamura et al., 1998).

### 2.3 Banana propagation and growth requirements

Edible banana are seedless and therefore are clonally propagated by tissue culture derived plantlets, suckers and split corms. *In vitro* propagation is the best choice because of rapid multiplication, uniformity and disease control (Kulkarni et al., 2007). *In vitro* plants have been produced from meristem culture (Swennen, 1990). Planting materials can also be collected from an existing old field, and or a multiplication plot maintained only for the production of suckers. Wild species of banana produce vast seeds from open pollinations and are propagated using seeds (Stover and Simmonds, 1987).

Banana is mostly cultivated between 30° N and 30° S of the equator with a mean monthly temperature of 27°C for optimal growth. Plantains grow well in lowlands
while the East African highland bananas (matooke) survive at altitudes between 1000 and 1800 metres above sea level. Banana require well distributed rainfall of an average of 2000 to 2500 mm throughout the year and short dry seasons. Banana can be grown on a wide range of soils, though the most ideal is deep well drained retentive loam soils, with high humus content (Zake et al., 2000). The major mineral nutrients required in bulk quantities for banana production to ensure maximal yield include; Nitrogen, potash and phosphorus.

2.4 Nutritive value of banana
The edible banana fruits constitutes; 74% water, 23% carbohydrates, 1% protein, 0.5% fats and 2.5% fibre (ED informatics, 2006). The vitamin content of ripe banana fruits include; carotene, vitamin E, thiamine, riboflavin, niacin, pyridoxine, folic acid, pantothenate, biotin and vitamin C (The banana nutrition group (UK); Dickinson, 2000). The mineral content of banana and plantain; sodium, potassium, calcium, magnesium, phosphorous, iron, copper, zinc, chloride, manganese and iodine (The banana group (UK); Dickinson, 2000).

2.5 Constraints to banana and plantain production
Banana production is threatened by both abiotic and biotic factors. Drought and declining soil fertility resulting from intensive land use have emerged as the major abiotic constraints to banana production (Okech et al., 1996). Biotic constraints to
banana production include pests and diseases. Banana is susceptible to a range of serious and debilitating diseases caused by fungi, viruses and bacteria.

2.5.1 Banana fungal diseases

2.5.1.1 Panama disease (*Fusarium wilt*)

The panama disease is caused by the fungus *Fusarium oxysporum f. sp. Cubense Foc*, a soil-borne fungus that infects the plant through the roots and blocks the xylem vessels resulting in a lethal wilt that devastates banana production (Moore *et al.*, 1995). A more recent virulent form of the pathogen, ‘Tropical Race 4’, has been associated with substantial losses to both subsistence and commercial growers since it attacks ‘Cavendish’ (Hwang and Ko, 2004). *Foc* ‘tropical race 1’ and ‘tropical race 2’ has been reported to affect bananas in Central and Eastern Africa (Tushemereirwe and Bagabe, 1998), while *Foc* ‘tropical race 4’ causes *Fusarium wilt* of ‘Cavendish’ (AAA genome) in South Africa (Viljoen, 2002).

*Fusarium oxysporum f. sp. Cubense Foc* is disseminated through infected planting material across banana growing areas (Ploetz and Pegg, 2000). It can also be disseminated through water, soil and field equipment. The fungus can survive as chlamydospores in the soil and organic matter for several decades. Control of the disease by use of chemicals such as fungicides or soil fumigants, or through cultural practices such as rotations or soil amendment is difficult. The only long-term option of ensuring continuous banana production is replacement of a susceptible variety with a resistant variety (Hwang and Ko, 2004; Daly and
Walduck, 2006). However, most commercial varieties are susceptible to ‘Tropical Race 4’ (Su et al., 1986). Resistant genes that may be useful in breeding or gene transfer programmes have been identified in some cultivars though these varieties cannot replace ‘Cavendish’ (Daly and Walduck, 2006).

2.5.1.2 Black leaf streak or black sigatoka
Black leaf streak also known as black sigatoka is an important fungal leaf disease of banana caused by *Mycosphaerella fijiensis*. The disease contributes to about 50% crop losses (Ferreira et al., 2004). The disease was introduced into Africa first in Gabon in 1978 and then in the island of Pemba off the Tanzanian coast in 1987 (Carlier et al., 2000). The fungus replaced a milder leaf disease of banana, yellow sigatoka leaf spot (caused by *Mycosphaerella musicola*), throughout tropical Africa (Carlier et al., 2000). Black sigatoka is severe and affects most banana cultivars, including EAHBs and plantains (Tushemereirwe and Bagabe, 1998). It is disseminated by infected planting material, by wind as ascospores or carried in wind and water as conidia (Carlier et al., 2000). Control by use of fungicides has been achieved in commercial plantations though not practical for smallholder producers in Africa.

2.5.2 Banana viral diseases
Viral diseases severely hamper banana production. Six viruses, namely *Banana bunchy top virus* (BBTV), *Banana streak virus* (BSV), *Banana bract mosaic virus* (BBrMV), *Banana mild mosaic virus* (BanMMV), *Cucumber mosaic virus* (CMV)
and *Banana virus X* (BVX) are currently known to infect banana worldwide (Swennen and Vuylsteke, 2001; Adams et al., 2004; Geering et al., 2005).

### 2.5.2.1 Banana bunchy top virus (BBTV)

Banana bunchy top disease (BBTD) caused by the Banana bunchy top virus (BBTV) is considered to be the most economically destructive virus disease affecting bananas worldwide causing complete yield loss in infected plants (Dale, 1987; Dale and Harding, 1998).

The disease is endemic in 11 countries in Sub Saharan Africa. It is widely distributed in the African continent, and affects most *Musa* cultivars (Lockhart and Jones, 2000; Thomas and Iskra-Caruana, 2000). Banana bunchy top virus is particularly severe in Southern African countries, such as Malawi where 40% loss of national banana production has been reported. The disease was first reported in Africa in 1901, however extensive spread into new production areas were observed during the last two decades. The disease has very recently invaded the Bénin (first reported 2011) and Nigeria (first reported in 2013) in West Africa. It is disseminated by the aphid vector as well as through infected planting materials.

New leaves emerging from mature infected plants appear narrow and wavy with yellow margins. The plants appear to be “bunched” at the top. Once established, BBTD is extremely difficult to eradicate or manage. Control of viral diseases can
be achieved by use of clean planting material, eradication of infected plants, control of vectors and proper sanitation practices (Lockhart and Jones, 2000; Thomas and Iskra-Caruana, 2000).

2.5.3 Banana bacterial diseases

Bacterial diseases afflicting banana production include the Xanthomonas wilt caused by *Xanthomonas campestris pv. Musacearum* and other bacterial diseases caused by *Ralstonia* spp. such as Moko, blood and bugtok (Swennen and Vuylsteke, 2001).

The most significant bacterial disease of banana in Africa is *Xanthomonas* wilt, caused by the bacterium *Xanthomonas campestris pv. Musacearum* (*Xcm*). The disease was first reported in Ethiopia on *Ensete* spp. (Yirgou and Bradbury, 1968) and banana (1974) but over the years it has slowly spread to other parts of the eastern region such as Uganda, Rwanda, Tanzania, DRC, Burundi and Kenya (Tushemereirwe *et al.*, 2003; 2004; Mbaka *et al.*, 2009; Tripathi *et al.*, 2009). The disease spreads rapidly leading to production losses and economic hardship to many small-scale growers in the banana growing regions (Tushemereirwe *et al.*, 2004).

Dissemination of *Xanthomonas* wilt is through traded products, infected plants, cutting tools and also by insects visiting flowers of infected banana plants.
(Tripathi et al., 2009). Symptoms of the disease are first seen on the male bud, spreading systemically in the stele to damage flowers and eventually infect the pseudostem and leaves. The disease can be effectively managed by early removal of the male buds, proper sanitation practices, and the use of clean planting material (Biruma et al., 2007).

Economic impact of the disease appears to be accelerated by farmers due to their inability to propagate and establish new banana planting sites free of the bacterium. Ultimately the disease leads to absolute yield losses (Tripathi et al., 2009). Although control of spread of disease by cultural practices is being attempted, a long-term solution may be achieved through resistant varieties. Genetic modification of banana to develop cultivars resistant to Xcm has been reported (Tripathi et al., 2010; 2014; Namukwaya et al., 2011).

2.5.4 Banana pests

2.5.4.1 Nematodes

Several nematode species that constrain banana production in Africa include; the burrowing nematode (Radopholus similis), the spiral nematode (Helicotylenchus multicinctus), the root lesion nematode (Pratylenchus goodeyi and Pratylenchus coffeae) and the root-knot nematode (Meloidogyne spp.) (Speijer and Fogain, 1998).
Nematodes are distributed in all banana growing regions across Africa, attacking all banana cultivars leading to plantations becoming of no economic gains and their being abandoned (Sarah, 2000). Nematodes are disseminated through infected planting material. Management practices aimed at control of nematodes include disinfesting suckers by paring and hot water treatment, using nematode-free tissue culture planting material, use of biological control agents such as fungal endophytes, and through the adoption of resistant and/or tolerant cultivars (Viaene et al., 2006). Use of nematicides is impractical or uneconomical to most small-scale farmers (Coyne et al., 2003; Hauser, 2007). However, a recent technology involving the use of cystatin and a repellent peptide has been reported in banana and plantain to develop nematode resistant varieties (Roderick et al., 2012).

2.5.4.2 Banana weevil

The banana weevil (Cosmopolitus sordidus), causes significant losses of bananas in Africa (Gold et al., 1994). The pest was introduced into the continent from Southeast Asia and has been disseminated to most banana growing regions through infected planting material. The larvae causes damage during feeding in the central cylinder and lower pseudostem (Gold and Messiaen, 2000), resulting in lower yields and snap-off of the entire plants. Management practices involve paring and hot water treatment of planting material, trapping, sanitation practices,
and the splitting and drying of harvested pseudotems. The pest can also be controlled by use of insecticides, pheromone traps, fungal biological control agents such as *Beauvaria bassiana* and non-pathogenic *F. oxysporum*. However, management is difficult due to the cryptic nature of the weevil larvae (Gold *et al.*, 2003).

2.6 Genetic improvement of banana and plantain through conventional breeding

Conventional breeding methods involving screening for seed-fertility, ploidy manipulations and interspecific hybridization (Rowe and Rosales, 1996; Vuylsteke *et al.*, 1997) have been reported in banana improvement programmes. Banana improvement programme through conventional breeding has been hampered by lack of useful genetic variability, low female fertility levels resulting from the triploidy nature of cultivated bananas and low seed set (Swennen and Vuylsteke, 1993). The improvement programme is further constrained by the long generation cycle of banana and plantain (Ortiz *et al.*, 1995). This calls for the integration of biotechnological tools into breeding programmes for banana genetic improvement.

2.7 Improvement of banana and plantain through genetic engineering

Genetic improvement of banana through mutation breeding and genetic modification can reduce the time and costs of conventional breeding substantially (Crouch *et al.*, 1998). Genetic engineering offers tremendous potential for
developing bananas resistant to pests and diseases (Crouch et al., 1998; Viljoen et al., 2004). Foreign genes conferring resistance to fungi (Sagi et al., 1998; Paul et al. 2011; Ghag et al. 2014), nematodes and viruses (Becker et al., 2000; Roderick et al., 2012), bacteria (Tripathi et al., 2010; Namukwaya et al., 2011; Tripathi et al., 2014), delayed fruit ripening (Balint-Kurti et al., 2001), tolerance to salt stress (Ismail et al., 2005) and the synthesis of therapeutic proteins (hepatitis B surface antigen; Sunil et al., 2005) have been introduced into banana.

2.7.1 Transformation using particle bombardment

Banana genetic transformation systems have been reported using particle bombardment. Sagi et al. (1995) reported transformation of embryogenic cultures and generation of transgenic plantlet using the biolistic particle delivery system. Cell suspensions of the hybrid ‘FHIA-21’ (Musa sp. AAAB) were transformed with gusA gene via particle bombardment (Daniels et al., 2002). Khalid and Jalil (2002) reported transient gus A gene expression in transformation of somatic embryos of Musa acuminata ‘Mas’ (AA) with both particle gun as well as Agrobacterium mediated transformation. The transformation efficiency was higher with the Agrobacterium mediated transformation compared to biolistic transformation system. Sreeramanan et al. (2006) reported successful recovery of transgenic banana, variety Pisang Rastali (AAB) transformed with gfp and gusA gene via particle bombardment.
The use of microprojectile particle bombardment has major limitations like, the shallow penetration of particles; inability to deliver the DNA in to the host systemically, delivery of large copy numbers of the preferred gene, cell damage due to high pressure applied and the equipment used are expensive. Despite cultivar-independent nature of biolistic transformation, *Agrobacterium*-mediated genetic transformation remains the method of choice due to high transformation efficiency and generation of transgenic plants.

### 2.7.2 Agrobacterium-mediated transformation

*Agrobacterium* mediated method uses the natural gene transfer mechanism of *Agrobacterium tumefaciens*, a common soil bacterium that causes crown gall disease in wounded plant tissue (Hooykas and Schilperoort, 1992). Transgenic banana plants have been obtained through *Agrobacterium tumefaciens*-mediated Shibata and Liu, 2000; transformation of Embryonic Cell Suspension (ECSs) (Ganapathi *et al*., 2001; Tripathi *et al*., 2010), intercalary meristems (Tripathi *et al*., 2005; 2008), shoot tips (May *et al*., 1995; Tripathi *et al*., 2002) and multiple buds (Yip *et al*., 2011). *Agrobacterium*-mediated transformation has several advantages over particle bombardment such as the defined integration of transgenes, potentially low copy number and preferential integration into transcriptional active regions of the chromosome (Hiei *et al*., 2000).
2.7.3 Genetic transformation using embryogenic cell suspension as explants

Embryogenic cell suspension (ECS) cultures have been developed for different genotypes of banana using different explants; basal leaf sheaths and corm section (Novak et al., 1989), highly proliferating shoot tip cultures (Dheda et al., 1991; Strosse et al., 2006), zygotic embryos (Marroquin et al., 1993), and immature male flowers (Escalant et al., 1994; Côte et al., 1996; Navarro et al., 1997; Becker et al., 2000). Genetic transformation of bananas using micro projectile bombardment of embryogenic cell suspensions has been reported for banana (Sagi et al., 1995; Côte et al., 1996; Becker et al., 2000). Agrobacterium mediated transformation protocols of banana using ECSs have also been developed (Ganapathi et al., 2001; Khanna et al., 2004; Kosky et al., 2010; Tripathi et al., 2010). Most of the transformation protocols for bananas rely on the use cell suspension, however, establishing cell suspension is a lengthy process and is cultivar dependent.

2.7.4 Genetic transformation using meristematic tissues as explants

The use of meristems is applicable to a wide range of banana cultivars irrespective of ploidy or genotype (Tripathi et al., 2003; 2005). This technique does not incorporate steps of generating embryogenic cell cultures but uses micro-propagation and the regeneration of homogeneous populations of plants within a short period of time. This procedure offers several potential advantages over the use of embryogenic cell suspensions as it is rapid and cultivar independent.
May et al. (1995) reported transformation of banana using meristems and corn slice explants from cultivar Grand naine (AAA). Tripathi et al. (2002) reported transformation of *Musa* varieties for sub-Saharan Africa (AAA, AAB as well as AAAA and AAAB) cultivars using shoot tips. Tripathi et al. (2005) standardized a genetic transformation protocol for plantain cultivar Agbagba (AAB) using apical shoot tips. *Agrobacterium* mediated transformation of highly regenerable single meristematic buds of banana cultivar, Rastali (AAB) has been reported (Sreeramanan et al., 2009). Establishing the MBC culture is easier and more effective because it is not lengthy compared to initiation of cell suspension. Generation of cell suspension requires 14-20 months to get explants ready for transformation. In addition, the process is labour intensive because of the several subcultures needed for cell suspension development. The time required to regenerate plants through MBC is comparatively shorter as compared with ECS. In addition, the procedure is easier and capable of reducing human power compared to cell suspension preparations (Yip et al., 2011).

Excising apical meristems from individual tissue culture plants requires skills whereas in MBC system, meristem from suckers can be excised easily. Use of MBC is also advantageous as numerous tiny and naked meristems could be induced directly from one single sucker collected from the field. However, transformation through meristemic tissue often leads to production of chimeric plants because multiple cells are involved in shoot development, while only a
proportion of them may be transformed. Manipulation of tissue culture procedures has been reported to generate uniformly transformed plants (Tripathi et al., 2005). Thus, meristematic tissue could be a substitution for plants where suspension culture is difficult to establish.

2.7.5 Sonication-assisted *Agrobacterium*-mediated genetic transformation

Sonication-assisted *Agrobacterium*-mediated genetic transformation (SAAT) is an efficient method of gene transfer especially in recalcitrant target plants (Trick and Finer, 1997). The method overcomes certain barriers such as the host specificity and the inability of *Agrobacterium* to access the target tissue by increasing the number of infection sites both on the surface and deep within the target tissue (Santare´m et al., 1998; Trick and Finer, 1998). The technique also enhances DNA transfer in diverse plant groups including dicots, monocots, and gymnosperms.

It involves subjecting the target plant tissue to brief periods of ultrasound while immersed in an *Agrobacterium* suspension (Liu et al., 2006). Sonication treatment produces micro wounds across the tissue and allows the *Agrobacterium* to penetrate deeply and more completely throughout the tissue than normal co-cultivation will permit (Trick and Finer, 1997; Tang et al., 2001; Liu et al., 2005), thus enhancing the bacterial colonization and infection of the tissue. This system has the potential to transform meristematic tissue buried under several cell layers (Trick and Finer, 1997). Sonication-assisted *Agrobacterium*-mediated genetic
transformation (SAAT) method has been successfully applied in soybean, pine, black locust, flax, and citrus (Santarem et al., 1998; Tang et al., 2001; Zaragoza´ et al., 2004; Beranova´ et al., 2008). Delivery of naked DNA into tobacco protoplasts (Joersbo and Brunstedt, 1990) and seedlings (Zhang et al., 1991) via sonication has been reported. Therefore, there is need to extend the application of SAAT to MBCs and meristematic tissues of bananas and plantains and evaluate the effect of sonication on transformation efficiency.

2.7.6 Vacuum infiltration Agrobacterium-mediated transformation

Agrobacterium infiltration is an effective method for rapidly transforming and inducing transient transgene expression in many plant species. The method delivers the gene of interest to the interior of the plant tissue (Bechtold and Pelletier, 1998; Tague and Mantis, 2006). Explants are submerged in a liquid suspension of Agrobacterium tumefaciens and then subjected to decreased pressure followed by rapid re-pressurization. The method has been successfully used for genetic transformation of several plants (Acereto-Escoffie´ et al., 2005; Canche-Moo et al., 2006; Gupta et al., 2006; Shrawat et al., 2007). Vacuum infiltration has been reported to increase transient expression levels in lettuce (Joh et al., 2005). Therefore, there is need to evaluate the effect of vacuum infiltration on the transformation efficiency of banana and plantain cultivars using MBCs and meristematic tissues.
2.7.7 Use of reporter genes in genetic transformation

Non-selectable marker genes or reporter genes have been used extensively as convenient markers to visualize gene expression and protein localization in prokaryotes and eukaryotes. Reporter genes have been used in the recovery of transgenic plants by allowing the visual detection of transformed tissues. Each reporter gene has specific, inherent characteristics that define its limitations and its applications. Examples of commonly used reporter genes include galactosidase, glucuronidase (gus), luciferase (Luc) and green fluorescent protein (gfp) (Helmer et al., 1984; Ow et al., 1986; Jefferson, 1987; Koncz et al., 1987; Ahlandsberg et al., 1999; Khan and Maliga, 1999).

