

**THE POTENTIAL OF HORIZONTAL GENE FLOW FROM TRANSFORMED  
EAST AFRICAN HIGHLAND BANANAS (*MUSA spp*) TO RHIZOSPHERE  
AND PHYLLOSPHERE INHABITING MICROORGANISMS**

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## DECLARATION

The work presented here is my own and has not been submitted to any other university for the award of a degree.

Signed.....  
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Date

This thesis is submitted with the approval of my supervisors

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## **DEDICATION**

To the good Lord whom I love

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## SUMMARY

Bananas constitute an important food security and household source of income for many farmers in Uganda, and are a major component of their livelihood strategies. Banana production is constrained by abiotic and biotic constraints that cause yield losses. The threat posed by these constraints is highest in Africa because of the relatively high costs of control that the farmers in most cases can not afford. Accordingly farmers have resorted to cultural control methods which provide a cheaper alternative but usually less effective control. However, other controls in the market include; chemical, biological and conventional breeding which have their shortcomings being inefficient at providing the requisite controls against pests and diseases. It is against this background that biotechnological approaches have been proposed to mitigate these biotic constraints.

Biotechnology in particular has been heralded as a very promising approach to these constraints. However its use has raised several biosafety concerns especially gene flow to microorganisms in the rhizosphere and phyllosphere. Fears have arisen over the potential negative effects on the major biological activities played by these microorganisms in case of gene flow, which could impact their ecological functions such as natural suppressivity roles played by fluorescent pseudomonads in soil. On the other hand, the fitness of the recipient microorganisms could be enhanced. This can result from directional selection that could lead to the emergence of new populations that are more pathogenic to the local landraces and other plants.

In this study, investigations were carried out on the potential horizontal gene flow to microorganisms in the rhizosphere of banana plantlets transformed with a  $\beta$ -

*glucuronidase* gene (*gus*) and a hygromycin (*hpt*) antibiotic resistance marker, driven by a *CaMV* 35S promoter and a nos terminator. The general objectives of this study were to generate information that would contribute to biosafety and handling of genetically modified banana under Ugandan conditions with respect to gene flow. The specific objectives of the study were to investigate the potential horizontal gene flow from transformed East African highland banana plants to soil inhabiting rhizobacteria and endophytes. The study hypothesis was that rhizosphere and phyllosphere inhabiting microorganisms experience horizontal gene flow and may therefore acquire transgenes from genetically modified bananas when exposed to them.

The microorganisms considered in the study were bacteria (*Escherichia coli*, *Agrobacterium tumefaciens* and bacteria naturally occurring in banana rhizosphere) and fungi (*Fusarium oxysporum*). In the first study, microorganisms were inoculated into the soil region surrounding the plants and left to acclimatise and colonise. The bacteria were re-isolated at intervals of time on non-selective and selective Luria Bertani (LB) media. The colonies growing on selective Luria Bertani media were subjected to a second round of selection prior to multiplication of the cells for DNA extraction. In the second study which investigated gene flow to fungi, the study microorganisms were inoculated into the banana plants by dipping the plantlets roots in a *Fusarium oxysporum* spore solution for four hours. The plantlets were grown in the screen house prior to collection of root samples at different time intervals. The study fungus was re-isolated on non-selective and selective potatoe dextrose agar. Colonies growing on selection media were sub-cultured on selective media afresh prior to multiplication and used for DNA extraction. DNA was extracted from the

hygromycin (*hpt*) resistant bacteria and fungi, and amplified using  $\beta$ -glucuronidase gene construct specific primers to confirm putative gene flow.

Results indicated that there was no uptake of the  $\beta$ -glucuronidase gene from the transformed bananas into the bacteria and/or fungi used in the study although the microorganisms demonstrated an ability to grow on hygromycin augmented media. Several factors are accountable for the non occurrence of gene flow including; lack of competence, differences in DNA amongst prokaryotes and eukaryotes in which the latter DNA has introns and are associated with histones interference of DNA uptake and recombination mechanisms of the bacteria. In fungi, though no gene flow was detected from the molecular analyses carried out, the microorganisms were observed to possess the ability to also grow on hygromycin augmented media. This was probably because of endogenous factors that enabled the fungi to degrade the antibiotic and thereby survive. In general, therefore, it can be concluded that the occurrence of gene flow from transgenic bananas to associated rhizosphere and phyllosphere microorganisms is limited with a very low probability of occurrence.

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## CHAPTER ONE

### BACKGROUND

#### 1.0 Bananas diversity and importance in East Africa

Bananas (*Musa* spp) are widely grown in East Africa and especially in Uganda where they are an important source of food security and household incomes for the local farming communities. Bananas are believed to have been domesticated in South East Asia with the East African highlands considered as the secondary center of diversity (Simmonds and Shepherd, 1955). The cultivated banana is both an allo- and autopolyploid, deriving genomes from *Musa balbisiana* (B) genome and *Musa acuminata* (A) genome. East African highland banana types (*Musa* spp. AAA genotype) are the most common cultivar group grown in many parts of Eastern Africa. *Musa* spp. AAA subgroup has several clones that fall in two types, viz cooking types (*matooke*) and the beer bananas (*mbidde*) (Rubaihayo and Gold, 1993). Non endemic bananas grown in Uganda include the desert types which were introduced from South-East Asia and comprise AB, ABB, AA or AAA genomes (INIBAP, 2007). The roasting bananas or plantains have the AAB genome while some newly developed tetraploid hybrids (AAAB, AAAA, AABB) are grown and used for beer brewing and cooking (Smale and De Groot, 2003). Taken together, the banana is a central part of thousands of rural and urban households' livelihoods not withstanding its role as the world's leading desert fruit.

#### 1.1 Banana production constraints

Banana production is affected by several constraints in both the abiotic and biotic categories. Drought has been identified as the main abiotic constraint to banana production (Smale and De Groot, 2003), while today, the major biotic constraint is

banana bacterial wilt disease (*Xanthomonas campestris* pv. *musacearum*), which destroys fruit bunches and causes total loss of infected plants (Eden-Green, 2004). Other major biotic constraints include arthropods such as weevils (*Cosmopolites sordidus*) which cause stunting of growth, disruption and delay of fruiting, production of small bunches and sometimes death, while heavily infested and damaged plants easily topple under mild wind; diseases such as black sigatoka (*Mycosphaerella fijiensis*) which reduce fruit numbers and weight per bunch and fusarium wilt (*Fusarium oxysporum* f. sp. *cubense*) which may cause total crop failure (Smale and De Groot, 2003). Nematodes constitute other important pests of the banana and include *Meloidogyne incognita* (root-knot nematode), *Pratylenchus coffeae* (banana root nematode), *Radopholus similis* (burrowing nematode), *Helicotylenchus multicinctus* (banana spiral nematode) and *Rotylenchulus reniformis* (reniform nematode) (Atkinson *et al.*, 2003; CABI, 2005).

## **1.2 Strategies to control diseases and pests of bananas**

Several approaches have been proposed for the mitigation of banana production constraints. Some of these have been used for a long time such as; host resistance, cultural methods and chemical control. Cultural control has been used successfully in the management of banana pests such as weevils. They are cost effective and affordable to the farmers. They involve standard agronomic practises used to alter the plant phyllospheres and rhizosphere to favour the plant rather than the disease (Okori, 2004). The commonly used cultural control practises include the use of clean planting materials, crop sanitation, weevil trapping using pseudostem and disc-on-stump traps and proper crop management practices such as timely weed control, pruning, de-trashing and debudding (Pinese and Piper, 1994; Gold *et al.*, 2001; Eden-Green, 2004; Biruma *et al.*, 2007). Other cultural controls have included the use of crop rotation

involving cassava and sweet potato in the control of nematodes, soil organic manure amendments as well as mulching. The latter two control practices enhance plant growth and vigour. Also use of ash and urine concoctions kill off pests and pathogens (Ploetz, 2004).

The use of destructive control measures such as chemicals today are one of the most widely used disease and pest management strategies (Morton, 1987; Romero and Sutton, 1997). For example chemicals have been effectively used to control pests and diseases of banana such as black sigatoka (Guzmán and Vargas, 2001), weevils (*Cosmopolites sordidus*) (Gold *et al.*, 2001), root nematodes (Chabrier *et al.*, 2002) amongst others. However, they are in general too expensive for resource constrained subsistence farmers and a danger to the environment (Carson, 1962). The need to conserve the environment has led to the deployment of Integrated Pest/ Disease Management strategies (Bolkan and Reinert, 1994).

The most widely used approach to control diseases is host resistance. Host resistance can be broadly classified as inducible usually under the control of several genes and non-inducible, usually based on plant architectural characteristics that can impede pest or pathogen attack (Agrios, 1997; Keen, 1999). Non-inducible host resistance and inducible resistance can both be bred for in crop plants. However, it should be noted that in both cases, resistance is a product of co-evolution between host (crop) and pathogen.

Host resistance is particularly cost effective for resource constrained farmers (Agrios, 1997). Host resistance is based on co-evolutionary history of plants and their pests or pathogens. Co-evolutionary processes especially directional selection which tends to

increase the frequency of selectively adapted alleles in populations and/ or episodic events, accounts in large measure for the distribution of pathogens and their hosts. Humans have also inadvertently contributed to pathogen, host distribution mainly by introductions. These are examples of geneflow, but more specifically genotype movement involving the transfer of individuals who carry selectively adapted alleles into new niches. Accordingly, the deployment of resistant lines provides the first line of defence in any agro-ecosystem against pests and diseases (Agrios, 1997). Of particular importance are induced forms of resistance which are widely viewed as responsive to pathogen micro-evolutionary processes (Parker, 2000). Examples of induced resistance include the hypersensitive reactions and other resistance gene induced reactions (Parker, 2000). The hypersensitive response is often associated with salicylic acid production and the broad spectrum systemic acquired resistance (Graham and Graham, 1999). Systemic acquired resistance has been extensively studied and used in crop protection, with various resistance genes mapped and cloned (Hammond-Kosack and Jones, 2000; Parker, 2000).

Induced systemic acquired resistance is another form of resistance based on the induction of plant defence genes especially jasmonic acid dependent pathways. It is induced when soil inhabiting plant growth promoting bacteria especially the pseudomonads infect plant roots (Tuzun and Bent, 2006). Such bacteria are always present in soil and presumably protect plants against soil inhabiting pathogens.

In the case of bananas, deployment of host resistance is compounded by slow breeding process caused by meiotic failures during gametogenesis (Ortiz *et al.*, 1995; Vuylsteke *et al.*, 1995). Additionally, the East African highland banana has a very

limited gene pool (Tushemereirwe *et al.*, 2004). These issues further reduce the possibilities to use conventional breeding to improve East African AAA bananas. It is against this background that emergent technologies such as biotechnology hold promise to manage pests and diseases of banana (Tripathi *et al.*, 2004). For example black sigatoka (*Mycosphaerella fijiensis*) resistance induction in bananas is being done using chitinases from rice and papaya (Swennen and Sagi, 1996). In AAB plantains and grande naine cultivars, anti-microbial proteins and chitinases are being used to induce resistance to the same pathogen. In rasthali cultivar (AAB), maganins are being used to induce resistance to *Fusarium oxysporum* f.sp. *cubense* and *Mycosphaerella musicola* (Atkinson *et al.*, 2003).

### **1.3 Biosafety issues associated with genetic engineering**

The main biosafety issue that has been associated with genetic engineering approaches in the improvement of crops against pests and pathogens has been geneflow (Cooper and Sweet, 2001). By definition, geneflow is the movement of genes from one organism to another (Eastham and Sweet, 2001). Geneflow can occur between species or within populations of the same species (Wong Kwan and Don-Soon, 2001). Geneflow has been considered as the main mechanism of gene escape into the environment. It occurs in three different ways; within species, between species (Jorgensen *et al.*, 1996) and between crops and microorganisms (Nielsen *et al.*, 1997). Geneflow within species of banana is of least concern because of the sterility nature of the crop. Geneflow between crops and microorganisms often referred to as horizontal geneflow or natural transformation raises several biosafety concerns especially with regard to its occurrence. It is of importance therefore to study the potential of its occurrence in agro-ecologies.

#### 1.4 Justification of the study

The banana (*Musa* spp.) crop is affected by many abiotic and biotic stresses (Smale and De Groot, 2003). Currently the most critical stresses affecting banana production are the banana bacterial wilt (*Xanthomonas campestris* pv *musacearum*) in the East African region (Eden-Green, 2004) and globally, fusarium wilt (*Fusarium oxysporum* f. sp *cubense*), black leaf streak also called black sigatoka (*Mycosphaerella fijiensis*) and the weevil (*Cosmopolites sordidus*) (Gold *et al.*, 2001). The threat by these diseases is higher in developing countries where farmers cannot afford many of the effective pesticides. Farmers therefore resort to the use of cultural control methods which in large measure have their limitations. Cultural control measures may not eliminate disease but reduce inoculum load (Campbell and Madden, 1990; Agrios, 1997). Thus ultimately, for the sustainability of banana production, the use of resistance is desirable.

