OPTIMAL GROWTH CONDITIONS OF *AGROBACTERIUM RHIZOGENES* FOR USE IN THE TRANSFORMATION OF BANANA (*Musa* spp.)

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A THESIS SUBMITTED TO THE DIRECTORATE OF RESEARCH AND GRADUATE TRAINING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTERS OF SCIENCE IN CROP SCIENCE OF MAKERERE UNIVERSITY

MARCH, 2014

DECLARATION

I, **Jimmy Moses Tindamanyire**, certify that this is my original work and that it has never been presented for examination for any award in any University.

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DEDICATION

I dedicate this thesis to my dear wife (Patricia), son (Jethro), my parents Hon. and Mrs. Gaudioso Tindamanyire, Mrs. Nice Kazooba and to my siblings (David, Sharon, Judith, Lillian, Agnes, Andrew, Maria and Monica).

ACKNOWLEDGEMENT

I am grateful to The Rockefeller Foundation which provided funds for this study through the National Banana Research Program of the National Agricultural Research Organisation (NARO).

My sincere appreciations go to my supervisors, Dr. R. Edema, Department of Crop Science, Makerere University and Dr. A. Kiggundu, National Agricultural Biotechnology Centre at the National Agricultural Research Laboratories, Kawanda. I also wish to thank Dr. W. K. Tushemereirwe, Head of the National Banana Research Program who allowed me to work under his overall oversight. This study was pioneered by Dr. A. Kiggundu in whose footsteps I followed.

The various *Agrobacterium rhizogenes* strains used in this study were kindly donated by Prof. Karl Kunnert`s research group at the Forestry and Agricultural Biotechnology Institute (FABI), Department of Plant Bioscience, of the University of Pretoria.

My appreciation also goes to Dr. Geofrey Arinaitwe whose invaluable contribution and criticisms made it possible to refine this study. I sincerely appreciate the help accorded to me by Alice N. Bukenya, Allan S. Male, Rebecca Nakacwa and Henry S. Mwaka and other colleagues at National Agricultural Biotechnology Centre (NABC), Kawanda.

Finally, I am forever grateful to God, the Almighty, who has successfully seen me though this study. God you are so good and your mercies endure forever.

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ABBREVIATIONS

ANOVA	Analysis of variance
AS	Acetosyrinone
OD	Optical Density
EAHB	East African Highland Banana
NABC	National Agricultural Biotechnology Centre
NARL	National Agricultural Research Laboratories
IAA	Indole acetic acid
6-BAP	6-Benzylaminopurine
DAI	Days after Inoculation
LB media	Luria Bertani media
YM media	Yeast Mannitol media
FABI	The Forest and Agricultural Biotechnology Institute
MUK	Makerere University Kampala
MS media	Murashige and Skoog media
MASL	Metres Above Sea Level
RPM	Revolutions per minute

ABSTRACT

In recent years, rapid procedures for obtaining transgenic roots have been developed using *Agrobacterium rhizogenes*, a soil pathogen which elicits adventitious and genetically transformed roots from a range of tissue types. The resultant plants are "composite" comprising a transgenic hairy root system attached to non-transformed shoot system. Hairy roots are important in the transformation process because of their first growth and high laterally branched nature. *Agrobacterium rhizogenes* strains vary in their virulence for induction of hairy roots in plants. The objective of this study was to 1) to determine the efficacy of *A. rhizogenes* ability to induce the hairy root trait in banana, 2) to determine optimal conditions (OD_{600nm} and inoculation time) required for effective induction of banana hairy roots using *A. rhizogenes*, and 3) to determine response of selected local East African Highland banana to transformation by *A. rhizogenes*.

Out of nine *A. rhizogenes* strains tested, only the agropine-producing strains, namely, LBA9402, 15835, A4 and PMP90 infected and induced varying amounts of hairy roots in banana corms, apical meristems and leaves of genotypes such as *M. balbisiana*, 9518S-12, 5610S-1, Calcutta 4, Kisansa, KM5 and 3165K-5, whereas the remaining 5 strains; LMG150, LMG151, PGR2260, NCPPB4042 and 5083, did not induce hairy roots when inoculated on the explants. An optical density (OD_{600nm}) 0.8 and inoculation time of 30 minutes was found to be optimum to induce hairy roots in the majority of the genotypes and explants that were able to respond to *A. rhizogenes* infection. The most responsive explants were apical meristems, followed by corms and finally the leaves. Beyond an optimal inoculation time of 30 minutes, overgrowth of bacteria was observed on infected sites leading to death and decomposition of explants and no transformation was achieved. At lower optical densities, only traces of hairy roots were produced while at higher levels such as OD_{600nm} 1.0 no hairy roots were produced. The explants (corms, apical meristems and leaves) used were obtained from cultivars that belong to 3 ploidy level groups; diploids (*M. balbisiana*, Calcutta 4, 5610S-1 and 9518S-12), triploids (Kisansa and KM5) and one tetraploid (3165K-5). Diploid *Musa* genotypes responded best with highest percentage hairy root induction frequencies observed in LBA9402 + *M. balbisiana*; A4 + *M. balbisiana*; LBA9402 + 9518S-12; PMP90 + *M. balbisiana*; 15835 + *M. balbisiana*; LBA9402 + 9518S-12; 15835 + *M. balbisiana*; 15835 + 5610S-1 with the highest %HRI as 12.5% (*M. balbisiana*) as compared to 2.08% (3162K-5). PCR analysis was performed on hairy root sets to

confirm presence and integration of *rol*A gene into the explants using Ri specific primers. Bands of similar expected size (300bp) were obtained from strains 15835, A4, PMP90 and LBA9402. This study demonstrated the susceptibility of banana to *A. rhizogenes* mediated transformation and hairy roots induction indicated that *A. rhizogenes* can be used to transform banana.