The β-glucuronidase gene from E.coli has been to measure the activity of gene regulatory elements in plants and for histochemical localization of marker gene expression (Jefferson, 1987). It has been extensively used in plants to follow gene transfer and monitor the genetic transformation (Martin et al., 1992). The enzyme uses the external substrates 4-methyl umbelliferyl glucuronide (MUG) for measurements of specific activity and 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) for histological localization resulting in a blue precipitate at the site of the enzyme activity (Jefferson, 1987).

The GUS enzyme is stable within plants, non-toxic when expressed at high levels and the β-glucuronidase assay is sensitive (Jefferson, 1987). Histochemical
localization of gene expression can be detected at the sub-cellular level in plastids (Daniell et al., 1991). The enzyme can be fused with selectable marker genes such as nptII to allow the visualization and selection in the process of transformation (Jefferson, 1987). The main advantage of using uidA gene as reporter system is the non-involvement of expensive equipment and ease of detection that involves visualization of the GUS expression (uidA) after treatment with the substrate, X-Gluc. However, the histochemical GUS assay is destructive for tissue and therefore not suitable for direct visual selection of transformed plants.

Luciferase ( luc) has been used in the transformation process as a visual marker and for manual selection of transgenic plants (Chia et al., 1994; Lonsdale et al., 1998). It has several advantages such as the capability to monitor gene expression patterns in a non-destructive way in real time with great sensitivity (Ow et al., 1986; Millar et al., 1992). This allows continuous monitoring of gene activity during development (Verhees et al., 2002).

The green fluorescent protein ( gfp) from jellyfish (Aequorea victoria) has become a powerful reporter gene compared to gus and luc in the transformation process. It is an ideal non-destructive visualization system that can facilitate the selection of only the transformed tissues (Able et al., 2001; Jeoung et al., 2004; Gao et al., 2005a; b; Gurel et al., 2009). Purified gfp protein has 238 amino acids (Prasher et al., 1992) and absorbs blue light (maximally at 395 nm with a minor peak at 470
nm) and emits green light (peak emission at 509 nm with a shoulder at 540 nm). Light is produced by the bioluminescent jellyfish *Aequorea victoria* when calcium binds to the photoprotein aequorin (Cody *et al.*, 1993). Though, the jellyfish produces green light, the activation of aequorin *in vitro* or in heterologous cells leads to production of blue light. This can be attributed to a second protein in *A. victoria* that derives its excitation energy from aequorin (Morise *et al.*, 1974).

Formation of the fluorescent chromophore does not require exogenous substrates or cofactors. However, molecular oxygen has been identified as a possible cofactor for chromophore formation (Heim *et al.*, 1994). The GFP chromophore is derived from the primary amino acid sequence through cyclization and oxidation of the protein’s own Ser-Tyr-Gly sequences at positions 65-67 (Cody *et al.*, 1993). GFP is stable and is only denatured under extreme conditions. The wild-type GFP has been used for expression studies in plants in various transformation systems or virus-based delivery systems (Haseloff and Siemering, 1998). It is not toxic to plant cells and there is direct visualization of GFP in living tissue in real-time without destruction of the plant (Newell, 2000).

GFP has been used extensively in studies involving; measurement of gene expression, cell labeling and in protein labeling localization (Rizzuto *et al.*, 1996; Yokoe *et al.*, 1996; Kohler *et al.*, 1997; Goetz-Zernicka *et al.*, 1997; Yano *et al.*, 1997; Tarasova *et al.*, 1997). Several mutants of GFP with increased stability and
enhanced fluorescence have been isolated (Davis and Vierstra, 1998; Haseloff and Siemering, 1998; Stewart, 2001). Use of GFP increases the efficiency of transformation (Jordan, 2000) and reduce the time required to produce a transgenic plants (Vain et al., 1998). However, there is need for costly equipment like fluorescent stereomicroscope for detection of GFP. It can also function as a protein tag to a broad variety of proteins which have been shown to retain their native function (Cubitt et al., 1995; Olsen et al., 1995; Moores et al., 1996). Therefore, the flexibility of GFP as a non-invasive marker in living cells allows for its wide range of applicability as a cell lineage tracer, reporter of gene expression and as a potential measure of protein-protein interactions (Mitra et al., 1996).

2.8 Banana improvement for disease and pest resistance using transgenic technologies

Sagi et al. and Remy et al. (1998) generated fungal resistant bananas exhibiting a broad spectrum antimicrobial activity against bacteria and fungus by introducing genes encoding antimicrobial peptides. The extracts from samples of the transformed plants were shown to suppress the growth of the fungus *Mycosphaerella fijiensis* responsible for black Sigatoka disease in bananas. Antimicrobial peptides in bananas have also been reported (Finalet et al., 2002). A synthetic substitution analogue of an AMP magainin, MSI-99 that was shown to inhibit the growth and spore germination of *Fusarium oxysporum f. sp. Cubense* was reported in banana transformation studies (Sunilkumar et al., 2003). The transgenic bananas generated were shown to confer resistance to *F. oxysporum*
f.sp. *Cubense* and *Myco-sphaerella musicola*. Becker *et al.* (2000) reported bombardment of Grand Naine ECS with Banana Bunchy Top Virus (BBTV) resistance genes (*BBTV intO1* and *BBTV utO5*). Shekhawat *et al.* (2012) reported transgenic banana plants exhibiting BBTV resistance. Enhanced fungal tolerance against *Fusarium oxysporum f. sp. Cubense* (Race 1) has also been reported in transgenic silk banana ‘Pisang Rasthali’ (Sreeramanan *et al.*, 2006c). Further studies on generation of fungal resistant bananas have been reported involving introduction of rice *chitinase* gene (RCC2) (Sreeramana, 2009). Sigatoka tolerance bananas expressing the endochitinase gene ThEn-42 have been developed (Vishnevetsky, 2011).

2.9 Hypersensitive response in plants

Plants employ a wide array of defense mechanisms against pathogen attack such as, hypersensitive response (HR). Hypersensitive response is an induced resistance mechanism characterized by rapid, localized cell death upon their encounter with a microbial pathogen (Goodman and Novacky, 1994; Alvarez *et al.*, 1998; Heath, 2000). The HR cell death forms a physical barrier thus preventing further pathogen infection. HR is often associated with activation of plant defense responses in the surrounding, and even distal uninfected parts of the plants leading to the development of systemic acquired resistance (Xie and Chen, 2000). The HR is associated with the production of reactive oxygen intermediates and the induction
of pathogenesis-related genes, and may be a form of programmed cell death (Morel and Dangl, 1997; Lam et al., 2001).

Several plant pathogenic bacteria not only cause diseases on host plants but also induce a hypersensitive response (HR) when inoculated to incompatible host or non-host plants (Goodman and Novacky, 1994). Plants expressing a resistance (R) gene have effectors capable of triggering HR by rapidly initiating defense responses upon contact with a pathogen expressing a corresponding avirulence (avr) gene (Flor, 1971); while the hosts lacking R genes are incapable of recognizing the effectors and thus become infected (Göhre and Robatzek, 2008). Klement et al. (1964) demonstrated the total collapse of non-host plant leaves following infiltration of pathogenic bacteria. Such recognition events will then activate defense signaling in the surrounding tissues, leading to enhanced disease resistance also in the uninfected parts of the plant (Goodman and Novacky, 1994; Dangl et al., 1996).

Other defense responses include ion fluxes, increased salicylic acid (SA) production, membrane depolarization, and pathogenesis-related (PR) gene induction (Goodman and Novacky, 1994; Dangl et al., 1996; Hammond-Kosack and Jones, 1996; Dangl and Jones, 2001). Thus, the understanding of the bacterium-plant interaction that results in the HR, and subsequent disease defence mechanisms in plants, may lead to the discovery of effective methods for disease
Hypersensitive response is always associated with active plant resistance against pathogens on non-host plants.

Resistance (R) genes detect the pathogen and change the membrane potential and ion permeability of the plasma membrane. Initially, the R genes trigger an increase in extra cellular pH and K\(^+\) (Orlandi et al., 1992), while eliciting an influx of calcium and hydrogen ions into the cell. The outward K\(^+\) and inward Ca\(^{2+}\) and H\(^+\) ion flux are dependent and trigger the HR, resulting in cell death and formation of local lesions, which contain antimicrobial compounds. Subsequently the cells undergoing the HR produce reactive oxygen species (ROS; oxidative burst), including super oxide anions, hydrogen peroxide, and hydroxyl radicals (Baker et al., 1993). Lipid peroxidation and lipid damage may be partially responsible for some of these cell changes and probably affect membrane function. Phenolics and phytoalexins, such as glyceollin (in soybean), and other compounds are synthesized in cells surrounding the lesion. Callose, lignin, are deposited and pathogen related (PR) proteins such as 1, 3-glucanase and chitinase are induced.

Hypersensitive response precedes the secondary resistance response, the systemic acquired response (SAR) and is characterized by necrotic lesions around the infection site; biochemical changes include generation of active oxygen species (oxidative burst), cell death, over production of lignin-related materials, and the induction of certain pathogen related (PR) proteins. SAR results in a long lasting
resistance in the uninfected parts of the plant against a further challenge by a broad range of pathogens (Ryals et al., 1996; Sticher et al., 1997) and is characterized by the expression of certain pathogenesis related (PR) genes, such as PRI (Ward et al., 1991; Uknes et al., 1992), also in distant parts of the plant. SAR induction is dependent on the accumulation of SA and requires the activity of the positive regulator NPR1 (Cao et al., 1994; Delaney et al., 1995).

Hypersensitive response-assisting protein (HRAP) is a novel plant protein isolated from sweet pepper (Capsicum annum). The protein can intensify the harpin_{psr}-mediated hypersensitive response in plants (Chen et al., 2000; Ger et al., 2002; You et al., 2003). Some harpins have been shown to elicit disease resistance (Huang et al., 1988; Wei et al., 1992; Wei and Beer 1996). The gene has been shown to delay the hypersensitive response induced by various pathogens like Erwinia (Pandey et al. (2005), Pseudomonas (Mur et al., 2008), Ralstonia (Peeters et al., 2013a) and Xanthomonas sp. in non-host plants through the release of the proteinaceous elicitor, harpin_{psr} in both dicotyledons and monocotyledons crops (Huang et al., 2004). The harpin of E. amylovora has been reported to activate disease resistance in Arabidopsis through salicylic acid (SA) and non-expresser of pathogenesis-related genes 1 (NPR1)-dependent signal pathways (Dong et al., 1999). The Hrap gene has been reported in tobacco with the resulting transgenic plants acquiring resistance to tobacco wildfire and soft-rotting bacteria (Ger et al., 2002). Pandey et al. (2005) demonstrated that constitutive expression of the Hrap
gene in *Arabidopsis* results in an enhanced disease resistance towards soft rotting bacteria pathogen, *E. carotovora* subsp. *carotovora*. *Hrap* gene is one of the most important hypersensitive cell death associated genes that can be utilized to protect plants from bacterial pathogen attack (Chen *et al.*, 2000). The gene has been shown to confer resistance against bacterial pathogens including *Xanthomonas* in monocot (rice). Tripathi *et al.* (2010) reported enhanced resistance to BXW in bananas transformed with *Hrap* gene under glasshouse and the resistance was further confirmed under field conditions (Tripathi *et al.*, 2014). These reports indicate that *Hrap* is one of the important hypersensitive cell death associated genes that could be utilized to protect plants from bacterial pathogen attack.

![Schematic representation of a hypersensitive reaction.](image)

**Figure 2.1**: Schematic representation of a hypersensitive reaction.
CHAPTER THREE
DEVELOPMENT OF Agrobacterium – MEDIATED TRANSFORMATION PROTOCOL FOR A BROAD RANGE OF BANANA AND PLANTAIN CULTIVARS

3.1 Introduction

Most of the transformation systems for generation of transgenic banana plants rely on the use of embryogenic cell suspensions (ECS) as the starting material (Sagi et al., 1997; Khanna et al., 2004; Tripathi et al., 2012). This is because the use of ECS leads to the development of uniformly transgenic plants without chimeras due to their unicellular nature (Escalant et al., 1994; Sagi, 2000; Ganapathi et al., 2001). However, establishment of ECS is a tedious and lengthy process, and is cultivar-dependent. In addition, most banana and plantain cultivars are recalcitrant to establishment of ECS. This presents a challenge in transformation of a wide range of banana and plantain cultivars. Therefore, there is need to develop alternative regeneration and transformation protocols to obtain transgenic banana plants within a short time and from a wide range of cultivars.

One of the alternative approaches is the use of meristemtic tissues as explants for transformation of a broad range of banana and plantain cultivars. Agrobacterium tumefaciens-mediated transformation of bananas using intercalarly meristemic tissues and corm slices, have been previously reported (May et al., 1995; Tripathi et al., 2008). The use of meristemic tissues as explants offers several potential advantages over the use of ECS in that it is rapid and cultivar-independent
(Tripathi *et al.*, 2002; Tripathi *et al.*, 2008). However, the use of meristems as explant for transformation results in low transformation efficiency and development of chimeras. The chimeras can be diluted and eliminated by tissue culture manipulations and optimization of selection procedures to develop uniformly transformed plants (Tripathi *et al.*, 2008).

Chimerism has been reported in *Agrobacterium*-mediated transformation of several herbaceous species, including tobacco (Schmulling and Schell, 1993), soybean (Christou, 1990), potato (Rackosy-Tican *et al.*, 2007), rice (Christou and Ford, 1995), flax (Dong and McHughen, 1993), strawberry (Mathews *et al.*, 1995), citrus (Dominguez *et al.*, 2004), *Vigna mungo* (Muruganantham *et al.*, 2007) and in woody fruit trees such as apple (Flachowsky *et al.*, 2008). High frequencies of chimeras were reported in studies on *Lesquerella fendleri* (Brassicaceae) (Skarjinskaia *et al.*, 2003; Wang *et al.*, 2008). High frequencies have also been reported in *Citrus*, for which escapes and chimeras account for 90% of regenerated lines (Costa *et al.*, 2002; Dominguez *et al.*, 2004). Several mechanisms have been proposed to explain the generation of chimeras, including the possibility of a shoot organ originating from a mixture of transformed and untransformed cells (Poethig, 1989; Zhu *et al.*, 2007); transformation effects in a cell or cells that either cease to divide or divide to daughter cells forming only a sector in a shoot and transient expression of a transgene occurring in many cells of a shoot. Chimera can be attributed to the challenge of cross protection where the untransformed cells could
be protected through efficient detoxification of the antibiotic by the transformed cells (Christou, 1990; Schmulling and Schell, 1993; Dominguez et al., 2004; Zhu et al., 2007) or if the selective agents in species with an endogenous tolerance is not effective (Rackosy-Tican et al., 2007). Transient expression of the marker gene during early stages of the regeneration process or the Agrobacterium cells recurrence in infected tissues may also be associated with the development of chimeras and escapes.

Several vegetative propagation cycles are required to dissociate chimeras in cultures which regenerate from multicellular origin (Novák, 1990). However, the number of vegetative generations, which are necessary to dissociate chimeras completely, is unknown. To establish the number of vegetative cycles required to eliminate chimerism, a system for chimera induction and detection is needed. Green fluorescent protein provides an excellent system for monitoring gene expression and protein localization in living cells. The gfp protein must be highly expressed in transgenic plants for them to fluoresce green under UV or blue light.

The aim of this study was to optimize the regeneration and Agrobacterium-mediated genetic transformation system for a broad range of banana and plantain cultivars using meristematic tissue explants. The effect of different concentrations of acetosyringone, infection time, sonication, vacuum infiltration and a combination of sonication and vacuum infiltration were tested in an effort to
determine optimum conditions for high transformation efficiency. The optimized parameters were used to develop an efficient system for chimera dissociation in *Musa*. Transgenic plants regenerated from multiple bud clumps (MBCs) or intercalary meristems were subcultured for several cycles to dilute chimeras and compared with control transgenic plants generated from ECS, based on the fact that uniformly transformed plants are generated from single cells (Tripathi *et al.*, 2012). The number of sub-culturing cycles required to obtain uniform transformation was determined using *gfp* reporter gene. The transgenic lines were also tested for the presence of transgene using PCR.

### 3.2 Materials and methods

#### 3.2.1 Plant material

Banana and plantain cultivars were selected on the basis of farmer’s preference for use in cooking, as a dessert and for brewing purposes. Ten cultivars both triploids categorized as desserts (Grande naine - AAA, Gross Michel - AAA), plantains (Gonja - AAB, Nusu Ngombe - AAB), cooking (Ngombe - AAA, Uganda green - AAA), beer brewing (Mpologoma - ABB, Kayinja - ABB) and diploids (Zebrina GF-AA and Calcutta 4- AA) were used. All the banana and plantain cultivars were obtained from International Institute of Tropical Agriculture (IITA) except Uganda green and Mpologoma, which were obtained from the field at Kenya Agricultural and Livestock Research Organization (KALRO) - Kisii.
3.2.2 Culture initiation from suckers

The outer layer of leaves and corm tissue of the suckers collected from the field were carefully removed ensuring the shoot apex remained intact to obtain a block measuring 2 - 4 cm long. The block was surface sterilized using 70% ethanol for 5 minutes and soaked in 15% sodium hypochlorite solution for 10 minutes. The leaf sheath and the base were trimmed to expose the meristematic region in the axial of the concentric leaves under aseptic conditions. This was followed by rinsing 3 - 4 times using sterile distilled water and soaking in 15% sodium hypochlorite for 10 minutes. The explant was then rinsed 3 - 4 times using sterile distilled water and placed on sterile paper using sterile forceps. The outer layers of corm and leaf sheaths were removed further to obtain the desired size of explants (approximately 2 cm) for inoculation. The explants were cut into two halves through the meristem using a sterile blade and each half was inoculated into proliferation medium in baby food jars. The explants were incubated at 26 - 28°C with a photoperiod of 16/8 hour. The cultures were regularly monitored and sub-cultured in fresh proliferation medium after every 2 weeks for two months. At each sub-culture the corm size was reduced, leaf sheaths removed, meristems injured separating the buds to multiply the shoots. Individual shoots were transferred to proliferation medium every 4 weeks for shoot multiplication.
3.2.3 Determination of effect of cytokinins (BAP and TDZ) on multiple bud induction

Different concentrations of TDZ, BAP and combinations of TDZ and BAP were evaluated to determine the optimal level for MBCs induction. Shoot tip cultures were inoculated in proliferation medium supplemented with TDZ (1 mg/L and 1.5 mg/L), BAP (2.5 mg/L and 5 mg/L), TDZ (1 mg/L) + BAP (2 mg/L), TDZ (2 mg/L) + BAP (2 mg/L), and P5 medium (Appendix 1). Ten explants were used for each concentration tested with three replicates. The scalp induction rate was recorded by counting the fleshy bulbous structures at the basal end of proliferating shoot tips divided by the total number of explants used. After one month, the shoot length of the plantlets at different concentrations of BAP and TDZ were measured. MBCs were selected, excised and transferred to fresh P4 medium (Appendix 1). The experiments were laid out in a completely randomized design and analysis of variance was done using Genestat computer program and comparison of means was tested for significance, using LSD test, at 0.05 level of probability.