Conventional breeding of resistant crops has been extensively used in disease management. With respect to bananas however, the use of conventional breeding is constrained by several factors including various levels of ploidy, long generation times, sterility of most edible cultivars and limited genetic variability (Pillay *et al.*, 2001). These breeding constraints have therefore made the use of genetic engineering approaches a more favourable alternative. Genetic engineering requires the inclusion of exotic genes into targeted genomes. These genes may be from various sources including microorganisms, plants or animals (Kleter *et al.*, 2006). They may also be developed by gene expression modifications such as down or up-regulation using various approaches including gene silencing (Chandler and Vaucheret, 2001). Organisms developed through genetic engineering are called transgenics, or

genetically modified organisms. The process of generating transgenics requires the use of reporter genes and selectable markers to identify cell lines or tissues expressing introduced genes for regeneration into whole plants using tissue culture (Bennett and Braun, 2007). Many selectable markers are usually not eliminated from transgenic lines because they are part of the transgenic gene construct and may also be used in the selection of transformed plants.

The use of genetic engineering approaches in the development of resistant crops in general raises several biosafety concerns deriving from geneflow (Cooper and Sweet, 2001; Smale and De Groot, 2003). Fears have arisen over the possibility of genetic pollution as a result of geneflow from genetically modified plants to wild relatives (Dale, 1999). There are efforts to develop transgenic varieties resistant to various biotic stresses (Atkinson *et al.*, 2003), given that cultivated bananas are sterile, the threat of geneflow from transformed plants to non transformed wild relatives is highly unlikely. However, geneflow to microorganisms resident in the rhizosphere cannot be precluded (Nielsen, 2003). Geneflow to microorganisms can have potential negative effects on their major biological and ecological functions. For example the natural soil suppressivity roles played by these microorganisms in soils may be affected by geneflow (Atkins, 2004). Moreover, if genes that have the ability to improve the fitness of the recipient microorganisms are taken up, directional selection may ultimately lead to emergence of new populations that may even be pathogenic to the landraces and other plants and disrupt the biological diversity in the environment (Cooper and Sweet, 2001; Atkins, 2004). Other threats from geneflow include the spread of acquired genes to microorganisms through conjugatory and transducing gene transfers with unknown effects in the recipient organisms (Nielsen, 2003).

Gene flow has been implicated in the creation of hybrid microorganisms including viruses; as well as the reactivation of dormant ones since the promoters of most of the transgenes in plants are of viral origin (Teycheney and Tepfer, 1999; Ho and Ching, 1999).

Banana plants provide a highly diverse habitat for a series of microorganisms such as bacteria, fungi as well as protozoa in their rhizosphere and phyllosphere (Harper, 1950; Rombouts, 1953). Several communities of both beneficial and non-beneficial bacterial and fungal endophytes are resident within the plant tissues, their phyllosphere and rhizosphere environments, often reaching very high densities per gram plant material or soil (Siciliano *et al.*, 1998). Given that transgenic approaches may be deployed in the control of various diseases of banana, it is imperative that studies on the potential gene flow to these organisms are done. In this study, rhizobacteria and fungal endophytes were used to investigate the potential gene flow from transgenic banana to endo and ecto- microorganisms of banana rhizosphere and phyllosphere.

## **1.5 General objective of the study**

To generate information that will contribute to biosafety and handling of genetically modified banana under Ugandan conditions with respect to gene flow.

### **1.5.1 Specific objectives**

- a) To investigate the potential horizontal gene flow from transformed East African highland banana plants to soil inhabiting rhizobacteria.
- b) To investigate the potential horizontal gene flow from transformed East African highland banana plants to endophytes in the plants.

### **1.5.2 Study hypothesis**

Rhizosphere and phyllosphere inhabiting microorganisms experience horizontal gene flow and therefore may acquire transgenes from genetically modified banana, when exposed to them.

## CHAPTER TWO

### LITERATURE REVIEW

#### 2.0 Background

Banana is an important staple food crop and a source of income for many smallholder Sub-Saharan farmers (Bagamba, 2007). Banana production is constrained by several abiotic and biotic factors. Among the biotic constraints are the banana bacterial wilt disease (*Xanthomonas campestris* pv *musacearum*), black sigatoka (*Mycosphaerella fijiensis* (M. Morelet), *Fusarium* wilt (*Fusarium oxysporum* f.sp. *cubense*), fruit anthracnose (*Collectotrichum musae*); viral diseases including bunchy top, banana mosaic, banana streak and bract mosaic; and arthropod pests such as the banana weevils (*Cosmopolites sordidus*) and nematodes (Gold *et al.*, 2001; Atkinson *et al.*, 2003; Eden-Green, 2004). Amongst the nematodes, the most important is the burrowing nematode (*Radopholus similis*) others include: *Meloidogyne incognita* (root-knot nematode), *Pratylenchus coffeae* (banana root nematode), *Helicotylenchus multicinctus* (banana spiral nematode), *Rotylenchulus reniformis* (reniform nematode) (Atkinson *et al.*, 2003). These pests and pathogens attack a wide range of banana cultivars, and left uncontrolled, can adversely affect growth and yields in bananas.

#### 2.1 Control and management of banana diseases and pests

Several strategies have been developed to control pests and diseases of banana, the most commonly used being cultural. Cultural control strategies mainly aim at reducing inoculum load by destroying pests and pathogens. In case of banana, the cultural control practices that rely on the principle of reducing the inoculum load include:

- Destruction of infected plant tissues by pruning, desiccation and burning as in the case of banana bacterial wilt, black sigatoka, banana weevil (CABI, 2005; Biruma *et al.*, 2007)
- Use of clean planting material disinfected by hot water treatment or pairing for the control of weevils and nematodes, banana bacterial wilt and banana rust thrips (Pinesse and Piper, 1994; Gold *et al.*, 2001; Biruma *et al.*, 2007).
- Minimising long distance and inter-plant transfer of inoculum by using clean planting material, clean tools (disinfection) and the removal of male inflorescences (Eden-Green, 2004).
- Regulatory measures especially quarantine have also been used to limit movement of planting materials from infected areas in the control of the spread of the banana bacterial wilt (Eden-Green, 2004).
- Weeding and fertiliser application to improve plant vigour and the removal of all crop residues of the previous crop from the field and the establishment of proper drainage channels in the control of the weevil, fruit scaring beetle and pseudostem borer (Munroe, 2007).

The measures to control banana pests however aim at containment of pests and maintenance of clean planting materials. They do not address the issue of developing long term solutions such as host resistance. Consequently banana pests and diseases continue to threaten production and hence the need for more durable solutions based on resistance (Tushemereirwe *et al.*, 2004). Transgenic approaches hold promise for developing resistance to pests and diseases of bananas. Various methods have been used to engineer resistance genes into plants (Atkinson *et al.*, 2003). For example in

bananas, genetic transformation can be achieved using *Agrobacterim* mediated approaches (Khanna *et al.*, 2004; Tripathi *et al.*, 2005) and microprojectile bombardment of embryogenic cell suspensions (Sagi *et al.*, 1995; Côte *et al.*, 1997). Some of the bananas that have been transformed include the AAB plantains and grande naine cultivars in which antimicrobial proteins and chitinases have been used to induce resistance to black sigatoka (*Mycosphaerella fijiensis*). However, the use of transgenic approaches in the development of host resistance has associated biosafety concerns mostly related to geneflow (Smale and De Groot, 2003).

## **2.2 Geneflow**

Geneflow from an evolutionary point of view refers to the introduction of new alleles or their combination into new niches (Hart and Clark, 1997). Geneflow may occur through sexual reproduction involving members of different populations especially when exotic populations are introduced in new niches. This form of geneflow is called gametic geneflow. It may also occur by exchange of individuals of different /or same species into new environments. In this case, the exotic individuals carry combinations of selectively adapted alleles (Hart and Clark, 1997; Okori, 2004). Geneflow especially among bacteria and microorganisms may occur through non-sexual exchange of genetic information between members of the same or different species (Eastham and Sweet, 2001). The occurrence of geneflow could potentially affect the roles played by various microbial species occurring in the environment that are of importance to man especially in the field of agriculture (Atkins, 2004). Amongst these are bacterial organisms living within the rhizosphere of banana plants and fungal endophytes that are soil borne but with numerous benefits to plants (Stoltzfus *et al.*, 1997; Niere *et al.*, 2002). Geneflow among microorganisms is known to occur in three different mechanisms (Wong Kwan and Don-Soon, 2001). These are conjugation,

transduction and natural transformation (Jackmann *et al.*, 1992; Normander *et al.*, 1998; Bertolla and Simonet, 1999).

### **2.2.1 Conjugation**

This is the most recently studied mechanism which involves cell-to-cell contact and the presence of conjugative plasmids to promote the transfer of plasmid and chromosomal genes (Paget and Simonet, 1994; Normander *et al.*, 1998). Conjugation can occur in soil microcosms between inoculated bacteria and the indigenous microfauna (Smit *et al.*, 1991). Its occurrence is facilitated by environmental factors which favour the survival and multiplication of microbial cells such as clay or organic matter (Richaume *et al.*, 1992). Conjugation has been of evolutionary significance to bacteria and indeed more than 16% of their protein-encoding genes have been obtained this way (Alberts *et al.*, 2002; Nielsen, 2003).

### **2.2.2 Transduction**

Transduction is a phage-mediated transfer mechanism thought to promote transfer between only closely related microorganisms (Jackmann *et al.*, 1992). Transduction occurs in soil when both recipient cells and phage particles are present (Germida and Khachatourans, 1988; Zeph *et al.*, 1988). However, there have been no reports on the occurrence of transduction between indigenous bacteria and other indigenous microflora under natural conditions (Paget and Simonet, 1994; Lorenz and Wackernagel, 1994).

### **2.2.3 Natural transformation**

Natural transformation is the process, by which a competent cell binds, assimilates, integrates and finally expresses exogenous DNA (Lorenz and Wackernagel, 1994; Bertolla and Simonet, 1999). Natural transformation occurs between plants and

bacteria than conjugation and transduction (Nielsen, 2002; 2003). Natural transformation occurs in several steps under natural conditions such as:

1. Development of the competence state which involves processes that make a microbial cell receptive to foreign DNA.
2. Efficient adsorption of the DNA from soil clods onto the cell surface of competent bacterial particles. The adsorption of foreign DNA occurs at specific recognition sites on the cell surface membrane.
3. Efficient DNA uptake: The adsorbed DNA passes through the membrane under the influence of a potential gradient followed by simultaneous hydrolysis of the complementary strand.
4. Chromosomal integration via recombination or autonomous replication resulting in the integration of DNA into the genome of the recipient cells.
5. The expression of transgenic genes by the recipient bacterium. In which the traits encoded by the integrated DNA are expressed in the phenotype of the recipient organism (Bertolla and Simonet, 1999).

### **2.3 Genetic modification of bananas**

Genetic modification of bananas by transformation can be achieved through methods such as microprojectile bombardment of embryogenic cell suspensions (Sagi *et al.*, 1995; Côte *et al.*, 1997) and *Agrobacterium* mediated approaches (Khanna *et al.*, 2004; Tripathi *et al.*, 2005). *Agrobacterium* mediated transformation methods involve co-cultivation of bacterium and banana plant tissues; depends on the bacterium naturally transferring transgenes into explants. They are more favourable and advantageous than the direct gene transfer approaches because of single copy transfer of DNA fragments at higher efficiencies, lower cost and with minimal genomic rearrangement (Hansen and Wright, 1999). Studies have demonstrated the possibility

of transforming bananas with *Agrobacterium tumefaciens*. Plant parts favourable for transformation have included the shoot tips and embryos. However, the shoot tips are more favoured since they can be applied on a wide range of cultivars irrespective of their ploidy status or genotype as compared to the embryo methods which are lengthy and cultivar dependent (Tripathi *et al.*, 2003; Tripathi *et al.*, 2005). Shoot tips also allow the regeneration of more homogenous populations of plants in a short period of time (Tripathi *et al.*, 2005).

## **2.4 Selection of transformed cells and tissues**

In order to identify transformed cells from a mass of tissue, there is need to deploy and use systems for selection (Bai and Qu, 2001). The most widely used selection systems involve the use of genes that encode for traits that permit the selection of transformants on selective media (Datta *et al.*, 2000). Such genes are called selectable marker genes. These genes are usually part of the transformation gene cassette that gets incorporated into target plants for transformation (White *et al.*, 1990). The most commonly used selectable markers in plants are antibiotic and herbicide resistance genes.