CHAPTER ONE

BACKGROUND

1.1 Uses of Banana

Banana (*Musa* spp*)* is the fourth most important food crop in the developing world after rice, wheat and maize (FAOstat, 2003; Swennen *et al.,* 2000). Bananas have several uses; ripe bananas are to avert various disorders like peptic ulcers, infant diarrheoa and coeliac disease (Sharrock and Lusty, 2000). Thus, making ripe banana a good source for nutrition of infants. Banana is also a good source of major elements like potassium, magnesium, phosphorous, calcium, iron and vitamins A, B_6 and C (Marriot and Lancaster, 1983). Its chemical composition is similar to the mucus of the stomach lining thus having a soothing effect on gastric ulcers and diarrhoea. Juice from bananas can be drunk fresh or fermented to make alcoholic drink. This beer is nutritionally rich in vitamin B due to the yeast content (Marriot and Lancaster, 1983) and bananas are easy to digest. Other uses include sap that can be used as a dye; as source of starch to make glue, shampoo (Marriot and Lancaster, 1983). Bananas and plantains are also a source of fibre that is extensively used in production of certain papers and art and crafts. Finally, banana leaves are frequently used for thatching (INIBAP, 1997).

1.2 Genetic improvement of banana

Banana can be improved via conventional breeding and biotechnology approaches (Swennen *et al*., 2000). Conventional breeding in banana is done by crossing wild male fertile diploid with female cultivated tripods. The derived F1 hybrids are mostly fertile tetraploids which are back crossed to triploid parent and the resulting F2 triploids are selected for further evaluation and selection. However, conventional breeding programs are slow and tedious; first bananas have a long generation cycle. Secondly, most cultivars are either male or female infertile or both making it difficult to use conventional breeding approaches. Thirdly, genetic improvement of banana is complicated by the complex banana genetic makeup (interspecific polyploidy). Fourthly, there is a lack of useful genetic variability mainly because of vegetative propagation of most widely grown clones (Tezenas *et al*., 1996; Rowe, 1984). To circumvent the later challenge, attempts

have been made to use wild bananas as parents in banana cross breeding programs. However, this presents a challenge due to genetic drag of unfavorable characteristics in the released hybrids and makes them extremely unacceptable to farmers.

Biotechnology approaches offer breeders several important tools which can be used to accelerate production of improved cultivars or genotypes in banana. Genetic engineering techniques can be used to introduce useful genes such as those for pest and disease resistance into popular varieties (Bosque-Perez, 2000). In principle these approaches allow introduction of valuable traits into the genetic backgrounds of farmer preferred and commercially acceptable cultivars. Thus, biotechnology approaches enable the restriction found in conventional breeding of banana to be overcome. Normally, transgenes are inserted into the nuclear genome of a plant cell and these cells regenerated into normal plants. Transgenic plants have been obtained using *Agrobacterium*-mediated DNA-transfer and direct DNA-transfer methods such as particle bombardment, electroporation and polyethylenglycol permeabilisation. One of the major challenges of the biotechnology approach is the identification of genes that will offer the desired phenotype in the transgenic plants.

1.3 Problem statement

Currently, banana transformation uses *A. tumefaciens* (Khanna *et al*., 2004) but requires the use of somatic embryogenic cell suspensions (ECS). However, generation of viable ECS is difficult and time consuming. Use of ECS is affected by cultivar and genotype which is also coupled by low transformation efficiency. Desired cultivars of banana cannot be easily transformed.

New technique employing of *A. rhizogenes* has been proposed and used in some plants (Xiang *et al*., 2008). A new technique that employs another species of Agro-bacterium known as *Agrobacterium rhizogenes* has been proposed (Xiang *et al*., 2008). *Agrobacterium rhizogenes* is a soil pathogen which elicits adventitious, genetically transformed roots (Ri T-DNA) that leads to the production of so-called “composite plants” comprising a transgenic hairy root system attached to non-transformed shoots and leaves. However, not much has been studied in bananas and plantains.

1.4 Rationale of the study

Currently, the preferred method of banana transformation is via *A. tumefaciens* infection (Khanna *et al*., 2004; Tripathi *et al*., 2005). In this procedure DNA transfer to somatic embryogenic cell suspension cultures are often difficult and time consuming (Zhi-Bi and Du, 2006; Xiang *et al*., 2008). Target cell suspensions have low viability leading to low transformation efficiency. Production is also affected by cultivar and genotype. Furthermore, the most desired cultivars of banana cannot be easily transformed.

The *A. rhizogenes*-mediated transformation makes it possible to co-transform plant cells with more than one T-DNA at the same time. The T-DNA containing the transgene of interest in a disarmed binary vector is (in case no use of wild *A. rhizogenes* strains) generally co-transformed with the resident *A. rhizogenes* Ri T-DNA containing the *root locus* (*rol*) genes (responsible for root proliferation).

The resultant transgenic hairy root induced can be used to provide root material for *in vitro* to evaluate effects of genes on biotic constraints such as weevils and nematodes (Li *et al*., 2008; Runo *et al*., 2010). Thus, this technique offers a new strategy for over expressing or suppressing endogenous genes focused on root biology and root microbe interactions. These cultures grow faster than the untransformed roots or shoots, and exhibit a high level of genetic (Aird *et al*., 1988) and biochemical stability (Mano *et al*., 1989). Some plants regenerated from Ri (root-inducing) -transformed roots, however, may display the "hairy-root syndrome" (Tepfer, 1984) caused by the T-DNA (transferred DNA)-borne genes (Schmulling *et al.,* 1988). To reduce effect, modification of the wild strains of *A. tumefaciens* is normally performed to “disarm.” This way, normal functional plants can be produced. Thus, there is potential for this method to become an alternative transformation protocol for recalcitrant species.

The use of *A. rhizogenes* is both rapid and technically simple. Secondly, plants can be regenerated from hairy root cultures either spontaneously (directly from roots) or by transferring roots to hormone-containing medium. Because of spontaneous shoot regeneration, the problematic callus phase and somaclonal variations often encountered in standard *A. tumefaciens* based procedures are avoided. Ri plasmid-based gene transfer also has a higher rate of

transformation and regeneration of transgenic plants; transgenic plants can be obtained without a selection agent thereby avoiding the use of chemicals that inhibit shoot regeneration; high rate of co-transfer of genes on binary vector can occur without selection (Estrada-Navarrete *et al.*, 2006).