3.2.4 Regeneration of multiple bud clumps (MBCs) and intercalary meristems (IM)

MBCs were cut vertically into 1 mm thick slices and used as explants to test for their regenerability. Ten MBCs of each cultivar were cultured in shoot induction medium (SIM, Appendix 1) and incubated in dark at 28°C for one week before incubating them in light at 16/8 hour photoperiod and 28°C. The number of shoots obtained from a single bud were recorded and used to determine the regeneration
frequency. Similarly, 10 IM were cultured in dark at 28°C for one week in proliferation medium and later transferred to light. The number of shoots obtained were recorded and used to determine the regeneration frequency.

3.2.5 *Agrobacterium* strains and plasmids

*Agrobacterium* strain EHA105 (Hood *et al.*, 1993) harbouring the binary vector pCambia2301 and pCambia2300-*gfp* were used in this study. T-DNA region of pCambia2301 binary vector harbours *nptII* encoding neomycin phosphotransferase as selectable marker and *gusA* encoding β-glucuronidase as reporter genes. Both *nptII* and *gusA* genes are driven by CaMV35S promoter, which favour the constitutive expression of transgenes in plants (Figure 3.1). Since the *gusA* gene is disrupted by a catalase intron, it can express only in cells that possess intron splicing mechanism. Hence, the integration and expression of the T-DNA in the plant tissues can be followed by GUS histochemical assay. T-DNA region of pCambia 2300-*gfp* binary vector harbours *nptII* encoding neomycin phosphotransferase and *gfp* encoding green fluorescent protein as a reporter gene. Both *nptII* and *gfp* genes are driven by CaMV35S promoter (Figure 3.1).

The plasmid was introduced into *Agrobacterium* strain EHA 105 through electroporation and the *Agrobacterium* strain harbouring the vector maintained on LB medium (Appendix 11) supplemented with kanamycin (50 mg/L) and rifampicin (50 mg/L). The bacterial culture was grown, harvested, inoculated and
used for the subsequent transformation experiments as previously described by Tripathi et al. (2012). *Agrobacterium* culture was grown in liquid 25 ml LB supplemented with kanamycin 50 mg/L and rifampicin 50 mg/L for 3 days at 28°C. A volume of 1 ml of the *Agrobacterium* suspension was inoculated into 20 ml LB supplemented with Kanamycin 50 mg/L and rifampicin 50 mg/L grown overnight at 28°C with gentle shaking. The overnight grown *Agrobacterium* culture was centrifuged for 10 minutes at 5000 rpm at 25°C. The pellet was re-suspended in 20 ml of liquid proliferation medium supplemented with the optimized acetosyringone concentration. The bacterial suspension was incubated at 28°C for 3 hours with shaking at 150 rpm. The optical density (O.D$_{600}$) of the bacterial culture was adjusted to 0.8 with liquid proliferation medium supplemented with the same concentration of acetosyringone.

![Figure 3.1: Schematic representation of T-DNA region of binary vector pCAMBIA2301 used for genetic transformation. RB; right border, TNOS; nopaline synthase terminator, gusA, 35SP; CaMV35S promoter, nptII; neomycin phosphotransferase gene, Nos PolyA; nopaline synthase terminator, LB; left border. (pCAMBIA2300-gfp has the same backbone except Gus)](image-url)
3.2.6 Optimization of parameters affecting transformation efficiency using meristems

3.2.6.1. Effect of sonication, vacuum infiltration, acetosyringone and co-cultivation period

The effect of different parameters including concentration of acetosyringone (100, 200, 300 and 400 µM) in both the inoculation and co-cultivation medium, length of infection time (5, 10, 15, 20, 25, 30 and 40 minutes), sonication time (1, 2, 3, 4 and 5 seconds) and vacuum infiltration time (1, 2, 3, 4 and 5 minutes) on the efficiency of *Agrobacterium* to transfer T-DNA to the explants and transformation efficiency was compared using transient and stable *gusA* expression. Ten explants were used in each experiment and replicated three times. Data collected was based on the number of GUS staining explants after co-cultivation for three days.

Explants (MBC and IM) from the same cultivar were transformed using the optimized parameters to evaluate their effect on the transformation efficiency. An experiment using one hundred MBCs per treatment and replicated three times, pre conditioned for two days was set up to investigate the effect of wounding on transformation efficiency using; the optimized sonication time, vacuum infiltration time, combined sonication and vacuum infiltration and a control (without wounding or sonication/vacuum infiltration) based on the putative transformants regenerated. Optimized parameters were used for the subsequent transformation experiments using various cultivars Mpologoma, Kayinja, Nusu

3. 2.6.2 Pre-conditioning of explants

Three to four months MBCs (measuring 1 by 1 cm) and 1 mm thick were excised and pre-conditioned by culturing them in pre-conditioning medium designated as PC medium (Appendix 1) in dark for two days. Pre-conditioning was done to maximize biochemical wounding responses before infection of explants with *Agrobacterium*.

Two fine cross sections (0.4 - 0.6 mm thickness) of IM tissues were excised from the corm after removal of the roots and leaves of *in vitro* plantlets. Intercalary meristems (IM) were pre-conditioned for two days in proliferation medium containing Murashige and Skoog medium (Appendix 1). One hundred (100) explants per experiment were immersed in *Agrobacterium* suspension and vacuum infiltrated for 5 minutes, followed by sonication for 2 seconds and incubated at room temperature for 15 minutes for MBCs and 30 minutes for IM with an optimized acetosyringone concentration of 100 μM and 200 μM for IM and MBC, respectively. A control underwent the same treatment but without *Agrobacterium* infection. The experiments were repeated three times for all the cultivars.
3.2.7 Co-cultivation, selection and regeneration of transgenic plants

The explants were inoculated with Agrobacterium suspension for 30 minutes. After inoculation, the explants were blotted dry on sterile filter paper to remove excess Agrobacterium and co-cultured on co-cultivation medium (CC, appendix 1), containing 100 μM and 200 μM of acetosyringone for MBCs and IM, respectively in 90 mm petri plates. The plates were sealed with cling film and co-cultivated for 3 days in the dark. The explants were transferred to recovery stage in resting medium designated as RM proliferation medium supplemented with 300 mg/L cefotaxime (Appendix 1) for 7 to 14 days depending on the cultivar and cultured in dark at 28°C. The regenerating explants were transferred to selection medium (SM 1); proliferation medium supplemented with 100 mg/L kanamycin and 300 mg/L of cefotaxime (Appendix 1). The cultures were maintained in SM 1 medium for two to three months with subculturing on the same medium after every two weeks. All the dead and bleaching buds were discarded during the subcultures. The multiple shoots obtained on selection medium were separated at every sub-culture until single plants were regenerated. The putatively transformed shoots were transferred to rooting medium containing indole 3-butyric acid IBA, 1 mg/L (RIM) Appendix 1) and in vitro plantlets were used for histochemical GUS assay and molecular analysis.

3.2.8 Transformation of embryogenic cell suspensions of banana cultivar Gonja manjaya

3.2.8.1 Co-cultivation
Embryonic cell suspension (ECS) of Gonja manjaya provided by IITA was transformation using protocol described by Tripathi et al. (2012). ECS were sub cultured in ZZ liquid cell suspension medium (Appendix 1) five days prior to transformation to increase cell competence and transformation efficiency.

The cells were divided equally into three sterile falcon tubes as per the experimental design (cells in 2 falcon tubes to be transformed each with EHA 105 pCAMBIA2301 and EHA105 pCAMBIA2300-gfp and cells in one falcon tube to serve as control). Agrobacterium culture was prepared as described in section 3.25. Agrobacterium suspension (10 ml) of O.D0.6 was added to non-transformed cells and ZZ medium. No Agrobacterium was added to the control. Pluronic F 68 detergent (110 µl) was added and the content centrifuged at 900 rpm for 3 minutes at 25°C. The cells were left at room temperature for 30 minutes. The cells were drawn from the Agrobacterium infection culture using a wide mouth pipette and transferred to sterile mesh placed on sterile tissue papers to drain off the excess culture medium. The 50 µM nylon meshes with the cells were transferred onto co culturing medium BCCM; A: 300 ml and B: 200 ml (Appendix 11) in petri plates. Acetosyringone was included in the infection media and co-cultivation media to enhance transfer of the T-DNA. The plates were sealed with cling film, wrapped with aluminium foil and co cultured at 22°C for 5 days.

3.2.8.2 Selection and regeneration of transgenic plants from Agrobacterium infected embryogenic cells
The *Agrobacterium* infected cells were washed 3 times in 50 ml falcon tubes containing ZZ liquid supplemented with cefotaxime 300 mg/L to reduce recurrence of the *Agrobacterium*. The cells were transferred to mesh and blot dried with sterile blotting paper towels. The meshes with the cells were transferred to selection MA3 medium supplemented with 300 mg/L cefotaxime (Appendix 1) to kill off bacteria and 50 mg/L kanamycin to select transformed cells. Transformants were maintained in the dark at 28°C in selective MA3 medium with fortnightly transfer onto fresh medium of the same type until embryos were obtained. Individual embryos were transferred onto selective RD1 medium for further embryo initiation (Appendix 1), until well developed embryos were obtained. The embryos were maintained in RD1 for one months. Mature embryos were transferred to selective MA4 media (Appendix 1) for embryo germination. Shoots developing on MA4 medium were transferred to antibiotic and hormone free semisolid proliferation media with half strength BAP for shoot multiplication (Appendix 1), with monthly subculture.

### 3.2.9 Histochemical GUS analysis

Histochemical GUS assay was performed using leaf and root segments of the putatively transformed and non-transgenic control plantlets according to the modified procedure of Jefferson (1987), as described by Tripathi *et al.* (2012) for banana. Explants (MBCs, IM, roots, leaves and cross section of the pseudostem) were immersed in a buffer containing 2 mM X - Gluc, 50 mM phosphate, 50 mM
potassium ferrocyanide and 5% Triton X-100 at pH 7.0. The tissues in the reaction mixture were vacuum - infiltrated for 10 minutes, and then incubated overnight at 37°C. Tissues containing chlorophyll were repeatedly soaked in 95% ethanol until chlorophyll was removed. Transient expression of gusA gene was examined after 3 days of co-cultivation, while stable expression of the reporter gene was examined in the successive stages of plant regeneration.

3.2.10 DNA isolation and Polymerase Chain Reaction (PCR) amplification

Genomic DNA was isolated from fresh leaf tissues of putatively transformed banana plantlets and non-transgenic control plants by using a plant genomic DNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. The quantity and quality of the DNA were analyzed using a Nanodrop 2000 spectrophotometer (Thermo Scientific). The isolated DNA was also assessed on a 0.8% agarose gel for purity.

Amplification by PCR using gusA and nptII gene specific primers was done to confirm presence of the transgene in transgenic plants. The gusA gene specific primers sequences were: GUS F: 5' AAAGTGTGGTGTAATAATCAGG 3' and GUS R: 5' ATGGATTCCGCGCATAGTTAAAG 3' and nptII gene specific primers are: nptII F: 5' GGGTGGAGAGGCTATTCGGCTATGA 3' and nptII R: 5' ATTCCGCAAGCAGCGCATC 3'. PCR amplifications were carried out in 25 μl reaction volumes containing template DNA (100 ng genomic DNA or 20 ng
plasmid DNA), 10 µM of each primer (forward and reverse), 0.2 Mm dNTP mix, 1.5 mM MgCl₂, 1 X PCR buffer, and 5 U Taq DNA polymerase (Qiagen). Reactions were programmed to an initial denaturation of DNA at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 63°C for 1 minute and extension at 68°C for 1 minute, and a final extension at 72°C for 7 minutes.

Presence of the transgene in the plant genome of transgenic plants transformed with pCAMBIA2300-gfp was confirmed by PCR amplification using gfp gene specific primers. GFP primers were GFP F: 5’TCTGTCAGTGGAGAGGGTG 3’, GFP R: 5’CTGGTAAAAGGACAGGGCCA 3’. PCR was carried out in a Gene Amp PCR systems machine (Applied biosystems) programmed with an initial denaturation of DNA at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 58°C for 45 minutes and 72°C for 1 minute, followed by a final extension at 72°C for 10 minute. Positive control (plasmid DNA) as well as a negative control (non-transgenic plant DNA) was included in each experiment. Amplified DNA fragments were analyzed by electrophoresis in a 1% agarose gel (Sigma, Aldrich, Spain) Appendix 11)), visualized by Gel red staining, and photographed by a Gel Documentation System (Syngene).

3.2.11 Manipulation of tissue culture and optimization of selection steps to dilute chimeras of transgenic lines
Selection steps were optimized to dilute chimeras of transgenic lines obtained using multiple buds and intercalary explants in order to get uniformly transform plants. Ten PCR positive *gfp* transgenic lines obtained from MBCs of cultivar Nusu Ngombe were tracked for *gfp* expression at different stages of transformation (transient, regeneration and selection stage). Transgenic lines obtained from ECSs were used as a positive control on the basis on the fact that uniformly transformed plants are generated from single cells (Tripathi *et al.*, 2012).

### 3.2.12 Visualisation of GFP fluorescence

GFP fluorescence was visualized using a fluorescence microscope (SMZ1500, Nikon) fitted with a 100-W mercury lamp and GFP2 filters (excitation filter at 450 – 490 nm, and long barrier filter at 505 nm). GFP expression in ECS and MBCs was analyzed 3 days after co-cultivation. Samples of fresh leaves, pseudostems and roots obtained from transgenic plants at different stages of development (regeneration stage, selection stage and further selection stage) were used for microscopy. The images were recorded in TIFF format using a digital camera and compiled using Microsoft PowerPoint program.

### 3.2.13 Molecular Characterization of transgenic lines

#### 3.2.13.1 Total RNA isolation and reverse transcriptase polymerase chain reaction (RT - PCR)

Ten PCR positive lines were randomly selected for RNA extraction using RNA Qiagen RNaeasy plant mini kit (Qiagen, Valencia, USA). Approximately 100 mg of banana leaf tissues were ground using mortar and pestle under liquid nitrogen.
The RNA was treated with DNase. One microgram of genomic DNA-free RNA determined on nanodrop 2000 (Thermo Scientific) was reverse transcribed using the First strand Revert Aid Transcriptase kit (Fermentas) as per the manufacturer’s instructions.

For RT - PCR analysis, 2 μL of the synthesized cDNA was used as template in a 25 μL reaction. The first strand (cDNA) was used as a template for the amplification of gusA and 25S (used as internal control) genes. The sequences of the gusA primers are given in section 3.2.7.1 and sequences for 25S are: Musa 25S F 5' ACATTGTCAGGTGGGGAGTT 3' and R 5' CCTTTTGTTCCACACGAGATT 3'. The temperature profile used for the PCR amplification was an initial denaturation of DNA at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 63°C for 1 minute and extension at 68°C for 1 minute, and a final extension at 72°C for 7 minutes. The expected 500 and 106 bp amplicons for gusA and Musa 25S were checked by loading ten microlitre of the PCR reaction onto 1.5% agarose gel stained with Gel red.

3.2.13.2 DNA extraction and southern hybridisation

The integration and copy number of the transgenes was analyzed using Southern blot analysis. Genomic DNA was isolated from the putatively transgenic plantlets using a modified hexadecyltrimethylammonium bromide (CTAB) extraction
method for *Musa*, as described by Gawel and Jarret (1991). About 1.5 g of young fresh banana leaf tissue from eight putative transformants was homogenized in liquid nitrogen and mixed in 3 ml of preheated (65°C) DNA extraction buffer (0.1M Tris-HCl, 20 mM Sodium Ethylene diaminetetra acetic acid (EDTA), 1.4 M NaCl and 20% (w/v) hexadecyltrimethyl ammonium bromide (CTAB) and 0.2% (v/v) β- mercaptoethanol (pH 0.8) in sterile 15 ml falcon centrifuge tubes and incubated at 65°C in a water bath for 60 minutes with occasional gentle swirling. The samples were mixed with chloroform (2/3 of the volume of CTAB buffer). The contents were centrifuged at 4000 rpm for 10 minutes at room temperature, and the aqueous phase was transferred to fresh sterile centrifuge tubes. DNA precipitation was done by the addition of equal volume of isopropanol to the supernatant and tubes were inverted about 20 times to mix. The contents were stored at -20°C overnight.

The mixture was centrifuged at 4000 rpm for 10 minutes to pellet the DNA. Isopropanol was discarded and pellet dried in laminar hood for 1 hour. The DNA pellet was dissolved in 400 μl double distilled water and transferred to 1.5 ml micro centrifuge tubes. Five microliters RNAse was added followed by incubation for 30 minutes in a heating block. The samples were mixed with 400 μl chloroform-isoamyl alcohol (24:1), mixed gently and centrifuged at 12000 rpm for 5 minutes. The upper layer was transferred into a new tube and mixed with 40 μl of 3 M potassium acetate. DNA was precipitated by adding 800 μl of absolute
ethanol, followed by incubation for 1 hour at -20°C. The mixture was centrifuged at 12000 rpm for 10 minutes and ethanol was discarded. The pellet was washed with 70% [v/v] ethanol and air-dried for 1 hour. The DNA pellet was dissolved in 200 μl of double distilled water. Genomic DNA was checked by running it on 1% agarose gel stained with gel red.

Genomic DNA digestion, hybridization and detection were carried out as previously described by Tripathi et al. (2012). Approximately 20 μg of genomic DNA was digested with HindIII followed by fractionation on a 1% agarose gel. The fractionating gel was incubated in depurination buffer (Appendix 3) on a shaker for 30 minutes, denaturation buffer (Appendix 3) for 30 minutes and neutralization buffer twice (Appendix 3) for 15 minutes each. The gel was rinsed after each incubation period with distilled water. The DNA was transferred to nitrocellulose membrane (Amersham Hy BOND) for 20 hours. Transferred DNA was cross linked to the membrane by UV light exposure for one minute.

Membrane prehybridization was done for a minimum of 1 hour at 42°C in DIG easy hybridization solution (Roche) in the rotor oven. Hybridization of the membrane was conducted at 65°C with DIG-labeled $^{35}$S probe for 20 hours. The DIG-labeled probe was heated to 95°C for 5 minutes prior to addition to the membrane. Following hybridization, the membrane was washed three times with W1 solution (Appendix 3) at room temperature for 10 minutes, followed by wash
using W2 solution (Appendix 3) pre warmed at 68°C in the shaking oven set at the same temperature. This was followed by a wash with W3 solution for 15 minutes at 68°C followed by a wash with WB at room temperature for 3 minutes. The membrane was incubated in B2 solution (Appendix 3) for 30 minutes on the shaker. DIG antibodies B2 solution was added to the membrane and incubated for 30 minutes at room temperature. This was followed by three washes in WB (Appendix 3) for 20 minutes each. The membrane was transferred to B3 and incubated for 5 minutes followed by addition of CDPstar for 5 minutes. The membrane was transferred to the film cassette and left overnight and developed in the dark room.