### **2.4.1 Antibiotic resistance genes**

Antibiotic resistance genes are the most widely used selectable markers in genetically modified organisms (Nielsen *et al.*, 1998). In genetically modified plants, mainly two types of antibiotic resistance marker genes have been used i.e. kanamycin resistance genes (*npt II*) and hygromycin resistance genes (*hpt*). These selection genes are driven by bacterial promoters and allow the selection of transformed plant cells (Bennett and Braun, 2007). Examples of selectable markers include; kanamycin (*nptII*), neomycine

(*aph(3') II*), hygromycin (*hpt*), ampicillin, streptomycin (*aadA*) and gentamycin (*aacCI-IV*) resistance genes (Nielsen, 2003).

#### **2.4.2 Herbicide resistance genes**

Most of the herbicide resistance genes have their origin in microorganisms that have been exposed to herbicides for more than 50 years (Don *et al.*, 1985; Mulwa and Mwanza, 2006). Examples of herbicide resistance genes that have been used in the genetic modification of plants include; the *nitrilase* gene which confers resistance to bromoxynil, *EPSPS* gene which confers resistance to glyphosate, *acetolactate synthase* gene that confers resistance to sulfonylurea, phosphinothricin acetyltransferase that confers resistance to phosphinothricin (Wong Kwan and Don-Soon, 2001). These genes like antibiotic resistance genes are part and parcel of the transgene cassette and are therefore incorporated in the genome of the transformants. The presence of such genes poses a threat especially if gene escape occurs to wild type weeds and to a limited extent pathogens of humans, crop plants and animals.

#### **2.5 Biosafety concerns of genetically modified organisms**

Organisms whose genomes possess intergrated exotic genomes are referred to as genetically modified organisms (GMOs) or living modified organisms (LMOs) (FAO/WHO, 2000; Roseland, 2001). Given the presence of selectable markers in GMOs, and, the likelihood of gene flow to non-target organisms, since the commercial cultivation of GMOs, has led to many biosafety concerns. The main biosafety concerns associated with the use of transgenic approaches are categorised into food and environmental safety. For example, the spread of genetically modified organisms in the environment and the loss of biodiversity are broad environmental issues (Beatrix *et al.*, 2002). Other biosafety issues that have been associated with transgenic

crops include the altered functionality of the transgenes (Dunfield and Germida, 2004).

### **2.5.1 Food safety concerns**

The main food safety concerns associated with transgenic products and foods relate to the possibility of increased allergens, toxins or other harmful compounds (Crawley *et al.*, 1993). For example the protein from the Cry9 gene has allergenicity concerns that have resulted in its commercial restriction to fodder crops (EPA, 2001). The snowdrop lectin (GNA) gene has toxicity properties that alter the enzyme activity in the intestinal brush border cells of rats (Pusztai *et al.*, 1996; Ewen and Pusztai, 1999). There are also concerns about other unintended changes in food composition that may occur during genetic improvement through gene technology (FAO/WHO, 2000).

### **2.5.2 Altered gene functionality and mutation of the inserted DNA constructs**

Many microorganisms in the environment for example fluorescent pseudomonads and non- pathogenic *Fusarium* perform important ecological functions such as suppressing soil borne diseases (Atkins, 2004). Uptake of exogenous DNA by these microorganisms undermines the ecological roles that they play in the environment (Atkins, 2004). This is because the insertion of transgenes is random and can occur anywhere in the genome of the recipient organism interacting with the crop plants (Ho and Ching, 1999). Ectopic gene insertion may result in gene silencing especially if insertion occurs within exons or open reading frames (Halpin, 2005). Insertion of several copies may equally lead to gene silencing (Chandler and Vaucheret, 2001). Altered gene functionality may influence selection frequencies leading to directional selection of species. Similarly, the introgressed genes in transgenic plants may be

altered changing their functionality and this may have negative effects on various metabolic processes and their products (Bradford *et al.*, 2005).

### **2.5.3 Evolution of superior pests and weeds**

New viral genomes with better competitive advantage than the native ones could result through recombination of the transgene and an infecting virus as a result of gene flow (Teycheney and Tepfer, 1999; Conner *et al.*, 2003). For example, the inoculation of transgenic *Nicotiana bigelovii* plants generated recombinant viruses that had a distinct competitive advantage in *N. bigelovii* when compared with the parental strain (Wintermantel and Schoelz, 1996). The observed gene flow was reported at low frequencies and was demonstrated under particular conditions (Wong Kwan and Don-Soon, 2001). In other studies, it was possible to reactivate dormant viruses that cause diseases since most of the transgenes in use are driven by virus promoters (Ho and Ching, 1999).

Gene flow associated with the movement of transgenes to non-target organisms may lead to alteration of their fitness and thereby promote directional selection and consequently ecological imbalances (Cooper and Sweet, 2001). This is especially true where the trait encoded by the transgene is selected and promoted naturally by the environment and consequently fixed in populations. In higher plants, the gene flow of the *CP4 EPSPS* gene or insect resistance (*Bacillus thuringiensis* toxin, Bt) genes may have the unintended effects of creating weeds or invasive species resistant to herbicides or their natural pests (Wong Kwan and Don-Soon, 2001). This is compounded by the fact that foreign genes behave differently from the plant genes and are therefore up to 30 times more likely to escape out of the plants and spread in the environment (Ho and Ching, 1999). This could be achieved through gene transfer

involving pollen via insects visiting the plants for pollen and nectar. The biodiversity of microorganisms in the natural environment could also be altered especially where they have introgressed exogenous plant DNA (Atkins, 2004). Introgressed genes may encode traits that are of high impact which directly affect the fitness of the recipient organisms with consequential increases in their frequencies in the environment due to directional selection to the detriment of other flora and fauna in the environment. Studies have revealed the ability of genetically modified microorganisms to survive in harsh environments because of ability to overcome natural barriers or controls due to improved fitness conferred by the introgressed gene (Beatrix *et al.*, 2002). These microorganisms can also spread to other environments with the aid of vectors like wind, water, animals including birds, protozoa and fungi, resulting in the contamination and pollution of underground water sources if these escape into water (Beatrix *et al.*, 2002).

## **2.6 Rhizosphere microbes**

Rhizosphere microorganisms are those that live within a volume of soil that is in direct contact with plant roots and thereby influenced. They comprise fungal, bacterial and protozoans amongst other microbial groups. Bacteria constitute an important component of these microorganisms and they have developed associations with plants which range from parasitic, symbiotic, epiphytic to endophytic associations. The diversity and development of these microorganisms is to a large extent stimulated by root exudates and other factors such as root exudation patterns, root structure, duration of the season/ growth, soil type, crop stage, cropping practices (such as tillage and crop rotation) and other environmental factors (Smalla *et al.*, 2001). Furthermore, species diversity of plants affects the amounts of root exudates and rhizo-deposition in the different root zones. This significantly affects the structural

and functional diversity of the rhizosphere microorganisms (Donegan *et al.*, 1999; Dunfield and Germida, 2004). Therefore the influence of plants on soil microbes is greatest in the rhizosphere and the magnitude of this influence is determined by the extent of microbial interactions and the plant (Kowalchuk *et al.*, 2003; Dunfield and Germida, 2004).

Changes in plants due to genetic modifications could have alterations on these effects. One such group of microorganisms are the Plant Growth Promoting Rhizobacteria (PGPR) which have been identified as having the potential to increase plant productivity by promoting growth or protection from pathogens (Vessey, 2003). The PGPRs are a heterogeneous group of bacteria which are active via a number of diverse mechanisms such as disease suppression mediated by iron-chelating siderophores; the release of plant growth regulators (auxins, gibberellins, cytokinins) or their homologues; and the production of anti-fungals (O'Sullivan and Ogara, 1992; Vessey, 2003). In some cases the mechanism of plant growth promotion is speculative. For example the ability to produce blends of volatile organic compounds which can act as plant signals (Ryu *et al.*, 2003). The PGPRs may be applied to a wide range of crops where they increase yields and reduce disease (Vessey, 2003). The relationship between plants and PGPRs is important and it need not be distorted.

## **2.7 Endophytes**

Endophytes generally refer to fungi inhabiting plant tissues. However, there are bacteria that live endophytically within the plant tissues where they play crucial roles (Stoltzfus *et al.*, 1997). Fungal endophytes play important roles in the growth of plants through the mutualistic associations. There are various types of endophytes, some occur among monocots while others occur among dicot plant species. The most

important genera that have been isolated from bananas are *Fusarium* and *Acremonium* (Niere *et al.*, 2002). Others include *Penicillium*, *Aspergillus*, *Gongronella* and *Trichoderma*.

*Fusarium* is the most dominant genus with *F. oxysporum* being the most widely spread species. *Fusaria* play important roles in improving plant growth through inhibitory effects on nematode and weevil development (Hallmann and Sikora 1994; Niere *et al.*, 1999). With respect to the environment, *Fusaria* unlike other biological control agents reduce the risk of side-effects on non-target organisms including crops and humans since they live inside plant tissues (Niere *et al.*, 2002). By virtue of their location, fungal endophytes are predisposed to DNA within the plant tissues as well as soil borne microbes which are similarly exposed to free lying DNA or DNA adsorbed onto the soil clods in the environment. Within soil, however, DNA released from plants is maintained or degraded in various ways and may play a role in horizontal gene flow.

## **2.8 Maintenance/ degradation of DNA in soils**

DNA from plants is released in the environment in many ways including, release through root exudates, lysis of the cells in the root regions as the plant root wades through the soil matrix. Other methods of release have included death and decomposition of plants in soil (Paget, 1994; Bertolla and Simonet, 1999). The released DNA may be destroyed by the nuclease enzymes within the soil unless it is adsorbed onto the soil (Paget and Simonet, 1994). The efficiency of DNA adsorption onto the soil components is influenced by factors such as the mineralogy of the sorbent, ionic strength, pH and molecular size (Paget, 1994). Divalent cations play a role in the adsorption of the DNA by acting as bridges in electrostatic interactions

which favour the adsorption of greater amounts of DNA especially on sand fractions of soil (Lorenz and Wackernagel, 1987) or clay (Paget *et al.*, 1998) than as monovalent cations where physical attraction forces prevail (Paget and Simonet, 1994). The availability of DNA for transformation purposes could possibly occur if desorption processes leading to the release of DNA occur. Chamier *et al.* (1993), have reported that a desorption process is not necessary in some cases since competent microorganisms directly take up DNA from the soil mineral particles. Some soils have been reported to significantly reduce the ability of adsorbed and bound DNA for use in transformation especially soils rich in the clay mineral montmorillonite (Khanna and Stotzky, 1992). In sandy soil grains, DNA is readily available for use in the transformation of competent cells (Lorenz *et al.*, 1988).

### **2.8.1 Competence induction**

This involves processes that prepare a cell to take up exogenous DNA (Nielsen *et al.*, 1998). It is one of the factors that affect the role of natural transformation amongst microorganisms. Competence development is enhanced by the availability of calcium ions, neutral pH and development on minimum media as compared to media rich in nutrients (Lorenz and Wackernagel, 1992). Competence induction in bacteria is also affected by the physiological state of the cells and/ or the accumulation of an environmental competence factor (Lorenz and Wackernagel, 1994). In *Bacillus subtilis*, competence is regulated by up to 40 genes which are activated only during active metabolic states (Hahn *et al.*, 1994). The genes work through a complex regulatory network to integrate a number of intra and extracellular signals to induce competence. The network can be divided into a sensory part governed by early competence genes like *comA*, *comP*, *comQ*, *comX*, *sinR*, *degU*, *comK* and *degS* that monitor cellular and environmental conditions and a structural part in which the late

competence genes like *comC*, *comE*, *comF*, *comG*, *recA* and *addAB* play a role (Dubnau, 1991).

### **2.8.2 DNA uptake and integration**

This is another step in the process of transformation where various forms of double stranded DNA can be taken up (Palmen and Hellingwerf, 1997). This DNA may have specific sequences that facilitate recognition and therefore uptake (Smith *et al.*, 1995). In other microorganisms, double stranded DNA are taken up regardless of whether they have consensus sequences or not (Palmen *et al.*, 1993). A distinct mechanism exists for the uptake of plasmid DNA into competent cells (Karudapuram *et al.*, 1995). However, in general, especially in *Actinobacteria calcoaceticus*, double stranded DNA is converted into a single strand with simultaneous hydrolysis of the complementary strand during the process of passing through the cell membrane prior to entry into the cytoplasm (Palmen and Hellingwerf, 1997). Once in the cytoplasm, DNA is bound to an eclipse protein which is expressed during competence (Morrison and Mannarelli, 1979). The eclipse protein binds the DNA and protects it from intracellular nucleases (Palmen *et al.*, 1993). DNA uptake may proceed in any direction. In *Streptococcus pneumoniae* and *Haemophilus influenza*, DNA uptake proceeds in the 3' to 5' direction while in *B. subtilis*, there is no observed directionality (Mejean and Claverys, 1988). The transformation process is completed after integration of the incoming single stranded DNA into the chromosome and expression of the genes encoded on this fragment. The integration of DNA into the chromosome is a recombination dependent process and requires the presence of a functional recombination system (Palmen and Hellingwerf, 1997).