However, the major limitation of this approach is that, since shoots are not transformed, sustained maintenance of composite plants is not possible either by vegetative propagation or self-fertilisation. Nevertheless, this limitation can now be circumvented since it has recently been shown that Ri T-DNA transformed *M. truncatula* roots can be successfully regenerated to plantlets (Crane *et al.*, 2006). The "hairy-root" syndrome caused by *A. rhizogenes* infection provides a convenient method for the growth of isolated root cultures (Kovalenko and Maliuta, 2003).

Transformation with a disarmed *A. tumefaciens* strain produces transgenic plants without such abnormal phenotypes; however, with disarmed *A. tumefaciens* species a precise regeneration selection system must be developed for each plant species or genotype. Although efficiency varies widely, *A. rhizogenes* has been used to produce various transgenic plants (Cao *et al*., 2009). Cao and others were able to regenerate shoots from callus tissues derived from the hairy roots, but did not propagate the shoots in soil or report on shoot morphology (Christey and Braun, 2004).

Transformed roots are able to regenerate whole viable plants; both the hairy roots as well as the plants regenerated from them are genetically stable. However, in some instances transgenic plants have shown an altered phenotype compared to controls (Estrada-Navarrete *et al*., 2006). The efficacy of transformation using *A.rhizogenes* depends on strain of the bacterium and cultivars used. Optimal conditions to transform important locally preferred cultivars in Uganda using *A. rhizogenes* are not known.

1.5 Objectives of the study

The main objective of the study was to determine the most suitable procedures to transform a set of banana (*Musa* spp) cultivars in Uganda using *A. rhizogenes*.

Specifically, this study sought to:

1. To screen *A. rhizogenes* strains for their ability to induce the hairy root traits
2. To determine optimal induction conditions (optical density and inoculation time) required for effective transformation of banana cultivars
3. To determine the effect of different banana genotypes on induction of hairy roots after co-cultivation of *A. rhizogenes*

1.6 Hypothesis

A. rhizogenes can successfully infect and transform locally preferred banana cultivars under optimum induction conditions

CHAPTER TWO

LITERATURE REVIEW

2.1 Techniques used in genetic transformation of banana

Although banana has been successfully transformed using *A. tumefaciens*, and particle bombardment, these techniques present some limitations. For example, *Agrobacterium* infection of plant tissues may in some instances result in plant tissue necrosis leading to poor transformation efficiency. In many instances T-DNA integration may remain the limiting step. But more importantly, plant genetic transformation using *Agrobacterium* is an integrated process which relies on many factors some of which are poorly understood. The efficiency of transformation is affected by the following factors; plant species, variety of cultivar, type of explant, bacterial strain, plasmid construct, culture conditions and contaminant (Cleene and Leu, 1981; De Bondt *et al*., 1994; Dong Cao *et al*., 2009). Efforts to improve gene transfer procedure via *Agrobacterium* are a continuous process in several plant species (De Bondt *et al*., 1994) including banana. In fact, several authors and scientists have recently begun to identify plant genes and protein products involved in the transformation process (Estrada-Navarrete *et al.,* 2006; Cao *et al.,* 2009). Manipulation of these genes during the *Agrobacterium*-mediated transformation process may thus be useful to increase both plant cell viability and transformation efficiency in plant species with an apoptotic response to *Agrobacterium* (Gelvin, 2003).

2.2.1 Particle bombardment

Particle bombardment (biolistic transformation) technique involves coating biologically active DNA onto small tungsten or gold particles and accelerating them into plant tissue at high velocity. The particles penetrate the plant cell wall and establish themselves within the cell where the DNA is liberated resulting in transformation of the individual plant cell in an explant. This technique is generally less efficient than Agrobacterium-mediated transformation, but has nevertheless been particularly useful in several plant species, most notably in cereal crops. Using this technique successful transformation of morphogenic tissues such as seeds, embryos or meristems has been made and transformed cells have been regeneration for rice, wheat, soybean and maize, thus demonstrating the enormous potential of this method (Fisk and Dandekar, 1993).

The major drawback of the biolistic method over *Agrobacterium*-mediated gene transfer is its ability to integrate too many copies of the foreign inserts into the plant genome, thereby increasing the risk of transgene rearrangements and gene silencing. The biolistic method is also considered to be comparatively an inexpensive method.

2.2.2 Electroporation and direct DNA entry into protoplasts

This method involves use of electrical discharge that enables the diffusion of macromolecules such as DNA through an otherwise impermeable plasma membrane and because the plant cell wall will not allow the efficient diffusion of many trans-gene constructs, protoplasts (cells without cell walls) must be prepared. This requirement presents a major obstacle for many applications as protocols making possible the regeneration of protoplasts into complete plants do not exist for many species so far hence the desire and need to find an alternative to this in the form of use of *A. rhizogenes*.

2.2.3 *Agrobacterium*-mediated gene transfer

Agrobacterium-mediated technique involves the natural gene transfer system found in certain bacterial plant pathogens of the genus *Agrobacterium*. *Agrobacterium* sp. infects dicotyledonous plants from over 90 different plant families including economically important fruit and nut crops, grapes, ornamental and landscape plants (e.g. rose, euonymous, dahlia, and chrysanthemum). Upon infection of plants, some strains of *Agrobacterium* incite abnormal cell proliferation (hyperplasia) which results in tumor formation in the case of the crown gall disease and excessive adventitious roots in the hairy root disease. Crown gall can also stunt mature plants by causing inferior development of the root system and/or disruption of vascular flow in the stem (Gelvin, 1990).