3.2.14 Data analysis

The experiments were carried out in a completely randomized design with three replicates. The regeneration frequencies and transformation frequencies from meristems of different banana and plantain cultivars were determined. The data on different stages of optimization during multiple bud generation, transformation and regeneration was subjected to ANOVA (p<0.05) and means were separated using Turkeys HSD test (p<0.05). The means and standard errors were calculated and analyzed using Genestat software.
3.3 Results

3.3.1 Initiation of shoot tip culture from field suckers

Following initiation of surface sterilised suckers on proliferation medium, small buds were induced after three to four weeks followed by massive shoot proliferation within 4–5 months (Figure 3.2). The buds were cultured in proliferation medium on monthly basis for multiplication to generate enough starting material.

Figure 3.2: Induction and proliferation of shoots from cultivar Mpologoma suckers. (A) Culture initiation from surface sterilised field suckers (B) Multiple shoots proliferation. Scale bars 5=cm.

3.3.2 Effect of different concentrations of cytokins on multiple bud induction

The effect of different concentrations of cytokinins (BAP and TDZ) were tested using banana cultivar Ngombe to determine their efficiency in multiple bud induction. The highest number of MBC was obtained using proliferation medium containing BAP (5 mg/L), a single shoot generating an average of five to six buds
An increase in the amount of TDZ from 1 mg/L to 1.5 mg/L increased the number of buds from 0 to about 2.2 generated from a single shoot. Further increase in the amount of TDZ beyond 1.5 mg/L, decreased the number of buds (Figure 3.4 A). No buds were obtained in medium supplemented with TDZ 1 mg/L, BAP 2.2 mg/L and TDZ 1 mg/L combined with BAP 2 mg/L, (Figure 3.3 A; Figure 3.4 A). Phenolics were observed in medium supplemented with combination of BAP and TDZ (Figure 3.3 E and F). Massive rooting was observed in medium containing BAP 2.5 mg/L compared to other media combinations (Figure 3.3 D). Proliferation medium supplemented with BAP 5 mg/L, generating maximum number of MBC, was thus used with the other cultivars for multiple bud induction. An Analysis of Variance (ANOVA) was performed to determine the effect of different concentrations of cytokininns on plant height and bud induction intensity. There were significant differences (p ≤ 0.05) across all the different concentrations of cytokinnins evaluated on MBC induction (Figure 3.4 A). Mean separation using Turkeys HSD test (95%) showed a significance difference between the different concentrations of cytokininns and the number of buds obtained.

Multiple buds arising from the base of the sucker were observed in a period of three to four weeks (Figure 3.3 A). These buds were transferred to P4 medium for proliferation and after three to four monthly subcultures on fresh P4 medium, highly proliferating multiple buds also called scalps consisting of numerous white
fleshy bulbous structures bearing tiny meristems were obtained in all the cultivars (Figure 3.5).

Figure 3.3: MBC induction intensity from Ngombe suckers in different concentrations of TDZ and BAP  (A) BAP 5 mg/L induced the highest MBCs (B) TDZ 1mg/L (C) BAP 2.2 mg/L (D) BAP 2.5 mg/L (E) TDZ 2mg/l +BAP 2 mg/L (F) TDZ 1 mg/L + BAP 2 mg/L. Scale bars 3=cm.
Figure 3.4: (A) Frequency of multiple bud clumps (MBC) induction and (B) shoot height from suckers of cultivar Ngombe in different concentrations of TDZ and BAP.
Shoot height for the different concentrations of cytokinin were also determined for the same cultivar after one sub-culture. There were significant differences (p ≤ 0.05) at the different concentrations of the cytokinins used (Figure 3.4 B). Mean separation by Turkeys HSD test showed a significance difference between the different concentrations of cytokinins and the shoot height. An increase in the TDZ concentration from 1 mg/L to 2 mg/L led to an increase in the shoot height. The longest shoots (5.5 cm) were obtained in proliferation medium supplemented with BAP 5 mg/L and the shortest (2.5 cm) in medium supplemented with TDZ 1 mg/L (Figure 3.4 B).

![Figure 3.5: Multiple bud clusters (MBCs) of various cultivars after two sub-culturing](image)

(A) Ngombe (B) Gonja manjaya (C) Nusu Ngombe (D) Mpologoma (E) Gross Michel (F) Uganda green. Scale bars=1cm
3.3.3 Regeneration from MBCs and IM

Shoot induction from the MBCs was observed after two weeks which was characterized by emergence of white shoots which turned green following transfer to the light (Figure 3.6 A-B). Well differentiated shoots formed after three months (Figure 3.6 C). Regeneration was obtained in all the cultivars except in the banana cultivar Gonja manjaya. A similar regeneration experiment was done using intercalary meristems (Figure 3.6 D) where shoot initiation was observed after two to four weeks (Figure 3.6 E) and well developed shoots regenerated after two months (Figure 3.6 F).

Cultivar Gross Michel had the highest number of shoots, approximately nine shoots arising from the intercalary meristems; followed by Grande Naine and Nusu Ngombe each producing eight shoots, Uganda green, Mpologoma and Ngombe had an average of seven shoots arising from a single IM. The number of shoots obtained from MBCs varied depending on the cultivar (Figure 3.7). The highest number of shoots were twelve obtained from the MBCs of the cultivar Grande naine and the least were ten shoots from cultivar Ngombe. Gross Michel, Uganda green, Mpologoma and Nusu Ngombe produced, on average, eleven shoots per a single MBC.
There were significant differences (p ≤ 0.05) between shoot induction from the MBCs and intercalary meristems, with the MBCs inducing more shoots than the intercalary meristems across all the cultivars tested (Figure 3.7).

**Figure 3.6: Shoot regeneration from MBCs and IM** (A) small pieces of MBC (B) regenerating MBC after 2 weeks of culture (C) shoots regenerated from MBC after 3 months (D) freshly excised IM (E) shoots initiation after 2 weeks of culture (F) shoots regenerated from IM after 3 months. Scale bars=1 cm
3.3.4 Evaluation of factors affecting transformation frequency

3.3.4.1 Effect of different infection time regimes on transformation efficiency

Effect of different infection time regimes on transformation efficiency were tested using MBCs and IM of five different cultivars. Transformation efficiency was determined based on transient GUS expression characterized either as spots or expressed on the entire surface of the explant. No transformation was recorded after infecting IM of the banana cultivar Mpologoma for 5 minutes. However, an increase in the infection time from 5 minutes to 30 minutes had a significant increase in the number of explants expressing GUS activity in Mpologoma with 30 minutes infection time giving the highest number of blue spots. An increase in infection time of Ngombe IM from 5 minutes to 20 minutes increased the number
of explants expressing GUS activity with the highest number obtained after infection for 20 minutes. Further increase in infection time beyond 20 minutes decreased the number of explants expressing GUS activity. The highest number of explants expressing GUS activity for the cultivars Nusu Ngombe, Kayinja, Ngombe and Zebrina was obtained after 10 minutes of infection, beyond which there was a decrease in the number of explants expressing GUS activity. There were significant differences (p ≤ 0.05) in the number of explants expressing GUS activity for the cultivars Mpologoma, Nusu Ngombe, Ngombe, Kayinja and Zebrina upon infection for 5, 20, 25 and 30 minutes. There were no significant difference for the cultivars Mpologoma, Nusu Ngombe, Ngombe, Kayinja and Zebrina after 10 minutes infection. Mean separation using Turkeys HSD test (95%) showed a significance difference between Mpologoma and Kayinja, Ngombe and Kayinja, Nusu ngombe and Kayinja, Ngombe and Mpologoma, Nusu ngombe and Mpologoma, Zebrina and Mpologoma, Nusu Ngombe and ngombe, Zebrina and ngombe and Zebrina and Nusu Ngombe upon infection for 5 minutes.

Mean separation showed significance difference between Mpologoma and kayinja, Ngombe and Kayinja, Mpologoma and Nusu ngombe and Mpologoma, Zebrina and Mpologoma and Nusu Ngombe and Ngombe after infection for 20 minutes. Mean separation after 25 minutes showed significance difference between Mpologoma and Kayinja, Ngombe and Kayinja, Ngombe and Mpologoma, Nusu ngombe and Mpologoma, Zebrina and Mpologoma, Nusu
Ngombe and Ngombe and Zebrina and Ngombe. The difference on mean separation were significant at 30 minutes infection time for the cultivars; Ngombe and Kayinja, Mpologoma and kayinja, Ngombe and Mpologoma, Nusu Ngombe and Mpologoma, Zebrina and Mpologoma, Nusu Ngombe and Ngombe and Zebrina and Ngombe. The optimal infection time for IM was 10 minutes for the cultivars tested except Mpologoma where maximum GUS activity was at 30 minutes and was thus used in the subsequent transformation experiments (Figure 3.8 A).

An increase in infection time from 5 minutes to 30 minutes increased the number of explants expressing GUS activity for cultivars Grande Naine, Nusu Ngombe, Ngombe, Kayinja and Zebrina using MBC. Explants had bacterial overgrowth upon infection for 40 minutes thus GUS assay was not performed. The highest number of explants expressing GUS activity was recorded for the cultivar Nusu Ngombe and the lowest for the cultivar Zebrina after infection for 30 minutes. There were significant differences (p ≤ 0.05) in all the cultivars after infecting the MBC for 5 minutes.

Mean separation using Turkeys HSD test (95%) at 5 minutes showed a significance difference between cultivars; Mpologoma and Kayinja, Ngombe and Kayinja, Nusu ngombe and Kayinja, Ngombe and Mpologoma, Nusu Ngombe and Mpologoma, Nusu Ngombe and ngombe, Zebrina and ngombe and Zebrina and
Nusu Ngombe. Mean separation at 20 minutes showed a significance difference between cultivars Ngombe and Kayinja, Ngombe and Mpologoma, Nusu ngombe and Mpologoma, Zebrina and Mpologoma, Nusu Ngombe and Ngombe and Zebrina and Ngombe. Mean separation at 25 minutes showed a significance difference between cultivars; Mpologoma and Kayinja, Ngombe and Kayinja, Ngombe and Mpologoma, Nusu Ngombe and Mpologoma, Zebrina and Mpologoma, Nusu Ngombe and Ngombe and Zebrina and Ngombe. Mean separation at 30 minutes showed significance difference between cultivars; Mpologoma and Kayinja, Ngombe and Kayinja, Ngombe and Mpologoma, Nusu Ngombe and Mpologoma, Zebrina and Mpologoma, Nusu Ngombe and Ngombe and Zebrina and Ngombe. There were no significant differences for infection time of 15 minutes for all the cultivars evaluated (Figure 3.8 B). The infection period of IM and MBC for 10 minutes and 30 minutes, respectively, showed significant differences compared to the other time regimes in terms of the frequency of the GUS positive explants for all of the cultivars and was thus used in the subsequent transformation experiments. Prolonged inoculation time adversely affected the explant because of overgrowth of *Agrobacterium*. 
Figure 3.8: Effect of infection time on transformation efficiency of various cultivars using (A) IM and (B) MBC
3.3.4.2 Effect of different concentrations of acetosyringone on transformation efficiency

Using the optimal infection time, *Agrobacterium* O.D$_{600}$ of 0.8 and co-cultivation period of 3 days, different concentrations of acetosyringone (0, 100, 200, 300, 400 μM) were tested during infection and co-cultivation to determine their effect on transformation efficiency. There was no *gusA* expression without incorporating acetosyringone in the media. The highest number of explants expressing GUS activity was achieved with 100 μM acetosyringone concentration for all the cultivars (Grande Naine, Nusu Ngombe, Ngombe, Zebrina, Mpologoma and Kayinja) tested; using IM explants for transformation. There were significant differences (p ≤ 0.05) across all the cultivars with the different concentrations of acetosyringone tested (Figure 3.9 A).

Mean separation using Turkeys HSD test (95%) at 100 M acetosyringone concentration showed a significant difference between cultivars; Ngombe and Grande Naine, Nusu Ngombe and Grande Naine, Ngombe and Kayinja, Nusu Ngombe and Kayinja, Zebrina and Ngombe and Zebrina and Nusu Ngombe. Mean separation at 200 μM acetosyringone concentration showed a significance difference between cultivars; Zebrina and Grande Naine, Ngombe and Kayinja, Nusu Ngombe and Kayinja, Zebrina and Kayinja, Zebrina and Ngombe and Zebrina and Nusu Ngombe. There was a significant difference between cultivars; Ngombe and Grande Naine, Ngombe and Kayinja and Nusu Ngombe and Ngombe at 300 μM acetosyringone concentration. At 400 μM acetosyringone
concentration, significant difference were between cultivars; Kayinja and Grande Naine, Ngombe and grande Naine, Zebrina and Grande Naine, Ngombe and Kayinja, Nusu Ngombe and Kayinja, Nusu Ngombe and Kayinja, Nusu Ngombe and Ngombe, Zebrina and Ngombe and Zebrina and Nusu Ngombe. Banana cultivar Nusu Ngombe had the highest number of explants expressing GUS activity at a concentration of 100 μM acetosyringone and cultivar Ngombe had the lowest GUS activity at the same concentration. Increased acetosyringone concentration above 100 μM decreased the number of explants expressing GUS activity for the cultivars; Grande Naine, Nusu Ngombe, Ngombe, Kayinja, Zebrina and Mpologoma. Thus, 100 μM acetosyringone was used in the subsequent transformation experiments on IM (Figure 3.9 A).

An increase in the concentration of acetosyringone from 100 μM to 200 μM led to an increase in the number of explants expressing GUS activity for the cultivars; Grande Naine, Nusu Ngombe, Ngombe, Kayinja and Zebrina. Further increase in acetosyringone beyond 200 μM decreased the number of explants expressing GUS activity. The highest number of explants expressing GUS activity was reported for the cultivar Ngombe, followed by Nusu Ngombe, Zebrina and the least for the cultivar Grande Naine and Kayinja (Figure 3.9 B). Mean separation using Tukeys HSD test (95%) at 100 μM showed a significance difference between cultivars; Nusu Ngombe and Grande Naine, Nusu Ngombe and Kayinja, Nusu Ngombe and Mpologoma, Nusu Ngombe and Ngombe and Zebrina and Nusu Ngombe. There
were significant difference between cultivars; Kayinja and Grande Naine, Mpologoma and Kayinja, Ngombe and Kayinja and Zebrina and Kayinja at 200 μM acetosyringone concentration. Significant difference at 400 μM acetosyringone concentration was observed between cultivars; Nusu Ngombe and Kayinja and Nusu Ngombe and Mpologoma. Thus, 200 μM acetosyringone was used in all transformation experiments on MBCs.
Figure 3.9: Effect of different acetosyringone concentration on transformation using (A) IM and (B) MBC
3.3.4.3 Effect of wounding on transformation efficiency

Different wounding methods were tested in order to determine their effect on transformation efficiency. Different sonication and vacuum infiltration times were tested using the optimised infection time and acetosyringone concentration using IM and MBCs. An increase in vacuum infiltration time from 1 to 2 minutes for the cultivars; Kayinja, Grande Naine, Nusu Ngombe, Zebrina and Ngombe transformed using IM increased the number of explants expressing GUS activity. Vacuum infiltration of more than 2 minutes for the same cultivars decreased the number of explants expressing GUS activity (Figure 3.10 A). The optimal vacuum infiltration time using IM was 2 minutes.

Mean separation using Turkeys HSD test (95%) after 1 minute vacuum infiltration showed a significance difference between cultivars; Kayinja and Grande Naine, Ngombe and Grande Naine, Nusu Ngombe and Grande Naine, Zebrina and Grande Naine, Nusu Ngombe and Kayinja, Nusu Ngombe and Ngombe and Ngombe and Zebrina and Nusu Ngombe. At vacuum infiltration for 2 minutes, significance difference was between cultivars; Kayinja and Grande Naine, Ngombe and Grande Naine, Zebrina and Grande Naine, Nusu Ngombe and Kayinja, Nusu Ngombe and Ngombe and Zebrina and Nusu Ngombe. There was a significance difference between cultivars; Nusu Ngombe and Grande Naine, Nusu Ngombe and Kayinja, Nusu Ngombe and Ngombe and Zebrina and Nusu Ngombe after 3 minutes vacuum infiltration. Mean seperation showed significance

An increase in vacuum infiltration time for MBCs from 1 to 5 minutes increased the number of explants expressing GUS activity for the cultivars; Grande Naine, Nusu Ngombe, Ngombe, Zebrina and Kayinja. There were significant differences \( p \leq 0.05 \) among the cultivars with Grande Naine recording the highest number of explants expressing GUS activity and Kayinja recording the least after vacuum infiltration for 1, 2, 3, 4 and 5 minutes. The highest number of explants expressing GUS activity was obtained after vacuum infiltration of MBCs for 5 minutes and was subsequently used as the optimal vacuum infiltration time for all the cultivars (Figure 3.10 B). Bacterial overgrowth was observed on explants after vacuum infiltration for more than 5 minutes.

Mean separation using Turkeys HSD test (95%) showed a significance difference between cultivars for the different vacuum infiltration time. The difference at 1 minute vacuum infiltration time were between cultivars; Kayinja and Grande
Figure 3.10: Effect of vacuum infiltration time on transformation efficiency using (A) IM and (B) MBC
An increase in sonication time from 1 to 2 seconds led to an increase in the number of MBCs expressing GUS activity in the cultivars Nusu ngombe, Ngombe, Kayinja and Zebrina. Further increase in sonication time for more than 2 seconds decreased the number of explants expressing GUS activity for cultivars Nusu Ngombe, Ngombe, Kayinja and Zebrina. There were significant differences (p ≥ 0.05) in number of explants expressing GUS activity of different cultivars at 2 seconds of sonication time. Mean separation using Turkeys HSD test (95%) at 2 seconds of sonication time showed a significance difference between cultivars; Ngombe and Kayinja, Nusu Ngombe and Kayinja, Zebrina and Kayinja, Nusu Ngombe and Ngombe, Zebrina and Ngombe and Zebrina and Nusu Ngombe. Banana cultivar Ngombe recorded the highest number of explants expressing GUS activity and Kayinja had the lowest after sonication for 2 seconds. The highest number of explants expressing GUS activity was obtained when MBCs were sonicated for 2 seconds and this was subsequently used as the optimal time for all the cultivars (Figure 3.11).
The effect of combined sonication and vacuum infiltration treatments on transformation efficiency was also tested using MBCs. The optimised parameters including infection time, acetosyringone concentration, sonication and vacuum infiltration times, together with combined sonication and vacuum infiltration times and a control (with no wounding effect) were used to compare the transformation efficiency based on the number of stably transformed events regenerated. There were significant differences \((p \leq 0.05)\) in GUS expression between the sonication, vacuum infiltration, combined sonication and vacuum infiltration and the control \(\textit{(Agrobacterium} \) infection alone). Mean separation using Turkey's HSD test (95%) showed a significance difference between control and combined sonication and vacuum, control and sonication, control and vacuum, sonication and combined vacuum and sonication and between vacuum and combined sonication and
vacuum. The intensity of GUS expression was higher after combined sonication and vacuum infiltration and no expression at all for the control with no wounding (Figures 3.12 and 3.13). Combined sonication and vacuum infiltration had the highest number of explants expressing GUS activity (13 MBCs), followed by only vacuum (10 MBCs) or only sonication (9 MBCs) and the control without sonication or vacuum had the lowest number of explants expressing GUS activity (5 MBCs) (Figures 3.12 and 3.13). Combined sonication and vacuum infiltration was used in the optimized transformation protocol.
Figure 3.12: Stable GUS expression for the cultivar Uganda green: (A) no wounding (B) sonication (C) vacuum infiltration (D) combined sonication and vacuum infiltration. Scale bars=1 cm
Figure 3.13: Effect of different wounding methods on transformation efficiency for the cultivar Uganda green

3.3.4.4 Effect of different explants on transformation efficiency

In this study, transformation efficiency of two explants (MBC and IM) of the banana cultivar Uganda green were compared using pCAMBIA2301 and pCAMBIA2300-gfp with the optimised transformation parameters (infection time, actosyringone concentration and combined sonication and vacuum infiltration). Analysis of variance showed a significant difference between (p ≤ 0.05) the two explants. Mean separation using Turkeys HSD test (95%) showed a significance difference between MBC and IM. However, there were no significant differences between the two reporter genes used to transform the same explant. MBCs had a significantly higher number of PCR positive plants expressing GUS activity as
compared to IM and was subsequently used in transformation experiments for all the cultivars (Figure 3.14).