## **2.9 Barriers to natural transformation amongst bacteria**

Several factors have been reported to affect the likelihood of the occurrence of horizontal gene flow to bacterial and other recipients in the soil and the phyllosphere in nature. These can be categorised into endogenous and exogenous factors.

### **2.9.1 Exogenous factors affecting natural transformation**

These comprise both abiotic and biotic factors and include availability of plant DNA for transformation and adsorption of DNA onto the cell surface (Nielsen *et al.*, 1998). Plant DNA is released into the environment through root exudation, wounds, death and decomposition of the plants (Bertolla and Simonet, 1999). The released plant DNA may persist in the soil environment for several months, however, its transforming ability is affected by the length of time spent in the environment (Nielsen, 2003). The adsorption of DNA onto the cell membranes of freely available microorganisms in the environment is the first step in the transformation process (Panjaa *et al.*, 2006). The mechanisms of interaction between transforming DNA and the cell walls of some bacteria may be influenced by the presence of unique recognition motifs. For example in *Neisseria gonorrhoeae* and *Haemophilus influenzae*, transformation depends on the presence of a recognition motif in the donor DNA (Palmen and Hellingwerf, 1997). In both bacteria, the specific sequences are a 9 base pair 5'-AAGTGCGGT-3' in *N. gonorrhoeae* (Smith *et al.*, 1995) and the 10 base pair 5'-GCCGTCTGAA-3' in *Haemophilus influenzae* (Elkins *et al.*, 1991). These sequences are repeated 1, 465 times throughout the *H. influenza* genome. Interestingly, in some bacteria, for example *B. subtilis*, transformation occurs between and among species (Palmen and Hellingwerf, 1997). The occurrence of recognition motifs facilitates transformation between same species and inhibits transformation amongst species.

### **2.9.2 Endogenous factors affecting natural transformation**

Endogenous factors affecting natural transformation amongst bacteria are broadly categorised into four including; the ability to translocate the DNA into the bacterial cytoplasm, DNA repair systems, internalisation of the transforming DNA, expression of the plant DNA and selection of the transformants (Nielsen *et al.*, 1998; Bertolla and Simonet, 1999). Bacteria often are not discriminative on the source type of DNA that they translocate into their cytoplasm and therefore, it is presumed that transgenic DNA would be translocated in much the same way as DNA from non-transformed plants. The uptake of DNA amongst competent bacteria involves a single strand step and for transformation to be successful, the terminal strands of the DNA are not cleaved by the DNA repair systems which involve restriction enzymes within the cell (Dreiseikermann, 1994). If the amounts of DNA are saturated or the restriction process itself is inefficient, transformation will also be successful (Bickle and Krüger, 1993). However, during the process of transformation, the DNA gets internalised by autonomous replication or illegitimate homologous recombination mechanisms (Bertolla and Simonet, 1999).

Homologous recombination is governed by factors such as sequence similarity, which require that the donor DNA and the host genome share DNA sequence similarities required to form DNA heteroduplexes. Studies have revealed that short sections of homology between the host genome and exogenous DNA facilitate the integration of non homologous genetic material at higher frequencies (Nielsen *et al.*, 2000). Extracellular DNA internalisation is also affected by roles played by the SOS system and the mismatch repair system (Bertolla and Simonet, 1999). The SOS system is a DNA repair system also referred to as the error prone repair in which apurinic DNA

molecules are repaired by incorporation of a base that may be the wrong one but that permits replication. The SOS system plays an important role in facilitating homologous recombination between the transforming DNA and the host genome. The mismatch repair system on the other hand inhibits the occurrence of gene transfers between phylogenetically distant organisms by way of inhibition of heterologous recombination leading to maintenance of cell integrity (Bertolla and Simonet, 1999). However, endogenous mutations have been shown in some commensal enterobacteria and pathogenic bacteria that instead facilitate recombination with heterologous transforming DNA (Leclerc *et al.*, 1996). For example, in *Azotobacter vinelandi*, when the recombination regulation processes are relaxed, the bacteria are more efficiently transformed by foreign DNA (Leclerc *et al.*, 1996).

The expression of the transformed DNA is dependent on a number of factors including codon usage, transcription and/or translation signals and the presence of introns (Bertolla and Simonet, 1999). However, since most of the transgenes that are being used are driven by promoters of prokaryotic origin, it is possible that these can be easily expressed in bacteria unlike transgenes that are driven by plant promoters. Upon expression of the exogenous DNA in the transformants, factors like selection and random genetic drift can act on the transformants thereby leading to their amplification and significance in the environment (Bertolla and Simonet, 1999).

## **2.10 Gene transfer in fungi**

In contrast to prokaryotic studies, approaches to investigate horizontal gene transfer to eukaryotic microorganisms are few (Zhang *et al.*, 2005). The uptake of DNA from dead plant material and its integration into the genome by the fungal saprophyte *Aspergillus niger* has been reported (Hoffman *et al.*, 1994). Most of the fungi grow in

intimate contact with or even within plants, which predisposes them to the uptake of plant DNA for example, endophytic fungi (Zhang *et al.*, 2005). In the phytopathogenic fungus *Plasmodiophora brassicae*, host plant DNA is taken up during the infection cycle (Bryngelsson *et al.*, 1988). While DNA integration preferentially occurs via homologous recombination in *Saccharomyces cerevisiae* or *Candida glabrata*, illegitimate recombination clearly dominates in *Histoplasma capsulatum* or *Blastomyces dermatitidis* (Magee *et al.*, 2003). Most filamentous fungi lack extra chromosomal plasmids and therefore incoming DNA is unstable unless it gets integrated into the host genome. They differ in their ability to integrate foreign DNA by either homologous or illegitimate recombination.

## **2.11 Model organisms for investigating horizontal gene flow**

### **2.11.1 *Agrobacterium tumefaciens***

*Agrobacterium tumefaciens* is a gram negative, motile, rod shaped bacterium which is non sporulating and is closely related to the nitrogen-fixing rhizobium species. The bacterium is surrounded by a small number of 5-11 circumtrichetal flagella (Sigeo, 1993). It causes crown gall disease on apples and infects many plants including monocot species of woody and herbaceous nature (Andrade *et al.*, 2003). Virulent strains of the bacterium contain one or more plasmids, one of which carries the genes for tumour induction in a plasmid known as the tumour inducing (Ti) plasmid (Chilton *et al.*, 1980). This plasmid also contains the genes that determine the host range and the infection symptoms. It can therefore be identified by the appearance of tumours or galls of varying size and shape on the lower stem and the main roots of the infected plants (Wright, 2006).

With regard to gene flow, some studies have been carried out using this bacterium especially in tobacco. In the study, transformed tobacco plants were used to investigate the potential horizontal gene flow to non-transgenic *A. tumefaciens* (Broer *et al.*, 1996). No transformants were reported from this study in spite of the provision of regions of shared DNA similarity between the transgene and the genome of the bacterium, due to the inability of the bacterium to develop a competent state (Mitten *et al.*, 1996). Since *A. tumefaciens* lives in association with plant roots as opposed to living ubiquitously within the soil environment, the unique environment provided in the rhizosphere region by plant exudates may induce competence (Bertolla and Simonet, 1999; Sigee, 1993). This association with the plant roots can therefore make *A. tumefaciens* susceptible to gene flow and hence amenable for studies in the rhizosphere (Sigee, 1993). In this study *A. tumefaciens* was used to investigate gene flow.

### **2.11.2 *Escherichia coli***

*Escherichia coli* is a common bacterium that normally inhabits the soil amongst other environments. It is the most common member of the genus *Escherichia*. *Escherichia coli* is a facultative anaerobe, gram negative rod-shaped bacterium propelled by long, rapidly rotating flagella (Chattopadhyay *et al.*, 2006). *Escherichia coli* has been used extensively in conjugative gene flow studies involving *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Heinemann and Sprague, 1989; Sikorski *et al.*, 1990). Gene transfers between eukaryotes and *E. coli* involving glyceraldehyde-3-phosphate dehydrogenase have been detected naturally in addition to the fact that *E. coli* releases large amounts of extracellular DNA (Doolittle *et al.*, 1990; Paget and Simonet, 1994). These make *E. coli* amenable for gene flow studies in the rhizosphere given that limited work has been done on its ability to take up exogenous DNA.

### 2.11.3 *Fusarium oxysporum*

*Fusarium oxysporum* is an imperfect fungus that produces three types of asexual spores; microconidia, macroconidia and chlamydospores which can be spread through running water, on farm implements and machinery (Agrios, 1997). There are various strains of *Fusarium oxysporum* some of which are pathogenic mainly causing vascular wilts. Some *Fusaria* are non-pathogenic and are used in the biological control of fungal and nematode pathogens (Niere *et al.*, 1998). The fungus normally lives endophytically in plants and since it is exposed to plant DNA, it is a candidate model organism amenable for use in geneflow studies. There are very few studies that have been done to investigate the occurrence of gene flow between plants and fungi. In other studies on geneflow, the mycorrhizal species *Amanita muscaria* was studied for the uptake of transgenes from poplar tree species carrying the hygrosopicus bar gene (Zhang *et al.*, 2005). No evidence of geneflow was found. Work has also been done using the filamentous fungus, *Aspergillus niger* and transgenic plants of the genus *Brassica* carrying the Hygromycin B resistance gene (Hoffman *et al.*, 1994). Fungal colonies resistant to hygromycin after co-culture with the transgenic plants were detected, but the hygromycin gene and unknown sequences detected in some resistant fungal clones were found to be unstable and rapidly lost. Only one *A. niger* isolate exhibited stable resistance, suggesting gene transfer (Bertolla and Simonet, 1999). Some studies have revealed that fungi release large amounts of DNA in the environment during their life cycles in addition to the fact that conjugatory gene transfers have been observed between fungi and bacterial communities (Paul *et al.*, 1987; Dunn-Coleman and Wang, 1998). Given that *Fusarium* is one of the most abundant organisms in endophytic and rhizosphere inhabiting organisms, their amenability for geneflow needs investigation.

## **2.12 Molecular tools for studying horizontal gene flow**

The polymerase chain reaction (PCR) has been developed as a specific and sensitive diagnostic method for the direct detection of microorganisms in aquatic environments, food and dairy products and clinical samples on basis of ability to amplify specific sequences of DNA (Simonet *et al.*, 1991). The method also finds wide applicability in environments including soils and sediments where it can be used to study the natural bacterial diversity in these complicated environments where *invitro* isolations of microorganisms for study result in low yields (Ward *et al.*, 1990; Torsvik *et al.*, 1990). The technique has found use as well in the detection of fastidious microorganisms that are difficult or dangerous to culture *invitro* in addition to determining the fates of selected or genetically engineered microorganisms and of particular, genes disseminated by transfer to indigenous microbes (Steffman and Atlas, 1988; van Elsas *et al.*, 1991). From soil samples however, the technique may be limited by the occurrence of phenols and humic acids which limit the extent of hybridisation that actually forms the basis of PCR. Soil borne nucleases may also destroy any DNA in the soil that may have been generated from the bacteria in the soil sample (Picard *et al.*, 1992). Such negative effects however, will be minimised by plating the samples in soil suspensions on Luria Bertani (LB) media from which colonies shall be derived for DNA extraction and subsequently used for PCR. In this study, the polymerase chain reaction will be used to identify putative transformants from among endophytic and rhizosphere inhabiting microorganisms. Primer sequences diagnostic for the target transgenes will be used.

## **2.13 Sectional conclusion**

Horizontal gene flow has been studied in many crops with varying results. The most studied systems have involved crops such as sugarbeet, rapeseed, tobacco, potato,

tomato, oilseed rape, maize, alfalfa, cotton and wheat (Kowalchuk *et al.*, 2003; Nielson, 2003). These studies investigated *in vitro* horizontal gene flow involving for example *Acinetobacter* spp. and transgenic sugarbeet and potatoes with a kanamycin (*nptII*) antibiotic resistance gene on agar plates. Transformant *Acinetobacter* spp. were observed when exposed to homologous chromosomal DNA, though none were detected under optimised conditions with excess plant DNA removed (Nielson *et al.*, 1997). Broer *et al.* (1996) investigated gene flow from T-DNA transformed tobacco with a gentamycin resistance selection marker to *Agrobacterium tumefaciens* but little success was reported. In potatoes, gene transfer to the pathogenic *Erwinia chrysanthemi* of an ampicillin resistance gene encoding  $\beta$ -lactamase has been investigated (Schlüter *et al.*, 1995). The study involved the injection of the bacterium into the potatoes, however, their results revealed no bacterial transformants. In tomatoes, horizontal gene transfer was investigated by availing *in vitro* purified DNA with a kanamycin (*nptII*) selectable marker and *in planta* during infection to *Ralstonia solanacearum* having a deleted *nptII* gene. No transformants were detected (Bertolla *et al.*, 2000). Taken together, these studies suggest a potential threat of gene flow especially to microorganisms. Given that efforts to develop transgenic banana with resistance to biotic and abiotic constraints are underway and the fact that banana is a perennial crop, potential gene flow can not be precluded.