Pathogenic strains of *Agrobacterium* share a common feature; they contain at least one large plasmid, the tumor- or root-inducing (Ti and Ri, respectively) plasmid. Virulence is determined by different regions of the plasmid including the transferred DNA (T-DNA) and the virulence (*vir*) genes. The virulence genes mediate transfer of T-DNA into infected plant cells, where it integrates into the plant DNA. Rhizogenic (root-inducing) Agrobacteria T-DNA contain *rol* (root locus) genes that render plant cells more sensitive to endogenous auxin. Although Ti and Ri

plasmids vary considerably between strains, they all carry similar *vir* genes (Gelvin, 1990). Virulence in *Agrobacterium* sp is conferred by a large bacterial plasmid designated the root inducing (Ri) or tumor inducing (Ti) plasmid. Portions of plasmid DNA are transferred into plant cells and integrated into their genome (Spano *et al*., 1982), and the transferred DNA (T-DNA) encodes genes which control tumor morphology (Garfinkel *et al*., 1981). Mutations in these genes result in tumors that form shoots called tumour morphology shooty (tms), roots called tumour morphology rooty (tmr), or that grow larger than wild type (tmi). The T-DNA also encodes genes which direct the synthesis of unique amino acid derivatives called opines, such as octopine (ocs), nopaline (nos), agropine (ags), and agrocinopine (acs) (Petit *et al.,* 1983). Another set of plasmid genes, which is essential for virulence (vir), is located outside the T-DNA; that is, it is not found in the tumor genome. In various studies and work performed, it has become apparent that *Agrobacterium* pathogenesis is a unique and highly specialized process involving bacterium-plant inter kingdom gene transfer. Crown gall and hairy root have been described as a form of 'genetic colonization' (Schell *et al*., 1979) in which the transfer and expression of a suite of *Agrobacterium* genes in a plant cell causes uncontrolled cell proliferation and the synthesis of nutritive compounds that can be metabolized specifically by the infecting bacteria. Thus, infection effectively creates a new niche specifically suited to *Agrobacterium* survival (Otten *et al*., 1984). In nature, *A. tumefaciens* and *A. rhizogenes* are the causative agents of the crown gall and the hairy root diseases, respectively. Therefore, *Agrobacterium* can transiently transform a number of these species efficiently, including agronomically important species such as maize, peanut and soybean (Madhumita, 2002).

2.3 Factors affecting *Agrobacterium tumefaciens* based transformation

Despite the great advances of increasing the number of plant species transformed and regenerated using *Agrobacterium*, many important species especially inbred lines or popular varieties remain highly recalcitrant to *Agrobacterium*-mediated transformation (Madhumita, 2002). *Agrobacterium*-mediated transformation offers remarkable advantages over direct gene transfer means by reducing the copy number of the transgene and potentially leading to fewer problems with transgene co-suppression and instability (Hansen and Wright, 1999). Many species, especially economically important monocotyledons such as cereals and *Musa* spp, do

not respond very well to *Agrobacterium*-mediated transformation since this method is both cultivar- and bacterial strain dependent (Bush and Pueppke 1991).

To achieve transformation using *Agrobacterium tumefaciens*-mediated, embryogenic cell suspensions (ECS) are required but unfortunately they have several limitations; (1) it takes a long time period to establish viable and sufficient ECS, (2) establishment of ECS is cultivar dependent and, (3) once *Agrobacterium tumefaciens* is used as wild strains without disarming it, no plant regeneration occurs from the cells that are transformed. Therefore, all tumor-inducing genes need to be removed and replaced by foreign genes prior to gene transfer so that disease symptoms are not induced. This process is called 'disarming' the wild *A. tumefaciens* strain. The process involves inactivation or removal of the native T-DNA genes involved in hormone synthesis renders the *A. tumefaciens* incapable of producing the crown gall disease symptoms. Despite the shortcomings with *A. tumefaciens* use, there is a high efficiency in new and useful genes being introduced into the plant being transformed genetically.

Recently attempts to use other species of *Agrobacterium* other than *A. tumafaciens* have been made and one such species is *A. rhizogenes*. This could potentially solve the problem of preparation of somatic embryogenic cell suspensions and not only improve transformation efficiency in banana but make transformation to the important cultivars currently not transformable yet preferred by farmers.

2.4 The use of *Agrobacterium rhizogenes* in plant transformation

Under certain conditions, certain bacterial strains of *A. rhizogenes* can cause the so-called "rooty tumor" disease resulting into adventitious (hairy) root formation at the site of infection. This causes certain biochemical changes in the plant metabolism. The molecular bases of this disease are strikingly similar to those of crown gall tumorigenesis. The *A. rhizogenes* has root inducing (Ri) plasmid that causes hairy roots at infection sites while its relative *A. tumefaciens* has tumour inducing (Ti) plasmid that causes crown gall tumours. Root induction is due to stable integration of the Ri-TDNA (transferred DNA) into the host plant genome and its subsequent expression. Hairy roots have several properties that have promoted their use for plant biotechnological applications and these include: - their fast growth, genetic and biosynthetic stability, offering an

additional advantage for their uses as an alternative to cell suspension cultures to produce secondary metabolites of interest.

The molecular bases of this disease are strikingly similar to those of crown gall tumorigenesis. Root induction is due to stable integration of the Ri T-DNA (transferred DNA) into the host plant genome and its subsequent expression (Hu and Du, 2006; Tepfer, 1984); unlike crown gall cells, transformed by the T-DNA of the related bacterium *Agrobacterium tumefaciens*, hairy root cells can regenerate whole fertile plants (Chilton *et al*., 1982). When the bacterium infects the plant, the T-DNA between the TR and TL regions of the Ri-plasmid in the bacterium is transferred and integrated into the nuclear genome of the host plant. The transformation process produces a valuable by-product, hairy root, which will form at or near the site of infection. In addition, opines are produced and serve as specific food for the bacteria (Chilton *et al*., 1982).