![Graph showing effect of different explants on transformation](image)

**Figure 3.14: Effect of different explants (IM and MBC) of the banana cultivar Uganda green on transformation using two constructs expressing different reporter genes based on PCR positive plants**

### 3.3.4.5 Regeneration of stably transformed plants

The optimized transformation protocol as described in figure 3.15 was used to regenerate transgenic plants for the 9 cultivars including Mpologoma, Kayinja, Nusu Ngombe, Grande Naine, Gross Michel, Ngombe, Uganda green and Zebrina using the two reporter genes. Pre-conditioned and wounded MBC were *Agrobacterium* infected (Figure 3.16 A - B respectively). The *Agrobacterium* infected explants were washed and cultured for resting for 7 days (Figure 3.16 C). After the recovery phase, MBCs were transferred to selection medium containing kanamycin 100 mg/L for 2 months (Figure 3.16 D - E), with 15 days sub-culture
intervals in the same medium, to allow the growth of transformed shoots. Bleaching was observed in the non-transformed shoots as well as in the control under selection. The shoots that survived on selection medium (Figure 3.16 F - G) were maintained in proliferation medium for multiplication and for further analysis.

The protocol was optimized further for dipoid banana Calcutta 4. To further optimize the protocol for generation of transformed events for cultivar Calcutta 4 and to enhance transgenic selection, the optimal inoculation density was lowered from 0.8 to 0.5. This is because at high O.D levels, the explant tissues were wholly colonized by Agrobacterium. In addition, the infection time for Calcutta 4 was reduced to 5 minutes and the recovery period was extended from 7 days (used for other cultivars) to 14 days to minimise death due to selection pressure. Selection pressure for Calcutta 4 was also reduced to Kanamycin 50 mg/L.

Analysis of variance showed a significant differences ($p \leq 0.05$) among all the different cultivars transformed with pCAMBIA2301 with highest transformation efficiency (13%) for the cultivar Nusu Ngombe and the least transformation efficiency (5 %) for the cultivar Kayinja (Figure 3.17). Mean separation using Turkeys HSD test (95%) showed a significant difference between cultivars; Gross Michel and Calcutta 4, Kayinja and Calcutta 4, Zebrina and Calcutta 4, Nusu Ngombe and Grande Naine, Zebrina and Grande Naine, Mpologoma and Gross
Michel, Nusu Ngombe and Gross Michel, Uganda Green and Gross Michel, Zebrina and Gross Michel, Mpologoma and Kayinja, Nusu Ngombe and Kayinja, Uganda Green and Kayinja, Zebrina and Kayinja, Zebrina and Mpologoma, Zebrina and Ngombe, Zebrina and Nusu Ngombe and Zebrina and Uganda Green. There were also significant differences (p ≤ 0.05) among the cultivars transformed with pCAMBIA2300-gfp. The highest transformation efficiency using pCAMBIA2300-gfp was 12% for the cultivar Nusu Ngombe and the least was 8% for the cultivar Kayinja. The highest transformation efficiency using the two reporter genes was Nusu Ngombe and Zebrina had the lowest transformation efficiency using pCAMBIA2301. There were no significant differences among the cultivars transformed with the two reporter genes. There were significant differences between the diploids (Calcutta 4 and Zebrina). A significant difference (p ≤ 0.05) among the beer cultivars (Mpologoma and Kayinja) was also recorded (Figure 3.18).
Figure 3.15: Schematic representation of *Agrobacterium* transformation of banana MBCs and their regeneration into whole transgenic plants
Figure 3.16: Schematic representation of transformation of Uganda green using MBC (A) pre-conditioned MBC (B) *Agrobacterium* infected MBC (C) *Agrobacterium* infected MBCs in the recovery stage (D) *Agrobacterium* infected MBC on selection (E) regeneration of putative transformants on selective medium (F - G) second step selection. Scale bars=2

3. 3.4.6 Histochemical GUS assay

Transient GUS expression characterized by blue colouration was observed in *Agrobacterium* infected multiple bud clumps (Figure 3.17 A). Stable GUS expression was observed in sampled parts (leaves, pseudostem, corm and roots) of transgenic plants tested (Figure 3.17: A, B, C, D, E, F and G). Histochemical GUS staining of the various tissues of transgenic plants demonstrated that they were fully transformed and no chimerism was observed. No GUS staining was detected in tissues of the non-transgenic control plants (Figure 3.17 H - I).
Figure 3.17: Transient and stable gus expression in sampled tissues  (A) transient gus expression on MBCs (B) stable gus expression in the cross section of the pseudostem (C, E, G) stable gus expression in the roots and (D, F) stable gus expression in the corm (F) stable gus expression in the intercalary meristem (H - I) control corm and root, respectively. Scale bars=2 cm
Figure 3.18: Transformation efficiency of different banana cultivars using two constructs (pCAMBIA2301 and pCAMBIA2300-gfp)
3.3.4.7 Transformation of embryonic cell suspension of banana cultivar Gonja manjaya

Cells subjected to Agrobacterium mediated transformation multiplied and distinct whitish cell clusters proliferated on kanamycin containing selection medium MA3 after three months, whereas the untransformed control cells turned brown (Figure 3.19 A). Transgenic embryogenic cells developed into embryos and germinated on selective medium MA3 medium (Figure 3.19 B - C). The embryos were then transferred onto selective semisolid RD1 media with subsequent sub-culturing every two weeks to allow embryo maturation (Figure 3.19 D). Embryos matured on selective RD1 after one month (Figure 3.19 E) and onto selective semisolid MA4 media. Mature embryos germinated into shoots after one month (Figure 3.19 F - G). The plantlets generated and were maintained and proliferated on proliferation medium until well developed shoots were formed (Figure 3.19 H). All plants regenerating from one cell clump were treated as clones and considered as one line. An average of 45 transgenic plants per 0.5 ml settled cell volume were obtained in this study. A single PCR positive line was sampled randomly and used as a positive control for gfp expression experiment.
Figure 3.19: *Agrobacterium* mediated transformation of plantain cv. “Gonja manjaya” using embryogenic cell suspension  
(A) *Agrobacterium* - infected cells proliferating on selective medium (B, C) Embryos on selective MA3 Embryo Development Medium (D, E) Embryos on selective RD1 Embryo Maturation Medium (F, G) Embryo germination on selective medium (H) Transgenic plantlets regenerated on selective medium. Scale bars=2 cm
3.3.4.8 Molecular characterization of transgenic plants

The regenerated putatively transformed plantlets were further analysed for the presence of transgene using PCR. Genomic DNA from kanamycin resistant shoots was subjected to PCR analysis for the presence of transgenes using gusA or gfp and nptII specific primers. The amplified products were observed in all the plants tested, confirming the presence of transgenes and no plant escapes. The expected 500 bp fragments of gfp gene was observed in plants transformed with pCAMBIA2300-gfp (Figure 3.20 A). All the tested plants also showed amplification using nptII gene specific primers (Figure 3.20 B). The expected 500 bp fragments of gusA gene were amplified in all the GUS positive plants transformed with pCAMBIA2301, which is the same as positive plasmid control (Figure 3.20 C). No amplified product was observed in case of untransformed plant (Figure 3.20 A - C).

RT-PCR analysis was performed on 9 randomly selected PCR positive transgenic lines to confirm the transgene expression. An expected 500 bp fragment was amplified from all the 9 tested transgenic plants using gusA specific primers (Figure 3.20 E), confirming the expression of gusA gene. However, the expression of gus A gene was variable among the transgenic lines tested. No RT-PCR product was detected in the non-transformed control plant (Figure 3.20 E). The expected 120 bp fragment using Musa 25S house keeping genes was detected in all the samples tested (Figure 3.20 F).
Figure 3.20: PCR and RT-PCR analysis of banana cultivar Nusu Ngombe and Gonja manjaya (A) gfp (B) npt II gene (C) PCR analysis of banana cultivar Gonja manjaya (positive control) using gfp primers. L is 1KB ladder and lane 1 is positive control, lane 2 is negative control and lanes 3 to 9 and 22 respectively were loaded with 10 μL of the PCR reaction of the putative banana plants. (D) PCR analysis of transgenic banana plants of cultivar Calcutta 4 to detect the presence of gusA (E) RT-PCR expression of gusA (F) RT-PCR expression of 25S gene. Lane P is Plasmid; Lane NT (Non-transgenic control plant); Lanes 1 – 24 (transgenic lines transformed with pCAMBIA2301)
3.3.4.10 Optimization of selection steps to dilute chimeras of transgenic lines

Chimerism was monitored at all stages of transformation in terms of gfp expression in the tissues sampled obtained from lines generated from MBCs of banana cultivar Nusu Ngombe and cell suspension of Gonja manjaya. There was uniform transient expression after the co-cultivation stage possibly due to the presence of *Agrobacterium* (Figure 3.21 A, B and C). Uniform gfp expression was observed in the positive control (ECS of Gonja manjaya) at all stages of plant transformation (transient, regeneration and selection). All the leaves, roots and cross section of the pseudostem sampled for gfp microscopy fluorescensed. The sampled event regenerated all stages of selection (Figure 3.21 D, E). No fluorescence was observed in the non transgenic plant (Figure 3.21 F).
Figure 3.21: *gfp* expression of the banana cultivar Nusu Ngombe at regeneration stage (A) transient (B – C) regeneration stage and (D – E) ECS of banana cultivar Gonja manjaya (positive control) (F) Non transgenic / Negative control. Magnification (×1000)
Uniform <i>gfp</i> expression was not always observed in plants regenerated from MBCs. Some leaves and roots sampled during the early regeneration stage did not fluoresce at all and some showed patchy fluorescence mostly in the vascular region (Figure 3.22 A and B). Significant fluorescence in shoots was detected with clearly visible <i>gfp</i> expression in lateral roots especially in the vascular system. Chimerism was observed during the regeneration step in medium containing Kanamycin 100 mg/L, characterized by patchy fluorescence in young leaves (Figure 3.22 A – D) and roots of the transgenic plants (Figure 3.22 B and E) as opposed to uniform expression in roots and leaves obtained from ECS (Figure 3.22 C, F and G). Chimerism was also observed in shoots during the selection steps characterized by white patches on the leaves (Figure 3.25). Some of the leaves and roots sampled from the same line showed uniform fluorescence or partial expression while others did not fluorescence at all. Such lines were subjected to further selection pressure and fresh leaf and root samples were monitored at in the subsequent sub-culture.
Figure 3.22: *gfp* expression at subculture 1 and 2 in MBC (A – B) sampled leaf and root, respectively (C) uniform expression at subculture 1 in ECS (DE – FG) expression at subculture 2 in MBC and ECS respectively. Magnification (×1000)

Partial *gfp* expression at subculture three was observed in leaf and root sample obtainend from MBC (Figure 3.23 A and B). There was uniform expression in the sampled leaf and root (Figure 3.23 C and D, respectively) obtained from ECS at subculture 3 and in the sampled leaf and root (Figure 3.23 G and H), respectively in subculture 4. Uniform expression in leaves and roots of plants generated from MBC was observed after selection step four (Figure 3.23 E, F, respectively).
Figure 3.23: *gfp* expression at sub culture 3 in MBC and ECS respectively (AB – CD) Expression at sub culture 4 in MBC and ECS, respectively (EF – GH). Magnification (x1000)

The plants that survived the selection pressure showed uniform expression at selection step four. Further selection step five and six were included, sampling all the leaves and roots to rule out any incidence of chimerism. All the leaves and roots sampled at sub culture five showed uniform *gfp* (Figure 3.24 A, B, respectively). A cross section of pseudostem from plants regenerated from MBCs at selection step five also showed uniform expression (Figure 3.24 C). The uniform green fluorescence observed at all stages is an indication that there was uniform expression of the target gene into banana tissues. Similar expression was
observed in the leaf, root and cross section of the pseudostem for the samples of the line generated from ECS (Figure 3.24 D, E and F).

Figure 3.24: *gfp* expression at subculture 5 in leaves, roots and a cross section of the pseudostem for the MBC (A, B and C, respectively) and ECS (D, E and F, respectively). Magnification (×1000)
Not all the plants survived the further selection pressure possibly due to the extent of chimerism. There was a decline in the number of transgenic plants with the subsequent sub culture in selection medium. However, there was no further loss after sub culture five (Figure 3.26). The sampled event from ECS regenerated all stages of selection. Each round of banana shoot regeneration under selection usually takes 2 weeks, thus the whole selection process can be optimized to 8 weeks, which is a reasonable length of time for transgenic selection.
Figure 3.26: Percentage of the PCR positive transgenic lines under selection in the successive subculture cycles

Stable integration of the *gfp* gene was further confirmed in 5 randomly selected gfp positive lines by Southern blot analysis using DIG labelled CaMV35S promoter as a probe. The results indicate that the T-DNA was inserted into the genomes of the transgenic plants. The copy number in the transgenic plants ranged from one to two, based on the number of bands present in each lane. Transgenic plants in lane 1 and 4 / 5 had one copy while transgenic plant in lane 2 had two copy numbers. A signal was detected in the positive control (P) and no signal in the negative control (lane 3) (Figure 3.27). The band size differed in the transgenic lines tested, confirming that the plants were the result of independent transformation events which are probably randomly located on the genome.
Altogether, the results of molecular analyses confirm that the $gfp$ gene is stably integrated in the plants.

Figure 3.26: Southern blot analysis of genomic DNA of transgenic lines digested with HindIII. Lanes 1, 2, 4 and 5, transgenic plant lines, lane 3 is non transgenic plant, P, plasmid construct DNA digested with HindIII
3.4 Discussion

The formation of MBCs is dependent on the banana cultivar and medium used (Sadik et al., 2007; Sholi et al., 2009). The present study evaluated different concentrations of TDZ and BAP to ascertain the optimal concentration for maximum number of MBC induction. Among the various concentrations evaluated, the optimal MBC induction was achieved with BAP at 5 mg/L. MBCs appeared as white fleshy bulbous structures (cauliflower like - structure) as earlier reported by (Dheda et al., 1991; Strosse et al., 2006; Sadik et al., 2007; Sholi et al., 2009). An increase in TDZ concentration was shown to increase the shoot height of banana plantlets in the present study instead of forming multiple buds. This could be attributed to the response of different genotypes to TDZ. Arinaitwe et al. (2000) reported high TDZ concentrations inhibit axillary shoot proliferation and stimulate formation of multiple bud clumps in bananas. High TDZ concentration has been reported to result in a higher number of extremely stunted and undifferentiated shoots in tobacco cultures (Thomas and Katterman, 1986). Other reports indicates that TDZ is resistant to cytokinin degrading enzymes, and is said to induce excessive suppression of lateral buds resulting in reduced proliferation rates (Hueteman and Preece, 1993).

The development of an efficient genetic transformation protocol is a pre - requisite for the application of biotechnology tools for the genetic improvement of crops. The objective of the present study was to develop an efficient Agrobacterium
mediated transformation using IM and MBCs of banana. In order to achieve the maximum transformation efficiency, a number of transformation parameters were studied and optimized including infection time, the concentration of acetosyringone, sonication, vacuum infiltration and a combination of sonication and vacuum infiltration. The effect of different types of explants (IM and MBC) on transformation efficiency was also investigated.

Infection time is one of the critical factors influencing the T-DNA transfer from Agrobacterium into the host plant genome. In this study, different inoculation times were tested and the optimal gusA expression was recorded after infecting MBCs for 30 minutes and IM for 10 minutes. Increasing the infection time above 30 minutes for MBC and 10 minutes for IM led to Agrobacterium recurrence and subsequent death of the explant. Our results are consistent with earlier reports by Lee et al. (2006) and Zhao et al. (2011) who recommended an inoculation period of 30 minutes in orchard grass and Chinese upland rice. However, Yip et al. (2011) reported 20 minutes infection time on banana MBCs. The difference from our results could be due to the use of different banana cultivars. Different inoculation periods have been reported by different researchers for different crops. Zhao et al. (2000) and Howe et al. (2006) in sorghum and Ishida et al. (2007) in maize, reported 5 minutes of inoculation period as optimal, while Sarker and Biswas (2002) reported 50 minutes of inoculation period as optimal along with 5
minutes vacuum treatment in rice. This suggests that infection time varies depending on the plant species used and also on the cultivars.

Acetosyringone, one of the phenolic compounds secreted by wounded tissues, is a potent inducer of *Agrobacterium vir* genes (Stachel *et al*., 1985), thus enhancing *Agrobacterium*-mediated transformation (Belarmino and Mii, 2000). Monocotyledons do not synthesize compounds such as acetosyringone known to induce the expression of *vir* genes. Addition of exogenous acetosyringone during transformation studies in monocotyledons is thus critical as it enhances gene transfer (Stachel *et al*., 1985; Hiei *et al*., 1994). Results of the present study indicate that inclusion of 100 µM and 200 µM of acetosyringone for IM and MBC, respectively, significantly enhanced the transformation frequency. These results suggest that inclusion of acetosyringone in co-cultivation medium is essential for successful transformation of bananas and plantains. This observation supports earlier reports in other crops showing that the addition of acetosyringone during co-cultivation increases the number of transformed cells in the target tissue in rice (Hiei *et al*., 1994; 1997), maize (Ishida *et al*., 1996), wheat (Cheng *et al*., 2003; Wu *et al*., 2003) and barley (Shrawat *et al*., 2007). The optimum concentration of acetosyringone obtained in this study concurs with that reported for banana transformation using MBC as explants (Yip *et al*., 2011). Significant transient GUS expression when the *Agrobacterium* inoculated explants were grown in the presence of acetosyringone in switch grass has been reported (Chen *et al*., 2010).
Exclusion of acetosyringone in the transformation of rice and onion resulted in low level of transient GUS expression without regeneration of any stable transformants (Hiei et al., 1997; Zheng et al., 2001). Khanna et al. (2004) reported an increase in the transformation frequency in bananas following the incorporation of acetosyringone in the medium. May et al. (1995) reported a similar 100 μM acetosyringone concentration in the transformation of banana shoot tips. However, Fang et al. (2002) reported that acetosyringone was not essential for Agrobacterium-mediated transformation of barley. Similar results were reported in Asparagus officinalis (Delbreil et al., 1993), Triticum aestivum (Cheng et al., 1997), Saccharum officinarum (Manickavasagam et al., 2004) and banana suckers (Subramanyam et al., 2011).