Using transgenic bananas with a  $\beta$ -glucuronidase gene marker, this study investigated the possibility of the occurrence of gene flow to *Agrobacterium tumefaciens*, *Escherichia coli* bacterial isolates occurring naturally in the banana rhizospheres and *Fusarium oxysporum* under natural conditions. .

## **CHAPTER THREE**

### **MATERIALS AND METHODS**

#### **3.1 Study site description and materials**

The study was carried out at the National Agricultural Research Laboratories Institute (NARLI) in Kawanda located about 13 km North of Kampala on latitudes 0°25'N 32°32'E and longitude 32°32'E at an altitude of 1190 metres above sea level. The materials used in the study included; transgenic banana plantlets from local cultivars (Mbwazirume and Mpologoma) transformed with  $\beta$ -glucuronidase (*gus*) reporter gene under the control of *CaMV35S* promoter and a hygromycin resistance gene as the selectable marker and terminated by a *nos* sequence, provided by IITA. The microorganisms tested were; *Agrobacterium tumefaciens* (EHA 105) resistant to the antibiotic gentamycin sulphate, *Escherichia coli* (DH5 $\alpha$ ) without any form of antibiotic resistance were provided by the International Institute of Tropical Agriculture (IITA) and naturally occurring soil bacteria obtained from the rhizosphere of banana plants.

#### **3.2 Experiment 1: Potential gene flow from transgenic banana to rhizosphere inhabiting bacteria**

##### **3.2.1 Introduction**

The objective of this study was to investigate the potential occurrence of horizontal gene flow to bacteria that are associated with banana plants in the rhizosphere. The microorganisms that were used in this study included *Escherichia coli*, *Agrobacterium tumefaciens* and other bacteria commonly occurring in the rhizosphere of banana plants. These studies tested the hypothesis that horizontal gene flow occurs between banana and rhizosphere inhabiting organisms. For this purpose, two

environments were used, *in vitro* media and the natural soil. The detailed procedure used to run the experiments are presented below.

### **3.2.2 Analysis of potential geneflow to rhizobacteria**

#### **3.2.2.1 Experimental design**

The experiment was set up following a Randomised Complete Block Design (RCBD). The treatments comprised of transgenic banana plantlets containing the  $\beta$ -*glucuronidase* gene and non transgenic control banana plants. These plantlets were inoculated with either *A. tumefaciens* or *E. coli*. The experiments were run for a period of 13-16 weeks during which normal agronomic practices were done to ensure proper growth. Each experiment had 5 transgenic plants and 2 controls. All the experiments were repeated three times. Re-isolation of the bacteria were from the rhizosphere region and were also done three times.

#### **3.2.2.2 Media preparation**

For culture of *E. coli* and natural soil bacterial isolates, LB media (Cools *et al.*, 2001) was used. The media was prepared by autoclaving constituted media for 15 min at 121°C and cooled prior to addition of a fungicide, cyclohexamide (150 µg/ml) (VWR International, England). The media was subsequently dispensed into petri-plates (90 mm). Selective LB media was prepared by adding an antibiotic hygromycin (50 µg/ml) (Duchefa Biochemicals, Netherlands) and a fungicide, cyclohexamide (150 µg/ml) (VWR International, England) to autoclaved and cooled media. The selective media was dispensed into petri-plates (90mm). Normal and selective media for *A. tumefaciens* re-isolation was prepared as for *E. coli* except that gentamycin sulphate (50 µg/ml) (Duchefa Biochemicals, Netherlands) was added as a selective antibiotic.

### 3.2.2.3 Re-isolation of rhizosphere inoculated bacteria

The bacterial inocula used comprised of *A. tumefaciens* and *E. coli* and were obtained from IITA, Kawanda. Dosage optimisation was initially done to establish the right amounts of bacteria to apply in the soil that would be easily re-isolated. The optimisation process involved the use of tissue culture banana plantlets (plate 1) grown in plastic pots (400 ml volume) in sterile soil for a period of 2 months. Some of the plants were inoculated with an overnight culture of *E. coli* (OD  $\approx$  0.80) using 1 ml, 2 ml, 3 ml or 4 ml. The other plants were inoculated with a 48 hour old culture of *A. tumefaciens* (OD $\approx$ 0.853) using the same volumes that were used with *E. coli*. The inoculation process was done by pouring the bacterial culture solution at the stem base region of the plants. The experiment was left to stand for 10 days prior to the re-isolation of the bacteria.

During the re-isolation of *A. tumefaciens* and *E. coli*, plantlets inoculated with the 2 ml and 3 ml bacterial inoculum dosages were initially used respectively. One gram soil samples were derived from each of the pots from the stem base region of the plant and vortexed for 2 min in 2 ml of sterile water in sterile 50 ml centrifuge tubes. The soil sample was subsequently serially diluted to  $10^{-2}$ ,  $10^{-4}$  and  $10^{-6}$ .



Plate 1: Banana plantlets used for the optimisation of the bacterial inoculum dosage

This procedure was followed for the rhizosphere soil samples that were respectively inoculated with *E. coli* and *A. tumefaciens*. 100 µl of the diluted solutions were spread on LB media (Cools *et al.*, 2001). The plates spread with *E. coli* were incubated overnight at 37°C while the *A. tumefaciens* culture plates were incubated at 28°C for two days. The dilution showing the most distinct and countable colonies was adopted for use in the experiment. Colony forming units (CFUs) were compared across the different plates for each of *E. coli* and *A. tumefaciens* and the inoculum dosage having the most distinct and countable re-isolated colonies were adopted for use in the experiments.

*E. coli* and *A. tumefaciens* were inoculated into the rhizospheres of the respective experimental plants using optimised inoculum volumes. This was followed with the re-isolation of the bacteria from the rhizospheres of transgenic and non-transgenic banana plantlets for the study of gene flow. Two 1 g soil samples collected three times at intervals of 10 days from the rhizosphere of each plant were used for re-isolation purposes. The intervals used were adopted to allow the acclimatisation of the microorganisms to the banana rhizosphere and adequate exposure to root exudates. The soil samples derived from the rhizosphere of plantlets inoculated with *A. tumefaciens* were suspended in sterile water and diluted to  $10^{-2}$  CFU. 100 µl of this solution was inoculated into LB plates containing selective media (2 LB plates containing 50 µg/ml hygromycin) and a control (without the antibiotic). A similar procedure was used for soil samples derived from the plantlet rhizospheres inoculated with *E. coli*. The exception was the initial dilution of the bacterial suspension to  $10^{-4}$  CFU. Both *A. tumefaciens* and other bacterial cultures were incubated at 28°C for 48

hours while the *E. coli* cultures were incubated at 37°C for 24 hours. Colony counts were taken and used to compute the colony forming units (CFU's).

#### 3.2.2.4 Molecular analyses for detection of gene flow

*Agrobacterium tumefaciens* bacterial colonies that grew on selective media were subjected to a second round of selection on plates containing hygromycin (50 µg/ml) (Duchefa Biochemicals, Netherlands) antibiotics to confirm their survival (plate 2). A similar procedure was used for *E. coli* and the natural soil bacterial isolates that grew on selective media. The selection plates used for *E. coli* and the natural soil bacteria isolates re-isolation had hygromycin (50 µg/ml) (Duchefa Biochemicals, Netherlands) as the selection antibiotic. In order to culture sufficient quantities of bacteria for DNA isolation, re-isolated colonies of *A. tumefaciens*, *E. coli* and natural soil bacteria were cultured in LB broth media. 25 mls of LB broth in 100 ml erlenmeyer flasks containing only hygromycin (*E. coli* and natural soil bacteria) or gentamycin sulphate and hygromycin (*A. tumefaciens*) was used to culture the test isolates. The flasks containing *E. coli* were incubated overnight at 37°C while those that had *A. tumefaciens* and natural soil bacterial isolates were incubated at 28°C for 48 hours respectively. Optical densities were taken at the end of the incubation period for all

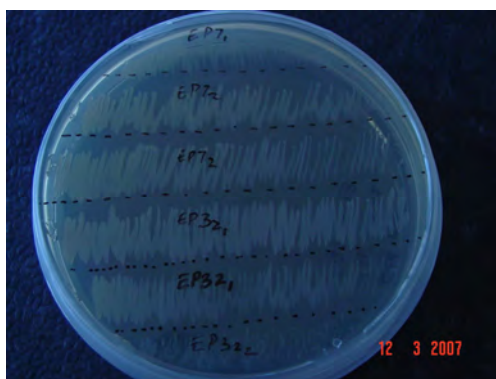


Plate 2: *A. tumefaciens* growing on selective media containing hygromycin and gentamycin sulphate

the bacterial cultures. The bacterial cells in solution were harvested by centrifugation (MIKRRO 250, Berlin Germany) at 6000 rpm for 15 minutes at 4<sup>0</sup>C in sterile 50 ml centrifugation tubes. Genomic DNA was extracted from the harvested bacterial cells (Mahuku, 2004). The DNA extraction buffer (Tris EDTA SDS (TES) contained: 0.2 M Tris-HCl [pH 8], 10 mM EDTA [pH 8], 0.5 M NaCl, 1% SDS. The quality of the extracted DNA was assessed by running 5 µl of the DNA and 2 µl of loading dye in a 1% agarose gel in TAE buffer. The gel was stained in 1% ethidium bromide for 20 minutes and visualized under an ultra violet trans-illuminator. The bands on the gel were documented using a gel documentation system. The tubes containing DNA were appropriately labelled and stored at -20<sup>0</sup>c for PCR. PCR was performed using primers specific to the *β-glucuronidase* gene. The primers used were primer 1- 5' TTTAACTATGCCGGGATCCATCGC 3' and primer 2- 5' CCAGTCGAGCATCTCTTCAGCGTA 3'. PCR involved a 2 min initial denaturation step at 94<sup>0</sup>C and 30 cycles consisting of 1 min denaturation at 94<sup>0</sup>C, 1 min primer annealing at 60<sup>0</sup>C and a 1 min extension at 72<sup>0</sup>C followed by a 10 min extension step at 72<sup>0</sup>C. The amplification products were size fractionated by agarose gel, electrophoresed in TAE buffer (Maniatis *et al.*, 2000) at 80 volts for one hour. The gels were stained with 1% ethidium bromide in an aqueous solution and were examined for amplification of the *β- glucuronidase* gene.

### 3.2.2.5 Statistical analysis of the results

The bacterial colonies that grew on media were expressed as colony forming units (CFUs) using the formula below;

$$CFUs = \frac{\text{Number of colonies}}{\text{Dilution factor}} \times \text{volume of sterile water used to dilute the soil sample}$$

The data collected from the optimisation experiments were too few to be statistically analysed using analysis of variance (ANOVA) therefore; appropriate T-tests were used for the comparison of means. The analysis for potential gene flow to rhizobacteria data were subjected to ANOVA using the GenStat Discovery Edition 3 (Lawes Experimental Trust Rothamstead Experimental Station UK). Where significant differences were found between treatment means, these were compared using Fishers Protected Least Significant Difference Test (Steel *et al.*, 1997).

### **3.3 Experiment 2: Potential gene flow from transgenic East African highland banana plants to fungal endophytes**

#### **3.3.1 Introduction**

The objective of this study was to investigate the potential gene flow from bananas to endophyte fungal organisms. A fungal endophyte *Fusarium oxysporum* (V5W2-9 NH3) was used. This isolate is being used to control banana nematodes (Niere *et al.*, 2002). Because of the free lying DNA within the tissues of plants, its possible that fungi living intimately within plant tissues interact with this DNA and ultimately take it up through horizontal gene transfer using mechanisms such as homologous and illegitimate recombination. Through these processes, natural functions performed by these fungi including the provision of nutrients and relief of biotic stresses to the plants could be lost due to the occurrence of horizontal gene flow. This study sought to investigate the occurrence of horizontal gene transfer of a  $\beta$ -glucuronidase gene in transgenic bananas to an associated fungal endophyte, *Fusarium oxyporum* (V5W2-NH3) under natural conditions.

### **3.3.2 Innoculation of plants with fungal endophytes**

The transgenic plantlets including the controls were established in a nutrient solution for 2 months for adequate root mass development (plate 3). At inoculation stage, pure *Fusarium oxysporum* (V5W2-9 NH3) cultures (Papar, 2008) raised on half strength potato dextrose agar (Duchefa Biochemicals, Netherlands) were used as inoculum. Spore counts were standardised using a haemocytometer. This was done by obtaining average scores of five spore counts and multiplying by a correction factor to express the counts in spores per ml. The spore solution that would provide the required concentration of spores in solution. Plants were exposed to the fungal inoculum by immersing them into ritar jar flasks containing  $1.5 \times 10^6$  spores/ml of fungal spore for four hours. Inoculated plantlets were later transferred to 10 L buckets having sterile soil. The experiment was set up following a Randomised Complete Block Design with three replicates (plate 4). The experiments were appropriately managed throughout the 14-16 weeks growth period to ensure normal growth.