Auxin synthetic genes recently have been shown to play a rather accessory role in hairy root induction in that they provide the auxin necessary to trigger differentiation of cells transformed by the TL-DNA whenever endogenous auxin levels are not sufficient. The TL-DNA region which is not yet functionally characterized confers the otherwise unresponsive cells the competence to respond to auxin and this is done by the differentiating roots (Cardarelli *et al*., 1987). In other words, TL-DNA-transformed cells appear to be far more sensitive to auxin than their untransformed counterparts.

Hairy roots have several properties that have promoted their use for plant biotechnological applications, including their fast growth, and genetic and biosynthetic stability. These offer an additional advantage for their uses as an alternative to plant cell suspension cultures to produce secondary metabolites of interest. Hairy roots can be obtained in various plant species with the aid of transformation using *A. rhizogenes* (Tepfer, 1984). This has been done on plants such as: - rape seed, *Medicago truncatula*, *Artemisia annua*, *Cichorium intybus*, opium poppy and *Arachis hypogaea* (Madhumita, 2002) and this has therefore, generated an interest to use this approach in bananas (*Musa* spp). “Hairy root” system has been utilized in many applications such as cytological investigations, biochemical production (Sasson, 1991) and (trans)-gene expression.

Plants can also be regenerated from the hairy root cultures spontaneously from roots or by transfer of the roots to hormone-containing media.

When *A. rhizogenes* strains harboring these mutations were inoculated onto the leaves of *Kalanchoe diagremontiana*, a number of phenotypes differing from the wild-type response were noted. The four genetic loci (termed *rol*A, *rol*B, *rol*C, and *rol*D) were defined according to the tumor morphology observed: - *rol*A mutants generated roots that were very straight (as opposed to the curled roots obtained using the wild-type T-DNA); *rol*B mutants were avirulent; tumors incited with *rol*C mutants showed attenuated root growth (although callus growth was normal), and *rol*D mutants showed the initiation, but subsequent retardation, of root growth. Thus, the importance of specific genes in the TL region of agropine-type Ri- plasmids was defined.

A number of different groups of Ri-plasmids have been characterized and the classification of these plasmids has to a large extent depended upon the types of opines (mannopine, agropine, cucumopine) that the T-DNA of these plasmids direct the infected plant to synthesize. Thus, there appears to exist two mechanisms of hairy root tumorigenesis: - one depends upon auxin overproduction directed by the TR T-DNA of certain *A. rhizogenes* strains, but the other is apparently independent of the transfer and expression of genes directing the biosynthesis of auxin. The first intensive genetic examination of *A. rhizogenes* T-DNA gene function was conducted by White *et al*., (1985) and these investigators generated a large number of transposon insertion and small deletion mutations in the agropine-type Ri-plasmid pRiA4 and it was discovered mutations in the TL region were especially interesting.

Transformed roots are able to regenerate whole viable plants; hairy roots as well as the plants regenerated from hairy roots are genetically stable. However, in some instances transgenic plants have shown an altered phenotype compared to controls (Vardja and Vardja, 2004). Plants can be regenerated from hairy root cultures either spontaneously (directly from roots) or by transferring roots to hormone-containing medium. The advantage of Ri plasmid-based gene transfer is that spontaneous shoot regeneration is obtained avoiding the callus phase and somaclonal variations. Ri- plasmid-based gene transfer also has a higher rate of transformation and regeneration of transgenic plants; transgenic plants can be obtained without a selection agent thereby avoiding

the use of chemicals that inhibit shoot regeneration; high rate of co-transfer of genes on binary vector can occur without selection (White *et al*., 1982; Vardja and Vardja, 2004). Further, *Agrobacterium rhizogenes* mediated transformation consistently yields only transformed cells that can be obtained after several cycles of root tip cultures whereas *Agrobacterium tumefaciens* mediated transformation results in high frequency of escapes.

These hairy roots can be maintained as organ cultures for a long time and subsequent shoot regeneration can be obtained without any cytological abnormality. Rapid growth of hairy roots on hormone-free medium and high plantlet regeneration frequency allows clonal propagation of elite plants. In *in vitro* cultures, the hairy root regenerants show rapid growth, increased lateral bud formation, and rapid leaf development; these regenerants are useful for micropropagation of plants that are difficult to multiply. Altered phenotypes are produced from hairy root regenerants and some of these have proven to be useful in plant breeding programs with the morphological traits with ornamental value are abundant adventitious root formation, reduced apical dominance, and altered leaf and flower morphology (White *et al*., 1982).

2.5 Applications of hairy roots in plant biotechnology

Plant roots are used as a source of pharmaceuticals, dietary products, agrochemicals, flavours, fragrances and many others especially chemicals. The limited number of medical plants has been manipulated by genetic engineering and very few pathways of secondary metabolism in them have been understood on molecular level. Hairy roots use in transformation process has significant advantages compared to use of *A. tumefaciens* use and thus having various application uses and purposes.

The “hairy-root” syndrome caused by *A. rhizogenes* infection provides a convenient method for the growth of isolated root cultures (Kovalenko and Maliuta, 2003) with these secondary metabolites normally biosynthesized in roots of differentiated plants (Saito *et al*., 1992). One recent advance in transgenic technology of potential value to pharmacognosy is an application of transgenic organ cultures such as hairy roots and shooty-teratomas to over-production and biotransformation of secondary metabolites. Many characteristics of these transformed tissue cultures are advantageous for plant metabolite production; first, these cultures grow faster than

the untransformed roots or shoots, and in many cases, their growth rates approach those of cell suspension cultures (Flores *et al.,* 1987). Second, the pattern of secondary metabolite production in these cultures is similar to that in the parent plants (Parr and Hamill, 1987). Third, these cultures exhibit a high level of genetic *(*Aird *et al.,* 1988) and biochemical stability (Mano *et al*., 1989). Plant cell culture techniques often provides an alternative means for secondary metabolite production, and the secondary metabolites yields in plant cell cultures have been found to be strongly dependant on the following aspects: these are nutrients components, size of inoculum, elicitors and biotransformation of the specific precursors to biosynthesis of necessary metabolite.