Delivery of T-DNA into the target plant tissue can be enhanced by exploring different methods of wounding to increase Agrobacterium infection. The essence of wounding is to allow the Agrobacterium to infect the target tissue as well as enhancing the production of inducers of the T-DNA transfer process from the wounded tissue (Stachel et al., 1985). There are different methods of wounding ranging from simple wounds made during the explant preparation (Horsch et al., 1985) to wounds generated using particle gun (Bidney et al., 1992). Syringes containing Agrobacterium can also be used for delivering the Agrobacterium to the target tissue (Chee et al., 1989). Wounding through sonication with naked DNA has also been reported (Joersbo and Brunstedt, 1990; 1992; Zhang et al.,
The enhanced transformation rates using sonication assisted *Agrobacterium* transformation results from micro-wounding both on the surface and deep within the target tissue. Therefore, this system has the potential to transform meristematic tissue buried under several cell layers (Trick and Finer, 1997). The duration of sonication had a dramatic effect on the number of GUS positive explants obtained in this study compared to the control (no wounding). A high GUS expression was observed after sonication of MBCs for two seconds. Sonication was reported to create microwounds through which *Agrobacterium* enters and adheres to the cell in embryonic suspension tissues of soybean hence the increase in transient GUS expression (Trick and Finer 1997; 1998). Subramanyam *et al.* (2011) reported maximum production of GUS positive shoots from banana suckers sonicated for 6 minutes.

Vacuum infiltration enlarges the contact area of the explants, allowing the transformation of cells found deeper in the scalps containing the meristematic cells as compared to the cells from the surface that are accessible by co-cultivation. Gu *et al.* (2008) suggested that a vacuum creates a negative pressure environment that in turn increases *Agrobacterium* volatilization, consequently enhancing the transfer of a foreign gene into plant cells. In the present study, when combined with optimized parameters (infection time, concentration of acetosyringone), vacuum infiltration assisted transformation of MBCs and IM of banana and plantain significantly enhanced the transformation frequency and efficiency. The
extension of vacuum infiltration time beyond 5 minutes resulted in complete colonization of explant tissues by *Agrobacterium*, making it difficult to eliminate and subsequently no regeneration and recovery of transgenic plants. Acereto-Escoffe et al. (2005) compared the effect of co-cultivation versus vacuum infiltration of meristematic banana tissues of *Musa acuminata* variety Grand Naine and reported a higher glucuronidase activity in infiltrated samples compared to co-cultivated ones. Vacuum infiltration led to higher transformation efficiency in partially germinated seeds of soybean (Ronde et al., 2001), cereals, (Dong et al., 2001; Amoah et al., 2001) and recalcitrant plants such as *Triticum aestivum* and *Pinus radiata* (Amoah et al., 2000; Charity et al., 2002). This could be attributed to a deeper bacterial penetration in the infiltrated tissues which increases the likelihood of a higher transformation of the banana tissues. Trieu et al. (2000) reported 3 minutes vacuum infiltration in the transformation of *Medicago truncatula* seedlings. Subramanyam et al. (2011) reported vacuum infiltration for 6 minutes in banana suckers. These variations in the optimum time vacuum infiltration could be due to the difference in cultivars and also plant species.

The study also evaluated the effect of combining sonication and vacuum infiltration time on transformation efficiency. Vacuum infiltration for five minutes and sonication for two seconds were applied to banana MBCs in an attempt to increase wounding and *Agrobacterium* infection and ultimately transient GUS expression. A higher transient GUS expression was obtained following a
combination of the two wounding methods than when sonication or vaccum infiltration were used individually. The results of this study are consistent with Shrawat et al. (2007) and Liu et al. (2005) who reported that vacuum infiltration combined with sonication assisted in Agrobacterium-mediated transformation of barley and kidney bean. Sonication for 2 seconds combined with vacuum infiltration for 10 minutes has been reported in production of transgenic citrus plants (de Oliveira et al., 2009). Park et al. (2005) reported 5 minutes sonication combined with 5 minutes vacuum infiltration in the transformation of radish plants.

The study also incorporated the pre-conditioning step for two days as earlier reported in the meristem transformation of bananas (Tripathi et al., 2008). Preconditioning of explants has been reported to not only inhibit necrosis but also increases the transformation efficiency. This was consistent with earlier works in Arabidopsis thaliana (Sangwan et al., 1992); tobacco (Sunilkumar et al., 1999); Brassica napus (Cardoza and Stewart, 2003) and Secale cereale (Popelka and Altpeter 2003). The improvement of the transformation efficiency as a result of preconditioning may be attributed to the initiation of cell division upon wounding; the improved binding of Agrobacterium to the newly synthesized cell wall at the wound sites and the production of vir-inducing compounds by the metabolically active dividing cells (Cardoza and Stewart, 2003). Actively dividing cells have been reported to be more responsive to delivery and integration of T-DNA into the
host (An, 1985). Pre-culture of explants for two days was also reported for *Picrorhiza kurroa* (Bhat *et al*., 2012). It has been proposed that during the preconditioning step, the plant cells undergo a series of physiological adaptation which enhances the competence for *Agrobacterium* transformation systems (Costa *et al*., 2002). In addition, several authors have suggested that the pre-cultivation step reduces plant-stress prior to *Agrobacterium* infection (Venkatachalam *et al*., 1998; Li *et al*., 2003).

In this study, *neomycin phosphotransferase* (*nptII*) was used as a selectable marker gene and kanamycin antibiotic (100 mg/L) was used as a selection agent for stable integration as stated in the previous study for meristem transformation (Tripathi *et al*., 2008). The effect of kanamycin during selection was evident due to bleaching of the non-transformed banana plantlets. However, Kanamycin at 100 mg/L exerted a lot of selection pressure on the cultivar Calcutta 4. This can be attributed to differential response among cultivars. Kanamycin exerts its effect on mitochondria and chloroplasts by impairing protein synthesis, resulting in chlorosis (Weide *et al*., 1989). Similar kanamycin effects were reported in transgenic papaya (Yu *et al*., 2003), transgenic pea plantlets (Bean *et al*., 1997; Nadolska-Orczyk and Orczyk, 2000) and transgenic bananas (Tripathi *et al*., 2008). At high concentrations, kanamycin is phytotoxic and causes shoot chlorosis and necrosis (Yepes and Aldwinckle, 1994). The inhibitory effect of kanamycin in the regeneration of plant transformation has been previously
reported for other crops (Yao et al., 1995; Bretagne-Sagnard and Chupeau, 1996). Rotting of the entire pseudostem for shoots under selection was observed that led to the eventual death of the plantlets. All the plants regenerated were transgenic confirming that kanamycin selection was efficient. This could have possibly been due to the continued use of cefotaxime. Similar effect of high toxicity associated with the use of cefotaxime has been reported in many different plant tissues (Lin et al., 1994; Antunez de Mayolo et al., 2003).

An efficient transformation system using appropriate starting explants is a prerequisite in the generation of transgenic plants (Ghosh et al., 2009). Different explants have different effects on transformation since different tissues have different endogenous hormone levels which are as important as exogenous hormone levels as they determine the rate of dedifferentiation and re-differentiation. The two processes of dedifferentiation and redifferentiation are equally critical for both direct and indirect shoot organogenesis (Trigiano and Gray, 2002). Agrobacterium mediated transformation has been successfully used to generate transgenic banana plants using different initial explants. In this study, MBCs of the different banana cultivars were transformed using Agrobacterium strain EHA105. Besides giving a higher transformation efficiency, generation of MBCs is cultivar independent, does not require a lot of starting material or expertise as in the case of IM. MBCs transformation in bananas has also been reported in banana cultivar Gros Michel (Yip et al., 2011). This method is
reproducible, less tedious and faster for the cultivars for which formation of ECS is not possible. May et al. (1995) used apical meristems, Tripathi et al. (2008) used intercalary meristems of East African highland bananas as initial explants to obtain transgenic plants. The use of ECS which are cultivar dependent and time consuming in banana and plantain transformation has also been reported (Tripathi et al., 2010; 2012).

ß-glucuronidase and green fluorescent protein genes have been incorporated with success into several plants and used as markers of gene expression (Martin et al., 1992; Stewart, 2001). Histochemical Gus assay was performed in the current study to assess the successful insertion of the gusA gene into the Agrobacterium-mediated transformed MBCs, sections of putatively transformed leaves, pseudostems and roots. High expression of gusA gene indicative of successful transfer of Agrobacterium into the target tissue was observed in some of the putatively transformed tissues sampled at the transient level. Stable gusA expression in mature tissues of bananas with no escapes confirmed the effectiveness of the optimized protocol. Similar results on apical shoot tips excised from in vitro regenerated shoots and successfully expressing the b-glucuronidase gene in plantain system have been reported (Tripathi et al., 2005). High transient and stable gusA expression in meristems and mature tissues of banana have also been reported by Tripathi et al. (2008) in EAHBs, Khanna et al. (2004) in Cavendish and Lady finger and Huang et al. (2007) in Musa acuminate cv. Mas.
Other reporters noted high GUS activity in initial transient assays of sorghum that could not be detected later (Casas et al., 1993; 1997). This was attributed to transgene methylation in sorghum cells that inhibited expression of the reporter gene. GUS expression seems to be very dependent on the plant developmental stage as reported in apricot plants where expression is uniform and strong in young regenerated buds but partial expression in the petioles and veins of leaves from micropropagated shoots (Petri et al., 2008). Differential expression may be found in different tissues or with plant development.

Most commonly used detection systems in visualization of gene expression require exogenous added substrates and co-factors, are expensive and time-consuming (Stewart, 1996). It is therefore necessary to have a detection system which is easy, simple, real-time, and non-toxic, does not require added substrates and one which could be applicable on any living tissues without impacting any damage. Such a detection system could be provided by GFP. GFP is a fluorescent protein isolated from coelenterates, such as the Pacific jellyfish, *Aequoria victoria* (Morin and Hastings, 1971) whose role is to transduce, by energy transfer, the blue chemiluminescence of another protein, aequorin, into green fluorescent light (Ward, 1979). Chalfie et al. (1994), demonstrated that GFP can be expressed as a functional transgene and thus opening new avenues of investigation in cellular, developmental and molecular biology.
The optimized protocol was used to generate transgenic lines of various cultivars of bananas and plantains like Mpologoma, Kayinja, Nusu Ngombe, Ngombe, Calcutta 4, Zebrina, Uganda green, Grande Naine and Gross Michel. The transformation efficiency varied among the cultivars. The differences in the transformation efficiency among the cultivars could be associated to genotypic variations. The present study reported transformation efficiency (8-12 %) depending upon cultivar, which is comparable to earlier report by Tripathi et al. (2008) who reported 10% transformation efficiency in meristem transformation. Wordragen and Dons (1992) suggested that differences in response among cultivars could be caused by differential response to the wounding stress. The protocol described here is applicable to both triploids and diploids and transgenic plants can be obtained within a short time.

Banana cells were subjected to heat shock prior to transformation as a way of minimizing cell death response resulting in recovery of a large number transformed plants following Agrobacterium infection. The inclusion of a surfactant, pluronic F68 during ECS transformation was to pre-induce the Agrobacterium (Cheng et al., 1997) and to further aid in the transformation of banana cells (Khanna et al., 2004). Acetosyringone was also used to enhance chemotactic movement and attachment of Agrobacterium tumefaciens to wounded tissues of banana cultivars (Hernández et al., 1999). Washing of Agrobacterium infected ECSs in liquid media supplemented with cefotaxime reduced the stress
associated with Agrobacterium recurrence and the effects of phenolics before transfer to selection media. After three subcultures Agrobacterium contamination was completely eliminated and cells proliferated vigorously. ECS turned brown due to necrosis and massive death of non-transformed embryogenic cells on selective MA3 media was evident after three weeks. Numerous whitish cell clumps appeared on the surface with dead cells in the background a month later. Similar results were reported by Arinaitwe (2008) and Ghosh et al. (2009).

The present study reported 40 to 50 transgenic plants per 0.5 ml settled cell volume, which is similar earlier report, where approximately 50 to 60 transgenic plants per 0.5 ml settled cell volume were regenerated for plantain cv. “Gonja manjaya” in about 4 to 5 months from the time of Agrobacterium inoculation of ECS (Tripathi et al., 2012). Ganapathi et al. (2001) reported production of up to 40 plants per 0.5 ml packed cell volume after Agrobacterium mediated transformation of shoot apex derived ECS of cv. “Rasthali” (AAB). Khanna et al. (2004) reported 25 to 65 plants per 50 mg of settled cell volume of embryogenic suspension cells of banana cvs. Cavendish and Lady Finger. Ghosh et al. (2009) obtained 30 transgenic plants per 50 mg of settled cell volume of embryogenic suspension cells of Cavendish cultivar.

Several authors have demonstrated the great potential in Agrobacterium mediated transformation studies based on the use of meristems as the explant source: Zhang
Transformation through the MBC system often results in the formation of chimeric plants because multiple cells are involved in shoot development, and only a proportion of them may be transformed (Yip et al., 2011). However, there are feasible techniques of avoiding this phenomenon. Zhong et al. (1996) multiplied plants derived from single transformed shoot tip and regenerated the shoots after selection to obtain uniform transformation.

In the present study, high gfp expression was observed especially in the vascular bundles. The high level of gfp expression would be explained by gene regulation by CaMV 35S promoter in cells of the vascular bundle (Benfey and Chua, 1990).

Chimerism can be diluted by multi-step selection and regeneration on selective medium. Uniform gfp expression in the tissues sampled for microscopy after the fourth to fifth selection step was reported for the present study. Tripathi et al. (2005), reported uniform GUS expression in apical shoot tips of plantain cultivar Agbagba (AAB) after two steps of selection and regeneration. However, not all the meristems isolated from putative transformants regenerated on selection medium. Similary in the present study, not all plants subjected to the selection pressure regenerated. Uniform GUS expression in two EAHBs transformed from intercalary meristems has also been reported after second step selection (Tripathi...
et al., 2008). Shoot tip culture has been used to regenerate non-chimeric plants in *Musa* though at a lower efficiency (Van Duren et al., 1996). Skarjinskaia et al. (2003) and Wang et al. (2008), reported a procedure of eliminating chimeras or producing isogenic transformants through multiple rounds of shoot regenerations in *Lesquerella fendleri* transformation. Two to three rounds of regenerations were recommended for chimera reduction in tobacco (Maliga and Nixon, 1998) and in strawberry (Mathews et al., 1995) whereas four rounds of regenerations were performed in *Lesquerella fendleri*. Yao et al. (1995) demonstrated that a two-step selection protocol increases the transformation efficiency and minimizes the incidence of escapes in addition to the use of a higher concentration of selective agent. Successive subculture and multiplication of the chimeric bananas resulted in the generation of newly formed progeny suckers expressing the transgene (Yip et al., 2011). Continual selection has been applied to eliminate chimeric clones (Finer and McMullen 1991).

### 3.9 Conclusion

Establishment of an easy, rapid and reproducible transformation system for banana and plantain is very important for crop improvement. Generating cell suspension in bananas is highly cultivar dependent thereby limiting the improvement of commercially important banana cultivars. However, the limitation can be addressed by seeking alternative explants for regeneration and transformation studies. The use of MBCs for non-responsive cultivars to ECS production can be
explored. MBCs have a high proliferating capacity and can thus be used for mass clonal propagation as well as target starting materials for genetic engineering. This study explored different parameters that affect transformation efficiency. These experiments optimized the effect of different wounding treatments, acetosyringone concentration and infection time for transient gene expression in bananas and plantains.

The optimized parameters resulted to higher transformation efficiencies compared to previous studies. The optimized protocol is cultivar independent, rapid and applicable to a broad range of bananas and plantains cultivars. Chimeras often give rise to non-transgenic gametes and progenies, thus generating a stable transformants remain a challenge. In this study, *gfp* was used for visual screening and documenting of chimeras. An efficient protocol was developed for purifying isogenic transformants through multiple rounds of shoot regeneration processes in order to eliminate the chimeras. The successive shoot regeneration process under selection as demonstrated in this study is a feasible strategy of diluting chimera. In conclusion the transformation system developed in this study provides an alternative method for banana improvement through introduction of traits of agronomic importance for cultivars recalcitrant to establishment of ECS.
CHAPTER FOUR

GENERATION OF TRANSGENIC PLANTS EXPRESSING SWEET PEPPER HYPERSENSITIVITY RESPONSE – ASSISTING PROTEIN (Hrap gene) FOR RESISTANCE TO BANANA Xanthomonas WILT

4.1 Introduction

The greatest threats to banana production in East Africa include, low soil fertility and banana Xanthomonas wilt (BXW) (Kalyebara et al., 2007). Banana Xanthomonas wilt (BXW) in banana growing regions has impacted negatively on both national food security and income generation (Tushemereirwe et al., 2003; 2004). The effects of BXW are both extreme and rapid as compared to other diseases which have caused gradual losses over years. BXW infects all Musa cultivars and can lead to 100% yield losses. This severely compromises livelihoods and food security to the banana farmers (Ssekiwoko et al., 2006a; b; Kagezi et al., 2006; Tushmereirwe et al., 2006). The economic impact of BXW is as a result of the death of the mother plant that would otherwise contribute to the ratoon plant production cycles (Tripathi et al., 2007). In addition, the farms infected with Xanthomonas campestris pv. Musacearum (Xcm) cannot be replanted with bananas for at least 6 months due to the carryover of soil borne inoculum (Turyagyenda et al., 2007).

The pathogen enters the banana plants through wounds on roots, pseudostems, leaves (Yirgou and Bradbury, 1974; Korobko et al., 1987), male buds as reported for the Moko disease (Korobko et al., 1987) and contaminated farming tools
(Yigou and Bradbury (1974). BXW is mainly transmitted by insects as they move from one plant to another looking for nectar in flowers (Sekiwoko et al., 2006). *Xanthomonas campestris* pv. *Musacearum* (*Xcm*) attacks the vascular system of both banana and enset (*Ensete ventricosum*) causing wilting and death of the plants. The management of BXW is a challenge due to continuous association of host and inoculum over a long period of time (Ploetz et al., 2007). Pathogen and pest dissemination can for instance be reduced by using clean tissue culture banana planting materials that have been properly indexed. Improved cultural practices including the use of clean planting materials, clean tools, de-budding, cutting and burying of the diseased plants, and crop rotation have been reported to reduce BXW incidences (Tushemereirwe et al., 2004; Rugalema and Bijukya, 2009).