### **3.3.3 Inoculated fungal endophyte re-isolation**

After the first eight weeks, two root samples were taken from each of the plants. The root samples were washed to remove soil and subsequently sterilised in 57% sodium hypochlorite (Reckit Bectinson, East Africa Limited Nairobi, Kenya) for 30 secs, 75% ethanol for one min and finally rinsed in sterile water for 1 min. Each root was cut into 12 small cylindrical discs and six root discs plated on normal half strength potato dextrose agar (PDA) (Duchefa Biochemicals, Netherlands) having streptomycin sulphate (Duchefa Biochemicals, Netherlands), penicillin (Duchefa Biochemicals, Netherlands) and chlorotetracyclin (Duchefa Biochemicals, Netherlands) antibiotics as well as potassium chlorate (Lab Express Inc New Jersey, USA). The remaining six root discs were plated on selective half strength PDA containing the above antibiotics

as well as hygromycin (50 µg/ml) (Duchefa Biochemicals, Netherlands). This procedure was repeated for each of the two roots excised from all the experimental plants. The plates were incubated for 7-10 days at room temperature. *Fusarium oxysporum* (V5W2-9 NH3) colonisation was assessed at the end of this period with major emphasis on its microscopic characteristics (plate 3). Fungal colonies growing on selection media were sub-cultured onto fresh half strength PDA plates and subsequently incubated for



Plate 3: A transgenic plant establishing in nutrient solution.



Plate 4: Experimental plants used in the study the National Biotechnology Centre in Kawanda.

7-10 days at room temperature for genomic DNA extraction. This was done to confirm the ability of the fungal colonies to establish on selection media.

### **3.3.4 Genomic DNA extraction and molecular analysis**

Genomic DNA was extracted from the mycelia of fungal colonies that grew on selection media according to Mahuku (2004). The TES extraction buffer (0.2 M Tris-HCl [pH 8], 10 mM EDTA [pH 8], 0.5 M NaCl and 1% SDS) was used. Sterile sea sand was used to crush the mycelia. The rest of the DNA extraction procedures were as described under the section on molecular analyses from rhizobacteria. PCR was performed with *gus* specific primers as described in the previous section.

### **3.3.5 Experimental design and statistical analysis of the results**

The experiments were set up in a Randomised Complete Block Design (RCBD). The treatments comprised of transgenic banana plantlets containing the  $\beta$ - *glucuronidase* gene and non transgenic control banana plants. Each experiment had five transgenic plants and two controls. All experiments were repeated three times. Reisolation of the *Fusarium oxysporum* from the roots was also repeated three times.

The data were subjected to analysis of variance (ANOVA) using the Statistical analysis Systems (SAS) (SAS Institute Inc, North Carolina, USA). Isolation frequencies of *Fusarium* spp among the plant roots were analysed using categorical logistic regression. Likelihood ratio tests were performed to investigate differences within factors (*Fusarium* spp and plant roots). In cases where significant differences ( $P \leq 0.05$ ) were detected, the Dunn-sidak correction factor was used as a posterior testing tool (Sokal and Rolf, 1995) using the SAS system (SAS, 1989).

## CHAPTER FOUR

### RESULTS AND DISCUSSION

#### 4.0 Experiment 1: Potential gene flow to rhizosphere inhabiting bacteria

#### 4.1 Re-isolation of rhizosphere inoculated bacteria

##### 4.1.1 Inoculum dosage optimisation

A comparison of best dilution volumes revealed significant differences at 5% level ( $P < 2.35$ ,  $t = 1.19$ ) in *Agrobacterium tumefaciens*. Distinct colonies were obtained with the 3 ml volume ( $13 \times 10^4$  colony forming units, (CFU) as compared to the  $8.60 \times 10^4$  CFUs,  $16 \times 10^4$  CFUs and  $26 \times 10^4$  CFUs obtained with the 1 ml, 2 ml and 4 ml volumes respectively. Subsequently, the 3 ml volume was adopted for use in the *Agrobacterium tumefaciens* experiments.

In *Escherichia coli*, the inoculum dosage used influenced the bacterial colony counts at re-isolation at the 5% level ( $P < 2.35$ ,  $t = 1.24$ ) with the highest colony count observed where the 2 ml dosage volume was used and the least in the 3 ml inoculum volume (Table 1). The 2 ml inoculum volume was therefore adopted for subsequent use in the study.

Table 1. Colony forming units from the plant rhizospheres inoculated with the different volumes of *E. coli*

<i>E. coli</i> inoculum volume (ml)	Dilution factor	Colony forming units (CFU) $\times 10^7$		
		1 <sup>st</sup> plate	2 <sup>nd</sup> plate	Average
1	$10^{-4}$	82.80	64.00	73.40
2	$10^{-4}$	94.20	88.40	91.30
3	$10^{-4}$	6.00	-	3.00
4	$10^{-4}$	40.60	41.60	41.10

#### **4.1.2 Inoculum re-isolation optimisation**

In *Agrobacterium tumefaciens*, significantly more colonies established with the  $10^{-2}$  dilution compared to other dilution factors ( $P < 6.31$ ,  $t=4.30$ ). About 83 colonies were obtained with the  $10^{-2}$  dilution where as 1 colony and none were observed for the  $10^{-4}$  and  $10^{-6}$  dilution factors respectively. Subsequently the  $10^{-2}$  dilution was chosen for use in the dilution of samples for all the *Agrobacterium tumefaciens* experiments.

Results from the re-isolations in *E. coli* revealed that all the dilutions gave similar results and none were significantly different ( $P \geq 0.05$ ). However, with the  $10^{-4}$  dilution plate, the colonies were more distinct compared to the  $10^{-2}$  and  $10^{-6}$  dilutions. The  $10^{-4}$  dilution was therefore used in the dilution of samples for all the *Escherichia coli* experiments.

#### **4.2 Analysis of potential geneflow from transgenic plants to rhizosphere bacteria**

The bacterial isolates obtained from the rhizosphere of banana plants were grown on both selection and normal LB media to determine the proportion of putative bacterial transformants. On plates, a total 1089 colonies (853 on normal media and 237 on selection media) were obtained from *E. coli* while in *Agrobacterium tumefaciens* and natural soil bacterial isolates, 12, 503 colonies (12,457 on normal media and 46 on selection media) and 615 colonies (564 on normal media and 51 on selection media) respectively were observed to grow. The colony counts obtained were expressed as colony forming units to enable working with the actual amounts of bacterial organisms that were in the sample of soil taken from the rhizosphere region of the experimental plants. Of the colonies that grew on selection media, a few colonies were randomly selected and grown on a second round of selection. These totalled to

36 bacterial isolates (8 from *E. coli*, 9 from *A. tumefaciens* and 22 isolates from natural soil bacteria). Generally more isolates from *E. coli* grew on selection media than from *A. tumefaciens* and natural soil bacteria. The lowest count of isolates that grew on selection was observed in *A. tumefaciens* (Figure 1, 2 and 3). The total colony forming units (CFUs) that grew on selective media were  $8.0 \times 10^6$  for natural soil bacterial isolates,  $4.26 \times 10^7$  for *E. coli* and  $5.80 \times 10^4$  for *A. tumefaciens*.

**CFUs of *E. coli* cultivated on both non-selective (Nm) and selective (Sm) LB media**

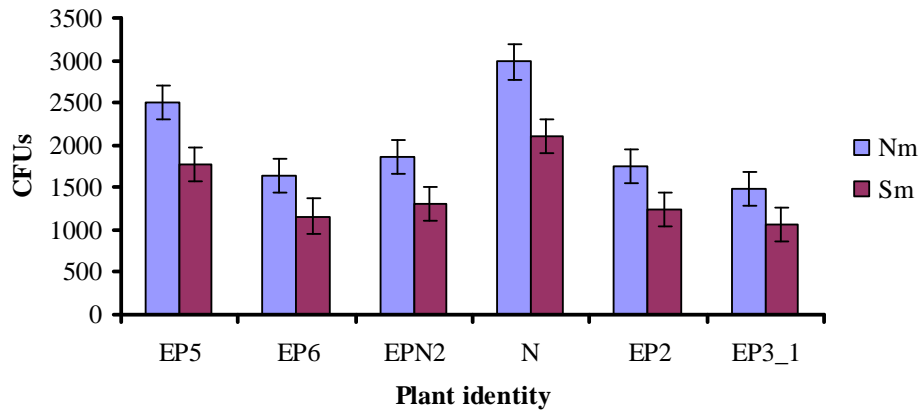


Figure 1

**CFUS of *A. tumefaciens* cultivated on both selective (Sm) and non- selective (Nm) LB media**

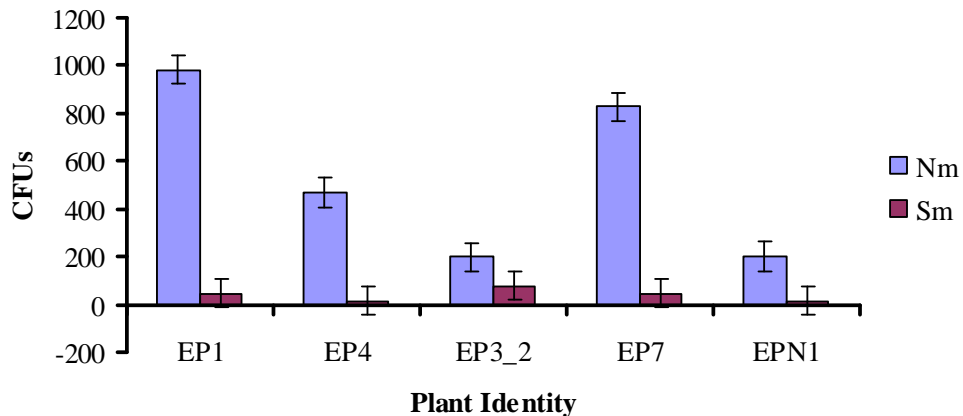


Figure 2

**CFUs of Natural soil bacterial isolates cultivated on both selective (Sm) and non-selective (Nm) media**

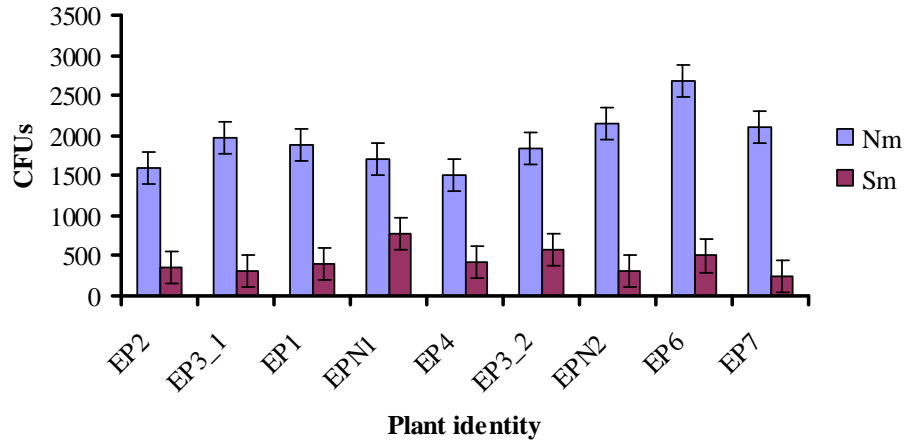


Figure 3

Figures 1, 2 and 3 represent respectively CFUs from *E. coli*, *A.tumefaciens* and natural soil bacteria cultivated on both non-selective and selective media. EP5, EP6, EP2, EP3<sub>1</sub>, EP1, EP4, EP3<sub>2</sub>, EP7 represent CFUs from the transgenic plants while EPN2, EPN1 and N represent those from the controls. Sm and Nm denote selective and non-selective media respectively. The histograms were generated using transformed data using the square root transformation (Sokal and Rolf, 1995).

There were highly significant differences ( $P < 0.001$ ) amongst means of the CFUs of *E. coli* that were re-isolated on both normal and selective media (Table 2). Marked differences ( $P < 0.001$ ) were similarly observed amongst the means of the CFUs derived from the different plants. However plant genotype effects on the re-isolation of *E. coli* were not observed ( $P \geq 0.05$ ) (Table 2). In general more bacteria were re-isolated from non-selective media (Table 3, 5 and 7). Interestingly, plants that supported high numbers of bacteria on non selective media similarly generated high numbers on antibiotic selective media.