Their fast growth, low doubling time, ease of maintenance, and ability to synthesize a range of chemical compounds, this offers an added advantage as a continuous source for the production of valuable secondary metabolites. To obtain a high-density culture of roots, the culture conditions should be maintained at the optimum level. Hairy root cultures follow a definite growth pattern, however, the metabolite production may not be growth related (Zhi-Bi and Min-Du, 2006). Hairy roots also offer a valuable source of root derived phytochemicals that are useful as pharmaceuticals, cosmetics, and food additives. These roots can also synthesize more than a single metabolite and therefore prove economical for commercial production purposes. Hairy root cultures produce secondary metabolites over successive generations without losing genetic or biosynthetic stability, therefore, this property can be utilized by genetic manipulations to increase biosynthetic capacity (Thimmaraju *et al*., 2005; Zhi-Bi and Du, 2006).

Secondary metabolite biosynthesis in transformed roots is genetically controlled but it is influenced by nutritional and environmental factors. The composition of the culture media affects growth and secondary metabolite production. The sucrose level, exogenous growth hormone, the nature of the nitrogen source and their relative amounts, light, temperature and the presence of chemicals can all affect growth, total biomass yield, and secondary metabolite production. Sucrose is the best source of carbon and is hydrolyzed into glucose and fructose by plant cells during assimilation; its rate of uptake varies in different plant cells. In hairy roots the source of new cells are in the tips so proliferation occurs only at the apical meristem and laterals form behind the elongation zone. Such a defined growth pattern leads to steady accumulation of biomass in root cultures. To obtain a high density root culture, the culture conditions should be

maintained at the optimum level. Hairy root cultures are able to synthesize stable amounts of phytochemicals but the desired compounds are poorly released into the medium and their accumulation in the roots can be limited by feedback inhibition. Media manipulations have been reported to aid in the release of metabolites.

2.6 Critical factors affecting efficacy of transformation using *A. rhizogen*es in plant transformation

To improve transformation rates or efficiency when using *A. rhizogenes*, the following factors were critical in this study. These covered two main areas, that is, manipulation of cultural conditions (bacterium and explants) and on *A. rhizogenes* genetic factors. Bacterial factors include, for example, testing a range of *Agrobacterium* strains and adding acetosyringone to the coculture media to increase *vir* gene expression. Plant factors include selection of a suitable genotype. In dicot plants such *Phaseolus vulgaris* (Estrada-Navarrete *et al*., 2006), soybean (Dang-Cao *et al*., 2009), cotton (Han *et al*., 1997), there are clear effects of species and cultivar on *A. rhizogenes*-mediated transformation rates.

Susceptibility of the chosen genotype to *A. rhizogenes* is an important prerequisite for the production of hairy roots. In addition, the actual explant used must be susceptible to *A. rhizogenes* to enable efficient transformation. In Agrobacterium-mediated transformation, several factors are important in the successful production of transgenics including explant source and age, cocultivation time, delayed introduction of selection, explant preculture (Christey *et al*., 2004). Manipulation of cocultivation conditions can also increase transformation rates. Increased rates of hairy root production have also been obtained by inclusion of 2,4-dichlorophenoxyacetic acid (2,4-D) in the medium used to resuspend the *A. rhizogenes* prior to inoculation. The rate of hairy root production across different plant species can be improved by the use of acetosyringone in the bacterial culture medium), and use of acetosyringone in the co-cultivation medium (Henzi *et al*., 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant materials used

The study was conducted at the National Agricultural Research Laboratories (NARL) at Kawanda. Eight banana genotypes were used, namely, *Musa balbisiana*, *Pisang lilin*, KM5, 9518S-12, Calcutta 4, Kisansa, 5610S-1 and 3162K-5, all found in the Kawanda Banana Germplasm. These genotypes represented both wild and cultivated banana each with varying ploidy levels, i.e., diploids, triploids and tetraploids.

Table 1: Banana cultivar, Ploidy level, Genotype and Trait of interest

Banana Cultivar	Ploidy Level	Genotype	Trait of Interest
Musa balbisiana	Diploid	2X (BB)	High Male and Female fertility
Pisang lilin	Diploid	2X (AA)	Sweet smell for dessert banana
KM5	Triploid	3X (AAA)	Strong root system; Tolerant to nematodes
9518S-12	Diploid	2X	Male and Female fertile
Calcutta 4	Diploid	2X (AA)	Resistant to Black Sigatoka disease
Kisansa	Triploid	3X (AAA-EA)	Male and Female sterile, Good matooke quality preferred by farmers
5610S-1	Diploid	2X	Improved Diploid
3162K-5	Tetraploid	4X	Big "*Matookeness*" bunch; Male and Female fertile

From these cultivars, 'explants' for use in the transformation studies were derived. The plant selected for use as 'explants' included leaves, apical meristematic tissues, and corms from tissue culture banana plantlets.

3.2. *In vitro* culture procedures

Preparation of the explants for *in vitro* culture procedures followed the protocol described by Vuylsteke (1989). Tobacco was included as a control since it is a plant that has been often used as a model crop for transformation studies.

3.3 Bacterial strains used

Nine strains were used in this study and these included: A4, 15835, NCPPB4042, LMG152, LMG150, 5083, LBA9402, PMP90 and PGR2260 (Table 2). Selection criteria of the strains were based on type of opines they produce. In this case, agropine-type strains included A4, 15835, LBA9404 and PMP90 while the mannopine types were PGR2260, 5083, LMG150, LMG152 and NCPPB4042 as earlier cited. These strains were obtained from FABI, Pretoria, South Africa, and stored at -80^{o}C freezer conditions as glycerol enriched stocks. Uniform culture media (LB solid media) was used to culture all the nine strains. Strains from the -80^{o}C freezer were streaked and cultured LB agar media containing plate (a nutritionally rich medium, is primarily used for the growth of bacteria) and incubated for 48 hours at 28^{o}C (Bio Concept FIRLABO incubator). A single colony was taken and grown overnight in 10 ml LB broth (starter culture) with shaking at 200 rpm (Stuart® Orbital incubator S150 incubator). After 24 hrs, the 10 mls of the overnight culture were added to 100 mls of fresh LB broth. The cultures were grown for about 2 hours until when the log phase was reached. Cells were collected by centrifugation at 4000 rpm for 10 min (MIKRO 22R Hettich Zentrifugen centrifuge) and stored in refrigerator for long term storage and later use. A summary is of these details is presented in Table 2.