Conventional breeding methods can be employed in the development of disease resistant cultivars subject to availability of resistant cultivars in bananas. Prospects of developing cultivars with resistance to bacterial wilt through conventional breeding are limited as no source of germplasm exhibiting resistance against *Xcm* has been identified in banana (Tripathi et al., 2009). Even with the availability of resistant germplasm, conventional breeding of banana is a difficult and lengthy process due to sterility of most cultivars coupled with long generation times. To overcome these difficulties, transgenic technologies for banana may provide a timely and cost effective alternative solution to the BXW pandemic. Biotechnology studies have unraveled several new options to manage bacterial
diseases in plants through manipulation of the host’s regulatory mechanisms or defense mechanism or inserting antimicrobial proteins (Tripathi et al., 2010).

One of the strategies that can be used to control BXW is the over expression of sweet pepper $Hrap$ gene in transgenic banana. HRAP, a novel plant protein isolated from sweet pepper ($Capsicum annum$) can intensify the harpinPss-mediated hypersensitive response in plants (Chen et al., 2000; Ger et al., 2002; You et al., 2003). The transgene has been shown to delay the hypersensitive response induced by various pathogens like $Erwinia$, $Pseudomonas$, $Ralstonia$ and $Xanthomonas$ sp. in non-host plants through the release of the proteinaceous elicitor, harpinPss in various crops including dicotyledons such as tobacco, potato, tomato, broccoli, orchids and monocotyledons like rice and banana (Huang et al., 2004).

The aim of this study was to test the optimized protocol described in Chapter three on a Kenyan banana cultivar, Mpologoma and generate transgenic lines expressing $Hrap$ gene previously used in other banana cultivars.

4.2 Materials and methods

4.2.1 Plant material

Banana cultivar Mpologoma obtained from KARLO (Kisii) was used in the generation of $Hrap$-transgenic plants.
4.2.2 *Agrobacterium* and plasmid

*Agrobacterium* strain EHA105 harboring the binary vectors pBI121-HRAP (Figure 4.1) was used in this study. The pBI-HRAP contains sweet pepper \( Hrap \) gene (obtained from Academia Sinica in Taiwan) regulated by CaMV35S promoter and contains \( nptII \) as a selectable marker. The vector was introduced into *Agrobacterium* strain EHA 105 (Hood *et al.*, 1993) through electroporation and the *Agrobacterium* strain harbouring the vector maintained on LB medium supplemented with kanamycin 50 mg/L and rifampicin 50 mg/L. The bacterial culture was grown, harvested, and used for the subsequent transformation experiments as previously described by Tripathi *et al.* (2012).

**Figure 4.1:** Schematic representation of the T-DNA region of the pBI121-HRAP construct used for plant transformation. RB, right border, PNos, nopaline synthase promoter; \( nptII \), neomycin phosphotransferase II gene; NosT, nopaline synthase terminator; PCaMV35S, CaMV35S promoter; \( Hrap \) (hypersensitive response-assisting protein isolated from sweet pepper); LB, left border

Bacterial cultures were grown in liquid LB medium supplemented with kanamycin 50 mg/L and rifampicin 50 mg/L with shaking at 150 rpm and 28°C until the \( O.D_{600} \) reached 0.8. The bacterial cells were harvested by centrifugation at 5,000 ×
g for 10 minutes at 4°C and re-suspended in 20 ml of liquid proliferation medium supplemented with 200 μM acetosyringone. The bacterial suspension was incubated at 28°C for 3 hours with shaking at 150 rpm. The optical density (O.D$_{600}$) of the bacterial culture was adjusted to 0.8 with liquid proliferation medium supplemented with 200 μM acetosyringone.

4.2.3 Transformation, selection and regeneration of transgenic plants

The optimized transformation protocol as described in chapter 3 was used to generate banana transgenic plants expressing Hrap gene using MBCs of cultivar Mpologoma.

4.2.4 Molecular characterization of putative transgenic plants

4.2.4.1 DNA isolation and PCR analysis

Genomic DNA isolation and quantification was done as described in section 3.2.7.1. Presence of Hrap gene in the plant genome was confirmed by PCR amplification using the Gene Amp PCR systems machine (Applied bio systems). PCR amplifications were carried out in 25 μl reaction volumes as described in chapter 3. The HRAP primer sequences were; forward: 5’ CAAGGTTGCCAAGATACAGG 3’, reverse: 5’ GGAGGACGAGGAACAATAATG 3’. The reaction conditions were; an initial denaturation of DNA at 94°C for 15 minutes, followed by 35 cycles of 95°C for
1.30 minutes 62°C for 1.30 minutes and 68°C for 2 minutes, followed by a final extension at 72°C for 10 minutes.

4.2.4.2 Total RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

RNA isolation was carried out as described in section 3.2.7.2. For RT-PCR analysis, 2 μl of the synthesized cDNA was used as template in a 25 μl reaction. The first strand (cDNA) was used as a template for the amplification of Hrap and 25 S (used as internal control) genes. The sequences of the HRAP primers are described in section 4.2.4.1 and sequences for 25 S are given in chapter 3 section 3.2.7.2. The temperature profile used for the PCR amplification was as indicated in section 4.2.4.1. The expected 500 bp amplicons was checked by loading ten microlitre of the PCR reaction onto 1% agarose gel stained with Gel red.

4.2.5 Evaluation of transgenic lines for resistance to BXW through In vitro screening

Twenty different transgenic lines containing Hrap gene were randomly selected and artificially inoculated with Xanthomonas campestris pv. Musacearum (Xcm) as previously described (Tripathi et al., 2008).

4.2.5.1 Preparation of bacterial suspensions for artificial inoculation

Xanthomonas campestris pv. Musacearum (Xcm) bacterial isolate was inoculated into 25 ml of YTS medium (Appendix 1B) using a sterile loop and cultured at
28°C with shaking at 150 rpm for 48 hours. The bacterial culture was centrifuged at 5000 rpm for 5 minutes and the pellet was re-suspended in sterile double distilled water. The optical density (O.D_{600}) of the bacterial suspension was adjusted to 1.0 with sterile water. Fresh inoculum was used for all the inoculation experiments.

**4.2.5.2 Inoculation of *in vitro* plantlets**

The bacterial inoculum was injected into the mid rib of the lower surface of single leaf *in vitro* plantlets as described by Tripathi *et al.* (2008). Three shoots per line were inoculated with the fresh culture of *Xcm*. Two non-transformed shoots were inoculated with water and another two with the *Xcm* to serve as controls. The plants were inoculated with 50 μl of bacterial suspension using a micro syringe fitted with a hypodermic needle. The inoculated plantlets were put into the baby jars fitted with lid and incubated in the growth room at 28°C for 60 days.

Inoculated plants were monitored and assessed for disease symptoms every day for 60 days. The occurrence and severity of infection was scored as previously described (Tripathi *et al.*, 2008; 2009). The disease symptoms included chlorosis, necrosis and finally complete wilting and death of plants. Observations were made regularly and the data was recorded on each plantlet. The relative resistance of transgenic lines to BXW was evaluated 60 days after inoculation. Plantlets were considered as resistant if a plant did not show any disease symptoms, partial resistant if a plant showed symptoms but the symptoms did not spread to another
part of the plant and was not completely wilted, and susceptible if a plant
developed symptoms and completely wilted after inoculation similar to control
non transgenic plants.

4.2.5.3 Data analysis

Data on disease incubation and complete wilting was collected based on chlorosis
and necrosis of leaves and complete wilting of plants, and entered into a table.
Average incubation and average wilting was computed based on number of days
for appearing of the symptoms and death of the plant. The means presented were
for three replicates. The resistance incidence was calculated as follows:

\[
\text{Resistance (\%)} = \left( \frac{\text{Reduction in wilting in comparison to control plants}}{\text{Total number of leaves wilted in control plant}} \right) \times 100.
\]

4.3 Results

4.3.1 Transformation, selection and regeneration of transgenic lines

The optimized protocol described in Chapter 3 was used to generate transgenic
plants of the banana cultivar Mpologoma expressing Hrap gene using MBCs
(Figure 4.2 A). The transformed explants started producing green shoot whereas
non-transformed MBCs did not showed any regeneration (Figure 4.2 B).
Putatively transgenic events were selected four times on medium supplemented
with kanamycin (Figure 4.2 C - D) to generate kanamycin-resistant banana plants
(Figure 4.2 E). The kanamycin-resistant events were analysed for the presence of
the transgene using PCR analysis. Eight transgenic lines per one hundred explants were obtained for the banana cultivar Mpologoma.

Figure 4.2: *Agrobacterium*-mediated transformation steps of banana cultivar Mpologoma using MBCs and *Hrap* gene; (A) *Agrobacterium* infected explants, (B) transformed explants regenerating on 1\textsuperscript{st} selection (C) transformed shoot sub-cultured on 2\textsuperscript{nd} selection (D) transformed shoot sub-cultured on 4\textsuperscript{th} selection step (E) regenerated uniformly transformed shoot. Scale bar=2 cm
4.3.2 Molecular analysis of transgenic lines

PCR analysis with *Hrap* gene amplified the expected 500 bp product size for all the samples tested. No amplification was observed in non-transgenic plant used as a control (Figure 4.3 A). RT-PCR using gene specific and housekeeping gene primers amplified the expected 500 bp and 100 bp product size, respectively (Figure 4.3 B and C).

![PCR and RT-PCR images](image)

**Figure 4.3:** (A) PCR analysis of transgenic plants using *Hrap* gene-specific primers (B) RT-PCR using *Hrap* gene-specific primers and 25 S gene-specific primers

4.3.3 *In vitro* screening of transgenic lines for BXW resistance

Twenty transgenic banana *in vitro* plantlets were inoculated with *Xcm* using the rapid bioassay, and the resistance responses were grouped into three categories: resistant, partially resistant and susceptible, respectively (Figure 4.4; Table 4.1, Figure 4.5). The non-transgenic control plantlets started showing symptoms
characterized by chlorosis and necrosis in about 15 days post-inoculation and completely wilting after 38 days. Out of the 20 transgenic lines evaluated, four lines did not show any symptoms up to 60 days post-inoculation and were classified as resistant lines (Figure 4.4 A; Table 4.1). Five transgenic lines showed symptoms in the inoculated leaf, but these did not spread to other leaves, and plants were never completely wilted, and were classified as partially resistant (Figure 4.4 B). The remaining eleven lines showed symptoms and completely wilted within 30 days post inoculation, similar to the control non-transgenic plants. These lines were classified as susceptible transgenic lines (Figure 4.4 D and F, respectively). The *in vitro* screening is very efficient especially when screening a large number of transgenic lines because highly susceptible lines can be identified and eliminated before any further glasshouse or molecular studies (Tripathi *et al.*, 2008).
Figure 4.4: *In vitro* screening of transgenic lines for resistance to bacterial *Xanthomonas* wilt. (A) inoculated transgenic plantlets showing no symptoms (B - C) inoculated transgenic plantlet showing partial resistance, (D) susceptible plantlet (E) non-transformed plantlet inoculated with water (F) non-transformed plantlet inoculated with *Xcm*. All pictures were taken at 8 weeks after artificial inoculation with *Xcm*. Scale bars=3cm
Table 4.1: Evaluation of transgenic lines for enhanced resistance to *Xcm* using *in vitro* plants

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Mean no. of days for appearance of disease symptoms</th>
<th>Mean no. of days for complete wilting</th>
<th>Resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control non-transgenic plant</td>
<td>13.3±0.9</td>
<td>38±0.6</td>
<td>0</td>
</tr>
<tr>
<td>Hrap 1</td>
<td>29±0.6</td>
<td>0</td>
<td>36.1</td>
</tr>
<tr>
<td>Hrap 2</td>
<td>28.3±0.3</td>
<td>0</td>
<td>76.7</td>
</tr>
<tr>
<td>Hrap 3</td>
<td>15±0.6</td>
<td>40±0.6</td>
<td>0</td>
</tr>
<tr>
<td>Hrap 4</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Hrap 5</td>
<td>21±1.5</td>
<td>39.7±0.3</td>
<td>0</td>
</tr>
<tr>
<td>Hrap 6</td>
<td>23±2.1</td>
<td>0</td>
<td>11.1</td>
</tr>
<tr>
<td>Hrap 7</td>
<td>22.7±2.1</td>
<td>13.3±13.3</td>
<td>9.5</td>
</tr>
<tr>
<td>Hrap 8</td>
<td>29.6±0.9</td>
<td>0</td>
<td>72.2</td>
</tr>
<tr>
<td>Hrap 9</td>
<td>14.7±0.7</td>
<td>39.3±0.3</td>
<td>0</td>
</tr>
<tr>
<td>Hrap 10</td>
<td>24±1.2</td>
<td>0</td>
<td>60.7</td>
</tr>
<tr>
<td>Hrap 11</td>
<td>23±0.9</td>
<td>40.3±0.9</td>
<td>0</td>
</tr>
<tr>
<td>Hrap 12</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Hrap 13</td>
<td>19±0.6</td>
<td>39.7±0.9</td>
<td>15</td>
</tr>
<tr>
<td>Hrap 14</td>
<td>16.7±1.8</td>
<td>39.3±0.7</td>
<td>0</td>
</tr>
<tr>
<td>Hrap 15</td>
<td>24±1.5</td>
<td>39.3±0.3</td>
<td>0</td>
</tr>
<tr>
<td>Hrap 16</td>
<td>22.6±2.3</td>
<td>13.3±13.3</td>
<td>28.6</td>
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<tr>
<td>Hrap 17</td>
<td>0</td>
<td>0</td>
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<td>24.3±1.2</td>
<td>35.3±1.2</td>
<td>6.7</td>
</tr>
<tr>
<td>Hrap 19</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Hrap 20</td>
<td>21.7±1.2</td>
<td>11±1.1</td>
<td>16.7</td>
</tr>
</tbody>
</table>
Control of bacterial diseases through genetic transformation has been reported in many crops (Huang et al., 2004). Tripathi et al. (2010b) advocated genetic engineering methods for Musa improvement. Several plant resistance (R) genes that mediate resistance to bacterial, fungal, viral and nematode pathogens have been cloned from several plant species (Bent, 1996). Such genes include, the Bs2 resistance gene of pepper specifically recognizes and confers resistance to strains
of X. campestris pv. vesicatoria (Xcv) (Wang et al., 1996) that contain the corresponding bacterial a virulence gene, avr Bs2 (Tai et al., 1999). Transgenic tomato plants expressing the pepper Bs2 gene have been shown to suppress the growth of Xcv. The Xa1 gene in rice confers resistance to Japanese race 1 of X. oryzae pv. oryzae, the pathogen associated with bacterial blight (Yoshimura et al., 1998). Sap1, an amphipathic protein isolated from the sweet pepper, Capsicum annum (Lin et al., 1997) has also been shown to delay the hypersensitive response induced by Pseudomonas syringae pv. syringae in non-host plants through the release of the proteineous elicitor, harpins (Lin et al., 1997). Sap 1 has shown enhanced hypersensitive response against various pathogens in many dicot and monocot crops (Tripathi et al., 2009; 2012).

Enhanced disease resistance towards soft rotting bacteria pathogen, E. carotovora subsp. Carotovora in Arabidopsis transformed with Hrap gene has been demonstrated (Pandey et al., 2005). Transgenic bananas expressing Hrap gene conferred resistance to BXW (Tripathi et al., 2010; 2014). These reports indicate that Hrap is one of the important hypersensitive cell death (HCD) associated genes that could be utilized to protect plants from bacterial pathogen attack.

In this study, Hrap gene conferred resistance to BXW in the banana cultivar Mpologoma. Similar reports were reported for transgenic plants of the dessert banana ‘Sukali Ndziizi’ (AAB genome) expressing the Hrap gene from sweet
pepper (*Capsicum annuum*), (Tripathi *et al.*, 2010a; 2014b). Transgenic bananas expressing *Xa21* gene were reported to confer resistance to BXW (Tripathi *et al.*, 2014a).

Rapid hypersensitive cell death in the leaves of tobacco resistant to bean pathogen *Pseudomonas syringae* at the site of infection, which prevents spread of the pathogen has been reported (Glazener *et al.*, 1991; Baker *et al.*, 1997; 1991; 1993). The *Hrap* gene was introduced into tobacco and the resulting transgenic plants acquired resistance to tobacco wildfire and soft - rotting bacteria (Ger *et al.*, 2002). Li and Fan (1999b) reported two highly resistant potatoes against *Phytophthora infestans* resistance out of 55 transformed plants with Harpin protein. Delayed occurrence of late blight symptoms in plants transformed with HR go gene has been reported (Zhang *et al.*, 2001). Li *et al.* (2002) demonstrated a significant difference in disease resistance compared to the control upon screening seven lines out of 24 positive PCR lines. Wang *et al.* (2007) reported that *Hrap* gene, integrated into a tomato plant, improved the plant’s resistance to *R. solacearum*. Enhanced resistance to *Phytophthora infestans* has also been reported in transgenic potatoes expressing *Hrap* gene (Li *et al.*, 2011). In this study, the hypersensitive response promotion protein gene *Hrap* was introduced into banana, resulting in transgenic banana plants that showed resistance to BXW similar to previous report of Tripathi *et al.* (2010). The results of this study indicate that transformation of *Hrap* gene into banana improved the ability of disease resistance.
4.5 Conclusion

Though banana is an important staple food, the high sterility of most commercial cultivars hinders breeding programmes. Conventional breeding programmes geared towards addressing some of the biotic stresses such as the challenge of BXW facing this crop have not been successful and hence the need to seek alternative approaches. Transgenic bananas expressing *Hrap* gene were generated and were shown to confer elevated resistance against BXW. In this study, MBCs of the banana cultivar Mpologoma were successfully transformed with the *Hrap* gene using the *Agrobacterium* mediated transformation method. PCR analysis of the selected transgenic lines of Mpologoma showed presence of the *Hrap* gene. The use of MBCs in this study offers an alternative and rapid method of generating transgenic plants and can thus be explored for the genetic improvement of *Musa* especially for cultivars recalcitrant to generation of ECS.
CHAPTER FIVE
GENERAL DISCUSSION

5.1 General discussion

In Africa, banana and plantain provide more than 25% of food energy requirement (Robinson, 1996) for more than 100 million people. Twenty million out of the one hundred million people, are from East Africa alone. Banana is one of the most important cash crops contributing up to 22% of national agricultural revenue (Kalyebala et al., 2007). However, banana production is affected by diseases of fungal, bacterial and viral origins. *Xanthomonas* wilt poses one of the greatest threats to banana production in Eastern Africa where banana is a staple food and it has the potential of destabilizing food security in the region. All banana cultivars and genome groups are susceptible to BXW disease though the prevalence rate varies depending on the cultivars. Control of bacterial diseases of plants once established is difficult due to lack of an effective chemical or other curative treatment. Early detection and destruction of the diseased plants is paramount in preventing disease spread (Karamura et al., 2005). Farmers are reluctant to employ labor-intensive disease control measures thus calling for the development of disease resistant banana cultivars.