Table 2. Analysis of variance for the means of the colony forming units (CFUs) of *E. coli* that established on non-selective and selective media

<i>Source of variation</i>	<i>Degrees of freedom</i>	<i>Mean square</i>	<i>F. pr</i>
Replicate	2	2480289	
Plant	5	1465884	<.001
treatment	1	3206770	<.001
Plant x Treatment	5	3206770	0.911
Error	22	146660	
Total	35		

Data were transformed using the square root transformation to normalise the variances (Sokal and Rolf, 1995)

Table 3. Mean colony forming units of *Escherichia coli* that established on both normal and selective media

<i>Plant</i>	<i>Treatments</i>	
	non-selective media	selective media
Transgenic plants		
EP2	1753	1240
EP3_1	1491	1054
EP5	2507	1772
EP6	1644	1162
Non-transgenic (controls)		
EPN2	1854	1311
LSD		
		264.7
CV%		
		26.1

Data were transformed using the square root transformation to normalise the variances (Sokal and Rolf, 1995)

In the naturally occurring re-isolated rhizobacteria, highly significant ( $P < .001$ ) differences were observed amongst the treatments though the re-isolates from the plants were not markedly different ( $P \geq 0.05$ ). Plant genotype did not influence ( $P \geq 0.05$ ) bacterial re-isolation (Table 4).

Table 4. Analysis of variance for colony forming units of natural soil bacterial isolates that established on non-selective and selective media

<i>Source of variation</i>	<i>Degrees of freedom</i>	<i>Mean square</i>	<i>f. pr</i>
Replicate	2	207515	
Plant	8	195282	0.748
Treatment	1	30380866	<.001
Plant x Treatment	8	252260	0.597
Error	34	310619	
Total	53		

Data were transformed using the square root transformation to normalise the variances (Sokal and Rolf, 1995)

The means among bacteria re-isolated from the rhizosphere of the transgenic plants were not significantly different although there were some differences between reactions of the isolates (Table 5).

Table 5. Mean CFUs of the natural soil bacterial isolates that established on both non-selective and selective media

<i>Plant</i>	<i>Treatment</i>	
	Non-selective media	Selective media
Transgenic Plants		
EP1	1886	404
EP2	1594	364
EP3_1	1965	316
EP3_2	1846	574
EP4	1512	428
EP6	2676	499
EP7	2095	236
Controls		
EPN1	1703	779
EPN2	2141	316
LSD		308.3
CV(%)		47.0

Data were transformed using the square root transformation to normalise the variances (Sokal and Rolf, 1995)

With *Agrobacterium tumefaciens*, no significant differences ( $P \geq 0.05$ ) were observed amongst the means of the CFUs of the isolates derived from the plants used in the experiments though differences ( $P \leq 0.05$ ) in treatments used were noticeable. The re-isolation of the *A. tumefaciens* was not influenced ( $P \geq 0.05$ ) by the genotype of the plants (Table 6). Amongst the means of the CFUs of *A. tumefaciens* from the rhizospheres of transgenic plants and the non transgenic plants (controls), no significant differences ( $P \geq 0.05$ ) were similarly observed though the some re-isolates reacted differently (Table 7).

Table 6. Analysis of variance for the colony forming units of *A. tumefaciens* isolates that established on non-selective and selective media

<i>Source of variation</i>	<i>Degrees of freedom</i>	<i>Mean square</i>	<i>f. pr</i>
Replicate	2	914930	
Plant	4	194385	0.583
Treatment	1	1840816	0.017
Plant x Treatment	4	192720	0.587
Error	18	266403	
Total	29		

Data were transformed using the square root transformation to normalise the variances (Sokal and Rolf, 1995)

Table 7. Mean CFUs of *A. tumefaciens* that established on both non-selective and selective media

<i>Treatments</i>	<i>Mean colony forming units</i>	
	Non-selective media	Selective media
Transgenic plants		
EP1	982	45.0
EP3_2	199	79.0
EP4	469	15.0
EP7	827	47.0
Control		
EPN1	202	15.0
LSD		396.0
CV (%)		105.0

Data were transformed using the square root transformation to normalise the variances (Sokal and Rolf, 1995)

#### 4.2.1 PCR screens for the $\beta$ - glucuronidase gene

A total 36 bacterial re-isolates were used for DNA extraction (8 from *E. coli*, 9 from *A. tumefaciens* and 22 isolates from natural soil bacteria) and PCR was performed using *gus* specific primers. Amplified fragment of about 500bp corresponding to the internal fragment of  $\beta$ - glucuronidase gene was observed in the positive control (plasmid DNA (pCAMBIA1201)) where as no amplified fragment was observed with DNA isolated from the bacteria re-isolated from the rhizosphere of the experimental plants (Plate 5, 6, 7 and 8).

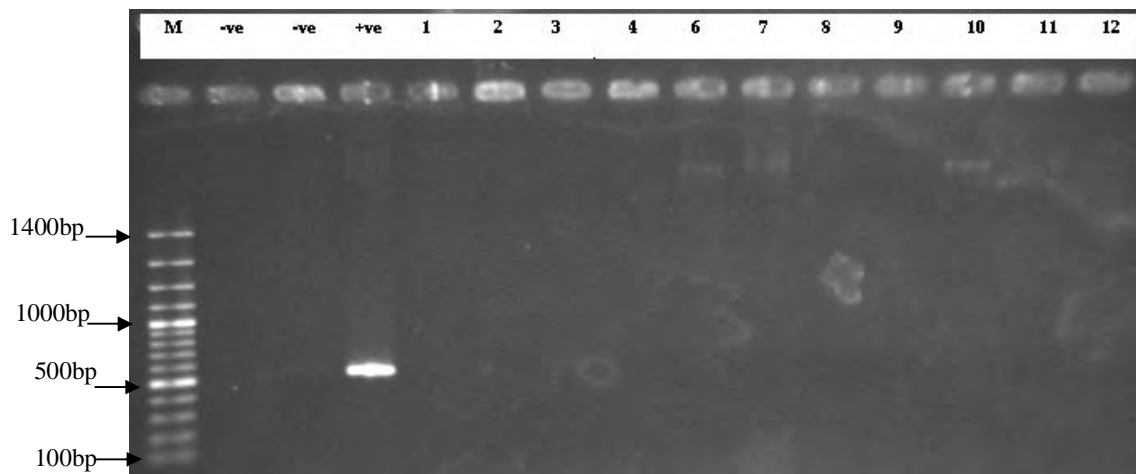


Plate 5: Electropherogram of PCR amplicons based on DNA isolated from re-isolated *A. tumefaciens*. Lane M is the molecular weight marker, lane -ve = negative control (water), lane -ve = negative control (EHA 105 DNA sample), lane +ve = positive control (plasmid DNA (pCAMBIA 1201)); lanes 4, 1-3 and 6-12 represent EHA 105 DNA samples from the rhizosphere of the transgenic plants. Lane 10 corresponds to the EHA 105 sample derived from the plant control.

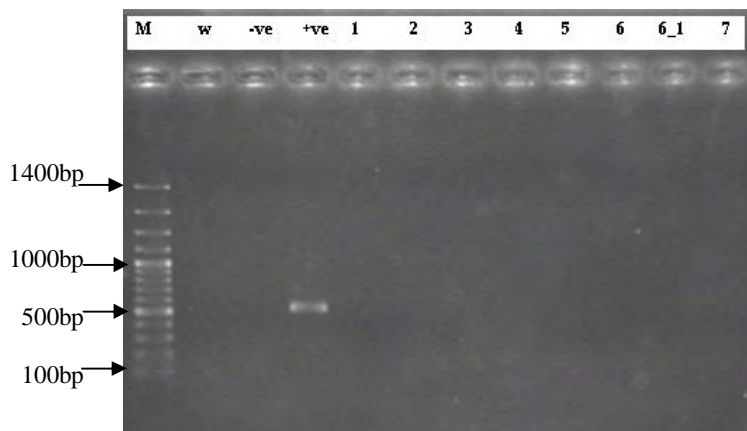


Plate 6: Electropherogram of PCR amplicons based on DNA isolated from re-isolated *E. coli* DNA. Lane M is the molecular weight marker; lane w = water control; lane -ve = negative control (pure *E. coli* DNA sample prior use in the experiments); lane +ve = positive control (plasmid DNA (pCAMBIA 1201)); lanes 1-7 represent *E. coli* DNA samples from the rhizosphere of the transgenic plants.

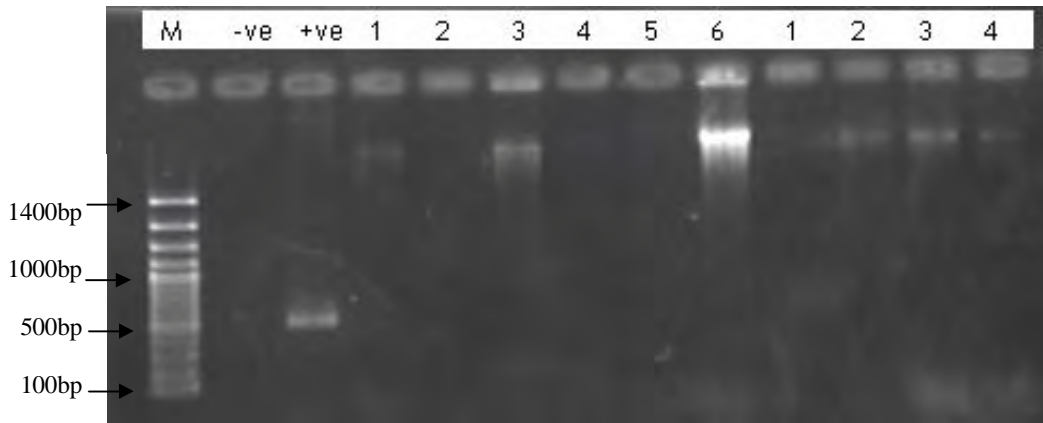


Plate 7: Electropherogram of PCR amplicons based on DNA isolated from re-isolated natural soil bacterial DNA. Lane M is the molecular weight marker; lane -ve = negative control (water); lane +ve = positive control (plasmid DNA (pCAMBIA 1201)); lanes 1-6 = natural soil bacterial isolates DNA (1<sup>st</sup> re-isolation), lanes 1-4 = 2<sup>nd</sup> re-isolate natural soil bacteria. The isolates were obtained from the rhizosphere of the transgenic plants.

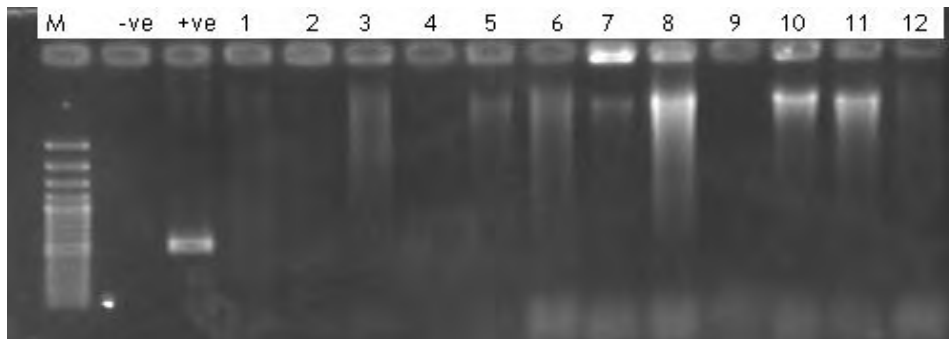


Plate 8: Electropherogram of PCR amplicons based on DNA isolated from re-isolated natural soil bacterial DNA. Lane M is the molecular weight marker; lane -ve = negative control (water); lane +ve = positive control (plasmid DNA (pCAMBIA 1201)); lanes 1-12 = 3<sup>rd</sup> re-isolate natural soil bacteria (sample 12 constituted DNA from the control plant). The isolates were obtained from the rhizosphere of the experimental plants.

### 4.3 Experiment 2: Potential geneflow to fungal endophytes

#### 4.3.1 Fungal endophyte re-isolation

A total of 1584 banana root cylindrical discs were screened for the presence of the inoculated *Fusarium oxysporum*. Plant colonisation was low with only 40 root

segments colonised by the fungus. A total 36 fungi grew on normal media of which 20 were from the transgenic plants and 16 from the controls. Four (4) fungi established on selection media of which 3 were from the controls and only one established from the transgenic plants. Overall, *F. oxysporum* colonisation averaged 27.3% in transgenic plants and 20.5% in the non-transgenic plants.

Plant colonisation by *F. oxysporum* was significantly influenced by the banana genotype ( $P \leq 0.034$ ). Although there was no effect of sampling time on colonisation ( $P = 0.076$ ) among plants of the same genotype, plant colonisation was significantly affected by the media composition ( $P \leq 0.0001$ ), with higher percentage colonisation observed for roots that were plated on non-selective media compared to those that were plated on selective media ( $P \leq 0.025$ ). There was no interaction between media composition and banana genomic structure ( $P = 0.31$ ) (Table 8).