Table 2. Bacterial strains used in this study

Strains	Source / Type of opines	Reference
LBA 9402	Agropine	Cleene and Leu, 1981
15835	Agropine	Cleene and Leu, 1981
A4	Agropine	Cleene and Leu, 1981
PMP 90	Agropine	Cleene and Leu, 1981
LMG 150	Mannopine	Cleene and Leu, 1981
LMG 151	Mannopine	Cleene and Leu, 1981
PGR 2260	Mannopine	Cleene and Leu, 1981
NCPPB 4042	Mannopine	Cleene and Leu, 1981
5083	Mannopine	Cleene and Leu, 1981

3.4 Preparation of bacterial culture media

Two types of media were used, namely, Luria Bertani and Yeast Mannitol were used. The former was used mainly to raise the bacterial cultures and the latter for screening purpose. The media were prepared as described by Murashige and Skoog (1962).(Appendix a) either in liquid or solid forms. Agar was included as a solidifying agent. Each type of medium was supplemented with various antibiotics as required and poured into petri dishes.

3.4.1 Inoculation of explants with *A. rhizogenes*, hairy root induction, culture and plant regeneration

Standard procedures as described by Khanna *et al* 2004; Kumar *et al* 2006 were used to inoculate selected 'explants' and regenerate the plants. Briefly, the following steps were undertaken:

The wounded explants were dipped into LB broth with acetosyringone (AS) and inoculated with specific *Agrobacterium* strain for 10, 20 and 30 min. thereafter, inoculation was done using two methods (dipping wounded explant into the bacterial culture and using of syringe to inject the culture into the explants). Co-culture period was also done between 10 and 20 days on AS containing hormone-free MS solid media and kept in the dark. The explants were later transferred to AS containing MS solid (hormone-free) media and observations were made over 48, 72, 96, 120, 144, 168, 192 hr. Explants were transferred to basal MS medium (hormone-free) with cefotaxime or timetin and observations done thereafter. The individual roots that emerged

from the wound sites were excised and subcultured onto hormone-free MS liquid medium (Appendix) in conical flasks and shaken in an orbital shaker at 90 rpm at 25 ^{o}C in the dark (aluminium foil was used to create the dark conditions). The roots were then subcultured onto the same medium every 4 weeks. After 4 months in liquid culture, hairy roots from each explant were observed for regenerants off them. The dark conditions were created by use of aluminium foil wrapped over and around the flasks containing the hairy roots. The roots were placed in hormone-free MS liquid media in which timetin (200 mg/ml) was added.

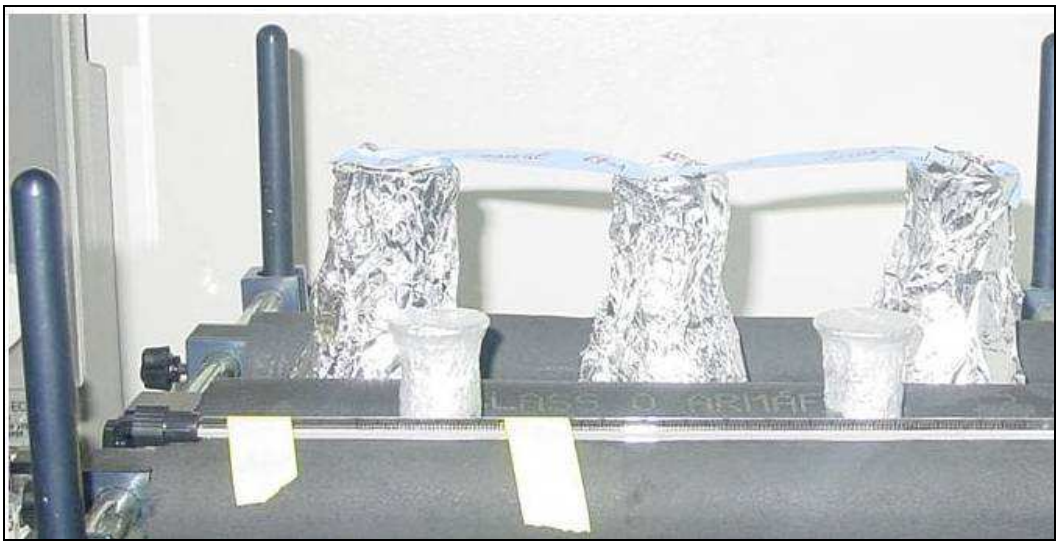

Figure 1: Conical 250 ml flasks containing hairy roots cultured in liquid MS medium in the dark on rotary shaker

To determine the ability to induce the hairy root trait in banana, plant explants were inoculated with the nine *A. rhizogenes* strains previously described above. At inoculation, acetosyringone, a phenolic compound was added to the liquid culture to enhance the interaction between the Agrobacterium cells and wounded site explant cells. Induction and growth of hairy roots was observed and recorded. The emerging hairy roots went through successive transfers on cefotaxime containing hormone free MS media (Murashige and Skoog) at intervals of 15 days. The hairy roots were washed with cefotaxime to remove excess bacterial growth.

This experiment was arranged as RBCD with three replicates per *A. rhizogenes* strain-explant treatment. Each replicate had the Agrobacterium strain with an explant (apical mersitem, leaf and

corm), all levels of OD_{600nm} (0.4, 0.6, 0.8 and 1.0), the varying infection times (10, 20 and 30 minutes) to give sufficient statistical inference. The number of explants (corms, apical meristems and leaves) was 20 for each treatment per experiment and 5 explants per cultivar/genotype were cultured as controls with no *A. rhizogenes* infections.