Genetic engineering has become an important tool for crop improvement. Different methods of transformation including electroporation (Sagi et al., 1995) and biolistics have been used for banana transformation (Becker et al., 2000).
However, *Agrobacterium* mediated transformation is the method of choice due to the high transformation frequencies (Khanna *et al.*, 2004) with fewer transgene copy numbers and significantly higher transient and stable gene expression compared to biolistics (Arinaitwe, 2008). The state of genetic engineering of banana for disease resistance and future possibilities have been extensively reviewed (Sagi *et al.*, 1998; Tripathi, 2003; Tripathi *et al.*, 2004; Tripathi, 2005). Tripathi *et al.* (2004), has reviewed a range of potential strategies for genetic engineering against banana bacterial wilt in bananas. The genetic transformation system developed in this study can be used for the production of transgenic bananas and has further been used for the production of bacterial wilt resistant varieties of banana.

Various parameters have been explored in order to increase the transformation efficiency and overcome problems associated with host / tissue specificity of *Agrobacterium* transformation including wounding treatments and use of compounds additives. The application of thiol compounds (Olhoft *et al.*, 2001; Olhoft and Somers, 2001), sonication-assisted *Agrobacterium* transformation (SAAT) (Trick and Finer, 1998), a combination of SAAT and vacuum infiltration (de Oliveira *et al.*, 2009), heat and separation by centrifugation (Hiei *et al.*, 2006), surfactants (Cheng *et al.*, 1997), use of super virulent *Agrobacterium* strains (Hood *et al.*, 1993; Torisky *et al.*, 1997) and mixing by vortex with carborundum (Somleva *et al.*, 2002) have been reported in a variety of explants of different
species in an effort to increase transient gene expression and hence stable transformation of plants. Pre-culturing explants before Agrobacterium inoculation has been reported to improve transformation efficiency (McHughen et al., 1989; Sangwan et al., 1991; Curtis et al., 1999).

This study described a method for routine Agrobacterium-mediated transformation of a broad range of bananas and plantains using npt II as the selectable marker. There are several reports on transgenic banana and plantain available based on the use of ECS as the explant source. The development of cell suspension and plant regeneration is time consuming and highly cultivar dependent, taking 14-42 months for bananas and 18-27 months for banana and plantains to produce cell suspension from callus induction to rooted plant regeneration (Strosse et al., 2006; Tripathi et al., 2012). This study therefore sought to explore alternative explant source that could be reproducible and applicable to a wide range of cultivars. The study tested the effect of two explants; (IM and MBC) and a range of parameters to develop a reproducible protocol. Transformation by use of MBC was more effective in terms of availability and the ease of explant preparation. MBC was thus used to produce independent transgenic plants in the banana cultivars Mpologoma, Kayinja, Ngombe Nusu Ngombe, Gross Michel, Grande Naine, Zebrina, Calcutta 4 and Uganda green. The use of MBC as opposed to the generation of ECS is rapid, cultivar independent and results in uniformly transformed plants upon optimization of the selection steps.
Different wounding methods were also tested and combined vacuum infiltration and sonication was reported as the most effective, producing much higher GUS expression than vacuum infiltration, sonication and no wounding.

Inclusion of acetosyringone in the infection and co cultivation medium for MBC transformation was also a prequisite for successful transient *gus* expression. Wounding helps in the secretion of some phenolic compounds such as acetosyringone and α-hydroxy acetosyringone from dicotyledonous plant explants, which subsequently enhances the attachment of *Agrobacterium* to the wound sites (Potrykus, 1990; Zambryski, 1992). These phenolic compounds in turn activates the *vir* genes present on *Ti* plasmid of *Agrobacterium tumefaciens* (Satchel *et al.*, 1985). Though dicotyledonous plants are known to secrete phenolic compounds naturally from wound sites, addition of exogenous acetosyringone (100 μM) was reported to enhance the transformation efficiency in *Datura sissoo*. Similar observations were reported in banana (Subramanyam *et al.*, 2011), *Malus* (Xu *et al.*, 2009) and vanilla (Retheesh and Bhat, 2011). This is in agreement with the findings of this study where inclusion of acetosyringone (200 μM) significantly increased the transformation efficiency. However, McGranahan *et al.* (1998) and Ducrocq *et al.* (1994) reported transformation of walnut and *Datura* without wounding. Mondal *et al.* (2001) reported tea transformation without wounding somatic embryos.
Stable GUS expression was evident in putative transgenic banana sections in all the cultivars assayed in this study. Alpeter et al. (1996) reported that transient gene expression was not correlated with stable transformation in wheat. However, in other studies, increased numbers of transgenic wheat and corn were regenerated from dissected explants after optimization of transient expression using reporter genes (Cheng et al., 1997; Frame et al., 2002). Trick and Finer (1998) reported increased transformation efficiencies of soybean and Ohio buckeye after optimization of transient expression.

Transgenic plants arising from multicellular origin often give rise to chimeras. This was evident in this study and further optimization of the protocol to dilute chimera was tested using banana cultivar Nusu Ngombe. Further selection in kanamycin containing medium was employed and gfp expression observed under a microscope at each sub culturing stage. Four or more round of further selection was found to be effective in chimera dilution. Mathews et al. (1998) and Husaini et al. (2010) reported that increasing antibiotic concentration diluted chimerism in strawberry.

An efficient Agrobacterium mediated transformation protocol using MBC as the source of explant such as that proposed in this study can facilitate further genetic improvement of this important banana cultivar. In addition, the optimized parameters were used to generate transgenic plants for a broad range of banana
and plantain cultivars. The optimized protocol is simple, reproducible and rapid. The optimized parameters were used to generate BXW resistant banana lines of banana cultivar Mpologoma expressing *Hrap* gene.
CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary of findings

The present study has effectively accomplished multiple shoot induction and regeneration from MBC as explants in bananas and plantains, which served as an explant source for Agrobacterium - mediated transformation. Establishment of an easy, rapid, and widely applicable transformation system for banana and plantains is very important for crop improvement and for study of gene function. Results of this study showed a successful transformation of a broad range of banana and plantain cultivars using MBCs upon optimizing parameters; acetylsyringone concentration, length of infection time, explant type and wounding effect via sonication and vacuum infiltration assisted inoculation and a combination of sonication and vacuum infiltration. In addition, the optimized protocol improved the probability of T - DNA delivery based on transient expression. The protocol was further optimized using banana cultivar Nusu Ngombe to dilute chimerism. Partial gfp expression was evident during regeneration stages and uniform expression was achieved after fourth and fifth sub culture under selection pressure. The protocol was used to regenerate kanamycin resistant transgenic plants. PCR and Southern blot hybridization analysis confirmed the presence and integration of gusA and gfp gene into the genome of the T0 generation transgenic plants. This study also confirmed that the optimized protocol can be used to generate transgenic with agronomic important traits like BXW resistant. In vitro evaluation
of randomly selected transgenic banana lines, containing \textit{hrap} gene from sweet pepper showed enhanced resistance against BXW confirming uniform transformation.

\textbf{6.2 Conclusions}

i. Multiple bud clumps can be used as an alternative starting explant for regeneration and transformation studies in banana cultivars recalcitrant to establishment of ECS.

ii. Successive shoot regeneration under selection is a feasible strategy of diluting chimera.

iii. Transgenic banana expressing \textit{Hrap} gene conferred resistance against BXW.

\textbf{6.3 Recommendations}

The optimized protocol described in this study can be used to introduce genes of agronomic importance for banana cultivars recalcitrant to generation of ECS for crop improvement to ensure food security.

\textbf{6.4 Suggestions for future study}

i. Use of optimized selection steps described in chapter three in other cultivars to rule out the effect \textit{gfp} expression on cultivar dependence.

ii. Screening of the transgenic events in the screen house and compare the resistance against BXW with the \textit{in vitro} screening results.
iii. Explore the effect of the different concentrations of cytokinins and TDZ suggested in this study on different cultivars in the development of MBC after induction.

iv. Further molecular characterization of the transgenic lines generated from MBCs to ascertain if indeed they were independent events based on the inserted copy numbers.

v. Employ a similar approach of chimera dilution as reported in this study in other transgenic crops generated from meristems.
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## APPENDICES

### Appendix I

Composition of various medium used for regeneration and transformation of MBCs and IM experiments

<table>
<thead>
<tr>
<th>Medium</th>
<th>Components</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation Medium (PM)</td>
<td>MS salts + MS vitamins pre mix</td>
<td>4.4 g/L</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>30 g/L</td>
</tr>
<tr>
<td></td>
<td>Ascorbic acid (10 mg/ml)</td>
<td>10 mg/L</td>
</tr>
<tr>
<td></td>
<td>BAP (1 mg/ml)</td>
<td>5 mg/L</td>
</tr>
<tr>
<td></td>
<td>Gelrite</td>
<td>2.4 g/L (pH 5.8)</td>
</tr>
<tr>
<td>Proliferation Medium (1/2 BAP)</td>
<td>As above except BAP</td>
<td>2.5 mg/L</td>
</tr>
<tr>
<td>P5 medium (P5)</td>
<td>As PM except BAP</td>
<td>2.273 mg/L</td>
</tr>
<tr>
<td></td>
<td>IAA(1mg/ml)</td>
<td>0.175 mg/L</td>
</tr>
<tr>
<td></td>
<td>Gelrite</td>
<td>3 g/L</td>
</tr>
<tr>
<td>P4 medium (P4)</td>
<td>As P5 except BAP</td>
<td>22.73 mg/L</td>
</tr>
<tr>
<td>Shoot induction medium (SIM)</td>
<td>As PM except BAP</td>
<td>2.5 mg/L</td>
</tr>
<tr>
<td>Pre conditioning medium (PC)</td>
<td>As PM except myo inositol</td>
<td>100 mg/L</td>
</tr>
<tr>
<td></td>
<td>Gelrite</td>
<td>3 g/L</td>
</tr>
<tr>
<td>Co cultivation medium (CC)</td>
<td>As PM. Supplement with acetosyringone</td>
<td>200 mM</td>
</tr>
<tr>
<td>Resting medium (RM)</td>
<td>As PM. Supplement with cefotaxime</td>
<td>300 mg/L</td>
</tr>
<tr>
<td>Selection medium (S1)</td>
<td>As PC. Supplement with Kanamycin and Cefotaxime</td>
<td>100 mg/L 300 mg/L</td>
</tr>
<tr>
<td>Selection medium (S2)</td>
<td>As S1 exclude cefotaxime</td>
<td></td>
</tr>
<tr>
<td>Rooting Medium (RM)</td>
<td>As PM without BAP.</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>--------------------</td>
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</tr>
<tr>
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<tr>
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<td>Gelrite</td>
<td></td>
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<td>Macro nutrients (10X)</td>
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</tr>
<tr>
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<td>Micro nutrients (100X)</td>
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<td>Fe 3+ (100X)</td>
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<tr>
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<td>Vitamins + myo-inositol (200X)</td>
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<td>ascorbic acid (10 mg/ml)</td>
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<td>(pH 5.8)</td>
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<td>Selective embryo formation media (MA3)</td>
<td>SH salts premix</td>
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<td>MS vitamins (200X)</td>
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<td>Biotin (1 mg/ml)</td>
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<td>Kinetin (1 mg/ml)</td>
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<td>2 – ip (1 mg/ml)</td>
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<td></td>
<td>Sucrose</td>
<td>45 g/L</td>
</tr>
<tr>
<td></td>
<td>Lactose</td>
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</tr>
<tr>
<td></td>
<td>Gelrite</td>
<td>3 g/L</td>
</tr>
<tr>
<td></td>
<td>Zeatin (1mg/ml) post autoclaving</td>
<td>0.1 mg/L</td>
</tr>
<tr>
<td></td>
<td>pH 5.8</td>
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</tr>
<tr>
<td>Germination medium (Ma 4 medium)</td>
<td>Macro nutrients (10x)</td>
<td>1X</td>
</tr>
<tr>
<td></td>
<td>Micro nutrients (100x)</td>
<td>1X</td>
</tr>
<tr>
<td></td>
<td>Fe 3+ (100x)</td>
<td>1X</td>
</tr>
<tr>
<td></td>
<td>Morel Vitamins (200x)</td>
<td>1X</td>
</tr>
<tr>
<td></td>
<td>IAA (1 mg/ml)</td>
<td>2 mg/L</td>
</tr>
<tr>
<td></td>
<td>BAP (1 mg/ml)</td>
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</tr>
<tr>
<td></td>
<td>Biotin</td>
<td>0.01 mg/L</td>
</tr>
<tr>
<td>Embryo maturation medium (RD1)</td>
<td>Sucrose gelrite pH 5.8</td>
<td>0.5X 1X 1X 1X 10 mg/L 30 mg/L 3 g/L</td>
</tr>
<tr>
<td>--------------------------------</td>
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<tr>
<td></td>
<td>Macro nutrients (10x)</td>
<td>1X 1X 1X 10 mg/L 30 mg/L 3 g/L</td>
</tr>
<tr>
<td></td>
<td>Micro nutrients (100x)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fe 3+ (100x)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ms Vitamins (200x)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ascorbic acid</td>
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</tr>
<tr>
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<td>sucrose gelrite</td>
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Appendix II
Bacterial culture media, extraction and electrophoresis buffers

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<thead>
<tr>
<th>Medium</th>
<th>Components</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Bacterial co-culture medium (BCCM A)</td>
<td>Sucrose</td>
<td>50 g/L</td>
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<tr>
<td></td>
<td>Maltose</td>
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<tr>
<td></td>
<td>Glucose</td>
<td>16.7 g/L</td>
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<tr>
<td></td>
<td>L- glutamine</td>
<td>166.7 mg/L</td>
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<tr>
<td></td>
<td>malt extract</td>
<td>166.7 mg/L</td>
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<tr>
<td></td>
<td>proline</td>
<td>500 mg/L</td>
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<tr>
<td></td>
<td>L– cysteine</td>
<td>666.7 mg/L</td>
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<tr>
<td></td>
<td>MS vitamins + myo inositol (200X)</td>
<td>400 mg/L</td>
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<tr>
<td></td>
<td>ascorbic acid</td>
<td>3.33X</td>
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<tr>
<td></td>
<td>biotin</td>
<td>1.7 mg/L</td>
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<tr>
<td></td>
<td>acetosyringone (400 mM)</td>
<td>1.7 mg/L</td>
</tr>
<tr>
<td></td>
<td>(pH 5.3, filter sterilize and store at –20°C)</td>
<td>400 µM</td>
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<tr>
<td>Bacterial co-culture medium (BCCM B)</td>
<td>Macro nutrients (10X)</td>
<td>0.25X</td>
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<td>Micro nutrients (100X)</td>
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<td></td>
<td>Fe 3+ complex (100X)</td>
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<tr>
<td></td>
<td>Gelrite</td>
<td>5 g/L</td>
</tr>
<tr>
<td></td>
<td>(pH 5.5, autoclave)</td>
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<tr>
<td>Bacterial re-suspension medium (BRM A)</td>
<td>Macro nutrients (10X)</td>
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<td>Micro nutrients (100X)</td>
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<td>Fe3+ (100X)</td>
<td>1.25X</td>
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<tr>
<td></td>
<td>Ms Vitamins (200X)</td>
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<tr>
<td></td>
<td>sucrose</td>
<td>85.5 g/L</td>
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<td>(pH 5.3, autoclave)</td>
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<td>Bacterial re-suspension medium (BRM B)</td>
<td>Thiamine</td>
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<tr>
<td></td>
<td>Cysteine</td>
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<tr>
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<td>Glucose</td>
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<tr>
<td></td>
<td>Acetosyringone (400 mM)</td>
<td>400 µM</td>
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<tr>
<td></td>
<td>(pH 5.2, filter sterilize and store at -20°C)</td>
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<tr>
<td>LB medium</td>
<td>Bacto-tryptone</td>
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<td>Yeast extract</td>
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<tr>
<td></td>
<td>NaCl</td>
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<td>Bacto agar</td>
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<td>YTS medium</td>
<td>Yeast extract</td>
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<td>Tryptone</td>
<td>Sucrose</td>
</tr>
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<td>--------------------------</td>
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<tr>
<td><strong>YTSA medium</strong></td>
<td>Yeast extract</td>
<td>Tryptone</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>micro agar</td>
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<tr>
<td><strong>TAE electrophoresis buffer (50x stock)</strong></td>
<td>Trisma base</td>
<td>Glacial acetic acid</td>
</tr>
<tr>
<td></td>
<td>EDTA (pH 8)</td>
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<td><strong>CTAB Buffer (DNA)</strong></td>
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<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>EDTA (pH 8)</td>
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<tr>
<td></td>
<td>Tris – HCl (pH 8)</td>
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<tr>
<td></td>
<td>Polyvinylpyrrolidone</td>
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<td><strong>Loading dye (6X)</strong></td>
<td>bromophenol blue</td>
<td>TE</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
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### Appendix III
**Southern blot analysis buffers and stocks**

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<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
<th>Amount</th>
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<tbody>
<tr>
<td>20x SSC</td>
<td>300mM Sodium citrate dihyd</td>
<td>88.23 g/L</td>
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<tr>
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<td>3M NaCl</td>
<td>175.32 g/L</td>
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<tr>
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<td>pH 7.6</td>
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<tr>
<td>20 % SDS</td>
<td>Sodium dodecyl sulfate</td>
<td>200 g/L</td>
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<tr>
<td>Depurination solution</td>
<td>250mM HCL</td>
<td>25 g/L 975 g/L</td>
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<tr>
<td></td>
<td>H\textsubscript{2}O</td>
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<tr>
<td>Denaturing solution</td>
<td>500 mM NaOH</td>
<td>20 g/L 87.66 g/L</td>
</tr>
<tr>
<td></td>
<td>1.5 M NaCl</td>
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</tr>
<tr>
<td></td>
<td>Autoclave</td>
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<tr>
<td>Neutralisation solution</td>
<td>500 mM Tris</td>
<td>60.57 g/L 87.66 g/L</td>
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<tr>
<td></td>
<td>1.5 M NaCl</td>
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</tr>
<tr>
<td></td>
<td>1 mM EDTA</td>
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</tr>
<tr>
<td></td>
<td>pH 7.2, autoclave</td>
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<tr>
<td>W1</td>
<td>SSC solution</td>
<td>2x 0.1 %</td>
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<tr>
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<td>SDS</td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>SSC solution</td>
<td>0.2x 0.1 %</td>
</tr>
<tr>
<td></td>
<td>SDS</td>
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</tr>
<tr>
<td>W3</td>
<td>SSC solution</td>
<td>0.1x 0.1 %</td>
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<tr>
<td></td>
<td>SDS</td>
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<td>WB</td>
<td>Tween 20</td>
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<tr>
<td>B1</td>
<td>100 mM Maleic acid</td>
<td>11.6 g/L</td>
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<tr>
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<td>-----------------------------</td>
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<td></td>
<td>150 mM NaCl</td>
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<tr>
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<td>pH 7.5, autoclave</td>
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<tr>
<td>B2</td>
<td>B1 solution</td>
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<tr>
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<td>blocking powder</td>
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<tr>
<td>B3</td>
<td>100 mM Tris- HCL (pH 9.5)</td>
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</tr>
<tr>
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<td>100 mM NaCl</td>
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</tr>
<tr>
<td></td>
<td>50 mM MgCl₂</td>
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