**Table 8. Logistic regression analysis for *Fusarium oxysporum***

<i>Source of variation</i>	<i>Degrees of freedom</i>	<i>Sums of squares</i>	<i>Probability</i>
Media	1	23.16	<0.0001
Treatment	1	4.48	0.034
Weeks	2	5.14	0.076
<b>Interactions</b>			
Media x treatment	1	1.04	0.308
Media x weeks	2	4.61	0.0997
Treatment x weeks	2	0.79	0.675

Various kinds of wild fungal isolates were similarly observed to grow especially on selective media as well as even on the non-selective media. The isolates that grew on selective media were subjected to a second round of selection with hygromycin (50 µg/ml), and all isolates were able to grow (plate 9).

### 4.3.2 Molecular analysis

Thirty fungal re-isolates (including *F. oxysporum* and the wild fungal isolates) from the transgenic plants and six fungal re-isolates from the controls that were able to establish on the second round of selection were used for DNA extraction. The DNA was amplified using  $\beta$ -glucuronidase gene construct specific primers and subsequently electrophoresed on a 1% agarose gel. No band was observed corresponding to the size of the  $\beta$ -glucuronidase gene construct used in the experimental plants (plate 10 and 11) however, an amplified fragment of about 500bp was observed with the positive control (plasmid DNA (pCAMBIA 1201)).



Plate 9: Fungal isolates growing on selection media.

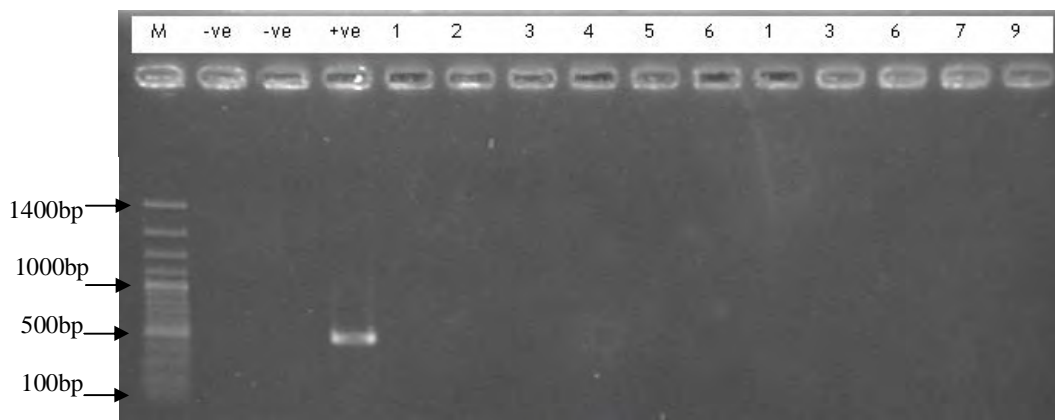


Plate 10: Electropherogram of PCR amplicons based on DNA isolated from re-isolated fungal organisms. Lane M – molecular marker; lane -ve = water control, lane -ve = *Fusarium oxysporum* control & lane +ve = positive control (plasmid DNA (pCAMBIA 1201)). Lanes 1-6 = replicate 1 - 1<sup>st</sup> re-isolates; lanes 1-3 = replicate 1 - 2<sup>nd</sup> re-isolates and lanes 6-9 = replicate 1 - 3<sup>rd</sup> re-isolates.

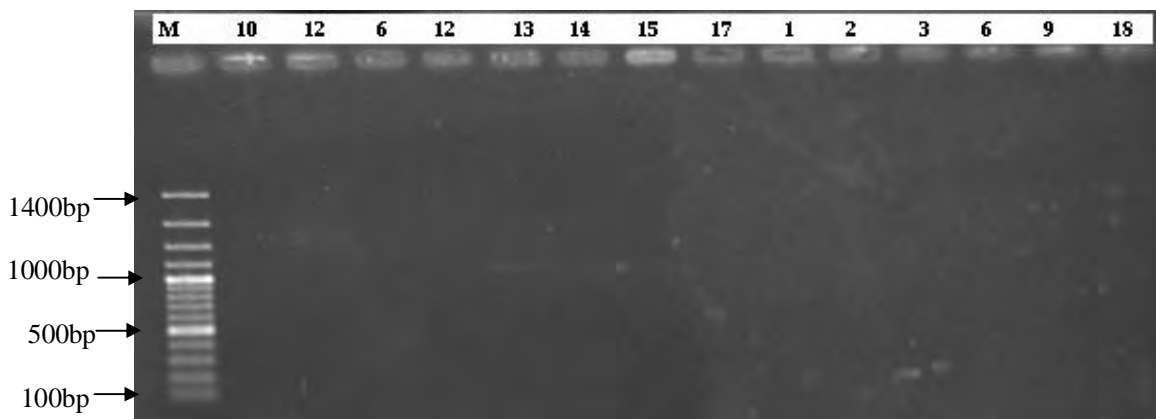


Plate 11: Electropherogram of PCR amplicons based on DNA isolated from re-isolated fungal organisms. Lane M is the molecular weight marker, lanes 10 & 12; 6 and 12-17 correspond to replicate 2, 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> re-isolates while lanes 1-18 correspond to replicate 3, 2<sup>nd</sup> re-isolates. This gel picture is a follow-up of plate 10.

#### 4.4 Discussion

The transgenic plants used in this study were transformed with a construct containing hygromycin resistance gene and  $\beta$ -glucuronidase gene. The bacteria (*E. coli*, *A. tumefaciens* and naturally occurring soil bacteria) from the rhizosphere of transgenic plants were grown on selective medium containing hygromycin. The bacteria (*E. coli*, *A. tumefaciens* and naturally occurring soil bacteria) growing on selective medium were used for PCR to test for the presence of the  $\beta$ -glucuronidase gene. The results from this study suggest no gene flow to the bacteria tested occurred. No significant differences in survival abilities of re-isolated bacteria suggests all re-isolated bacteria from the rhizosphere of transgenic plants reacted in a similar manner to the bactericide and were thus non-transformed. Interestingly for all bacteria re-isolated, some survived on selective media suggesting uptake of the transgene. However, even bacteria from natural soil survived on selective media (Tables 5, 7). The absence of PCR amplicons of the  $\beta$ -glucuronidase gene in all the re-isolated bacteria surviving on selective media provides support for absence of gene flow and for theory that even the bacteria used in this study possess endogenous capacity to degrade antibiotics. Indeed, other studies on *E. coli* have revealed the presence of a plasmid encoded

antibiotic resistance gene to hygromycin (Rao *et al.*, 1983). Elsewhere studies have demonstrated the occurrence of antibiotic resistance genes on plasmids (Riesenfeld *et al.*, 2004). Natural horizontal gene transfer events have been detected in *E. coli*. Studies by Doolittle *et al.* (1990) revealed that *E. coli* integrated a second glyceraldehyde-3-phosphate dehydrogenase gene from a eukaryotic host. In case of *A. tumefaciens*, similar response was found on both selective and non-selective media suggesting absence of gene flow from bananas. Similar reports have been made in other studies involving *A. tumefaciens* (Broer *et al.*, 1996).

The absence of gene flow could be attributed to various reasons. Firstly, transformation of bacteria requires that cells be competent (able to take up exogenous DNA). The ability to naturally develop competent cells has been reported among bacteria (Palmen and Hellingwerf, 1997). However, this capacity may be compounded by physiological state of the cell and influence of environmental factors (Lorenz and Wackernagel, 1992). In other bacteria, competence “state” development is influenced by absence or presence of certain amino acids and glucose availability which modulate DNA-binding and uptake machinery (Palmen and Hellingwerf, 1997). Availability of amino acids and glucose are inadvertently influenced by rhizosphere conditions especially enzyme activities that may enhance bio-degradation of both DNA and these ingredients (Nielsen *et al.*, 1998; Bertolla and Simonet, 1999). The bio-degradation process may also degrade DNA reducing dosages for adsorption and ultimately uptake (Bertolla and Simonet, 1999). In this study, bacteria were inoculated into the rhizosphere and grown in soil for 13 weeks. The failure to uptake DNA from plants (banana) could thus be attributed to in availability of transgenic DNA or more importantly, the lack of competent state

limited gene transfer. These studies on *Agrobacterium* and other soil inhabiting bacteria suggest a very low probability of gene flow from banana.

Also notable for mention is the fact that eukaryotic DNA molecules are usually associated with proteins such as histones which condense DNA and could therefore drastically interfere with the uptake and recombination mechanisms of bacteria (Bertolla and Simonet, 1999).

In the potential gene flow study to fungal endophytes, the fungal isolates re-isolated from transgenic banana phyllospheres were not transformed with the  $\beta$ -glucuronidase gene. This study however showed that fungi especially *Fusarium oxysporum* can breakdown antibiotics deployed against them. An earlier study during optimisation had demonstrated that *F. oxysporum* can survive on selective media containing 50  $\mu\text{g/ml}$  of hygromycin. As in the case of bacteria, the test fungi were able to survive on selective media suggesting endogenous capacity to degrade the antibiotics. However, attempts to amplify the transgene were negative with no amplicons affirming that *F. oxysporum* re-isolates were indeed not transgenic. Among other fungi, it has been demonstrated. Whereas gene flow may occur through homologous and illegitimate recombination (Magee *et al.*, 2003; Ruiz-Duiz, 2002), up taken DNA are rapidly destroyed as in the case with Hoffman *et al.* (1994). This phenomenon may explain in part the survival of re-isolates on selective media and failure to detect the  $\beta$ -glucuronidase gene. Nevertheless, the survival of re-isolates even prior to exposure to transgenic banana suggests these fungi (*F. oxysporum*) possess mechanisms to degrade hygromycin. This study thus found no evidence for gene flow. These results are in agreement with those that were obtained by Zhang *et al.* (2005). The study failed to show any gene flow of the *Streptomyces hygrosopicus* bar gene under the

control of a *Cochliobolous heterostrophus* glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter in transgenic poplar to *Amanita muscaria* ectomycorrhizas. However other studies have detected horizontal gene flow events to *Aspergillus niger* in soil microcosms after co-cultivation with transgenic plants of *Datura innoxia* expressing the hygromycin B gene under the control of the 35S promoter (Hoffman *et al.*, 1994). This is interestingly one of the few reports of horizontal gene flow to fungal organisms. Taken together, this study finds no empirical evidence of horizontal gene flow from transgenic banana to root endophytic *Fusarium* spp.

## CHAPTER FIVE

### CONCLUSIONS AND RECOMMENDATIONS

#### 5.0 Conclusions

Studies have been carried out to investigate the occurrence of horizontal gene flow from plants to microorganisms. Some of these studies have been performed under optimised conditions in the laboratory while others in the field. In both cases, the studies reported either positive results for bacteria and fungi studied or negative results. For example studies on fungi that detected gene flow to *Aspergillus niger* in soil microcosms after co-cultivation with transgenic plants of *Datura innoxia* (Hoffman *et al.*, 1994; Bertolla and Simonet, 1999) these were followed by other studies that failed to detect gene flow to *Amanita muscaria* of a *Cochliobolous heterostrophus* glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter from transgenic poplar (Zhang *et al.*, 2005). In bacteria, studies with *Agrobacterium tumefaciens* have failed to detect any gene flow events while others have detected natural gene flow events in *Escherichia coli* especially of a glyceraldehyde-3-phosphate dehydrogenase gene from a eukaryotic host (Doolittle *et al.*, 1990). On the whole, most studies have reported the absence of gene flow events to the microorganisms used in the studies (Nielsen *et al.*, 1998). The major reasons advanced for the absence of gene flow have been exogenous plant and environmental conditions as well as endogenous factors including DNA repair mechanisms and lack of competence.

This study sought to investigate the occurrence of horizontal gene flow events to bacteria and fungi associated with banana rhizosphere and phyllosphere under field conditions. However, no evidence for horizontal gene flow were observed. With the

exception of *A. tumefaciens* which is not known to develop competence, the competence status of *E. coli*, *Fusarium oxysporum* and the natural soil bacterial isolates used in the study were not known. However, from the results, it's probable that they were not competent since they failed to uptake the foreign DNA from the bananas. Given that different crops differ in the nature and patterns of root exudation coupled with their attendant influences on the potential to induce competence amongst the microorganisms that are associated with them, it would be imperative that geneflow assessments be done perhaps for a longer time. Assessments in the field would be most preferable given that the interactions in the natural environments may influence the natural transformation of microbial organisms that will be associated with the plants.

## **5.2 Recommendations**

The non occurrence of horizontal geneflow from bananas to the associated rhizo and phyllosphere bacteria and fungi, suggests that with genetically modified bananas, the occurrence of geneflow will be minimal or non-existent. However, given the diversity of bacterial species in the country that are associated with the banana crop whose competence status is not known, studies geared towards establishing the entire complement of these bacterial organisms could be conducted as well as the performance of experiments that will establish whether they have the abilities to develop metabolic states that would enable them to take up exogenous DNA from plants that they may be associated with, is worthwhile.

In conclusion, from a biosafety point of view, the lack of evidence for geneflow in this study suggests that deployment of transgenic bananas may not be a threat to the

microorganisms in the environment. Judicious deployment of such bananas coupled with more sensitive detection tools are worthwhile pursuing.

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