To determine the optimal concentration and inoculation time to enable efficient transformation of banana, four strains, namely, A4, LBA9402, PMP90 and 15835 were selected and used to infect the following *Musa* cultivars, *Musa balbisiana*, *Pisang lilin*, KM5, 9518S-12, Calcutta 4, Kisansa, 5610S-1 and 3162K-5. Experimental set was as previously described. Briefly, bacterial culture was raised in falcon tubes, each containing 10mls to a growth log phase and an OD_{600nm} 1.0. Thereafter these were diluted using LB broth media to attain required concentration (OD). The final working volume (V_F) of LB broth was determined using the formulae below:
Formula, $\mathbf{V_T} = [(OD_1)\ X\ (V_O)] / (OD_w)$ and $\mathbf{V_F} = [(V_T) - (V_O)]$

Bacterial concentration was measured using OD measurements of 0.4, 0.6, 0.8 and 1.0 was with the spectrophotometer (Bio Mate ™3 Series Thermo Electron Corporation) set at 600nm, while inoculations were done at three different times, that is, 10, 20 and 30 minutes after Agrobacterium infection of the wound explants.

Inoculation was done using dipping wounded explants into culture or use of injection by syringe was used to bring bacterial inoculum into contact with the explants. The explants (apical meristems, corms and leaves) were co-cultured with *A. rhizogenes* diluted with MS liquid medium (Murashige and Skoog 1962). Hairy roots obtained were cultured in hormone-free MS liquid media under dark conditions and these hairy root cultures were placed on a rotary shaker at 90rpm.

To confirm the absence of bacteria in root cultures, root pieces were cultured on solid yeast-mannitol (YM) medium (with 10 g/l agar at 25 °C under dark conditions). After purification from bacteria the root cultures were maintained and propagated for several subcultures in liquid hormone-free MS medium. To induce shoot regeneration, segments of each root clone with a length of about 0.5-1.0 cm were cultured on liquid MS liquid media with no hormones included.

This experiment was repeated four times with 10 hairy root explants per treatment. The shoots regenerated from hairy roots were micro propagated and maintained on standard MS medium without any hormone.

3.4.2 Elimination of *A. rhizogenes* from hairy roots

The cultures were incubated in the dark for 24 hrs and later transferred to fresh hormone-free MS medium containing cefotaxime 200 mg/ml and persisting *A. rhizogenes* contamination was eliminated by frequent subcultures on antibiotic containing media. The hairy roots were maintained in 50 ml hormone free MS liquid media containing flasks and kept in dark conditions. We observed growth of the hairy roots in liquid MS medium devoid of growth regulators and antibiotics hairy roots were transferred to fresh media after 40 and 80 days respectively.

3.5 Data collection and analysis

Frequency of hairy root formation for each treatment was scored from 14 days onwards after co-cultivation. In this case, the number of emerging hairy roots per explant was counted and recorded. In this case, successful infection of some species was achieved by the addition of acetosyringone. Once good root growth was noted from the explants, the roots were excised from the explants using sterile blades and transferred to individual baby jars and clearly labeled with a specific number. Once established, these hairy roots were subcultured by cutting a 1.5 cm square of culture and transferred to fresh medium every 2 weeks. Some root tissue was cut and collected into sterile 1.5mL eppendorf tubes for DNA extraction for further PCR analyses for *rol*A gene presence. This process was followed for every strain and cultivar that produced hairy roots. Data collection involved counting the number of hairy roots obtained by each Agrobacterium strain on a given *Musa* cultivar.

3.5.1 Statistical analysis of results

All statistical analyses were performed using Analysis of Variance (ANOVA) (SAS Institute, Cary, NC, USA, © 1999-2001). The results were expressed in percentage Hairy Root Induction frequency (%HRI). $$\textbf{\%HRI} = \frac{\text{Number of explants with hairy roots X 100}}{\text{Total number of explants infected with } \textit{A. rhizogenes}}$$

3.6 Molecular analysis and molecular confirmation of putative transgenics

3.6.1 Transformation detections

To detect the success of genetic transformation in plant cells, polymerase chain reaction (PCR) was used. This confirmed the presence of *rol*A, or *rol*B or *rol*C gene in roots. Standard extraction procedures were followed to isolate and purify DNA from hairy roots prior to molecular analysis (Gawel and Jarret, 1991). This was a Modified CTAB DNA Extraction Procedure for *Musa* spp adopted from Gawel and Jarret, 1991. Briefly, contaminant bacteria was removed from the hairy roots by washing in NaOH, 200 mM followed by SDS (1% w/v) for 10 minutes and then rinsed in sterile de-ionised water. The roots were dried on sterile filter paper, quickly frozen in liquid nitrogen and ground to a fine powder in a mortar containing liquid nitrogen The bacterial plasmid DNA was extracted and purified using the QIAprep Spin Miniprep Kit and a Microcentrifuge, (QIAprep Miniprep Handbook, 2nd Edition, June 2005), QIAGEN®. Plasmid DNA from *A. rhizogenes* strain 15835 was used as a positive control.

Isolated DNA was analysed by PCR for the presence of the *rol*A gene in the Ri T-DNA plasmin integrated and incorporated into the plant genome. The oligonucleotide primers used to amplify *rolA* gene on the T-DNA of *A. rhizogenes* were obtained from Inqaba Biotechnical Industries (Pty) Ltd, South Africa. A band in the region of 300bp *rol*A gene fragment was amplified by using the following primer sets. The primers designed to amplify *rol*A were forward primer 5`-AGAATGGAATTAGCCGGACTA-3` and the reverse primer 5`-GTATTAATCCCGTAGGTTTGTTT-3`. The PCR mixture (25µl) contained 50ng of DNA prepared from normal and the putative transformed hairy roots respectively as the template, 5X PCR buffer (Promega, USA), 10 pmoles of each primer (Inqaba biotec, South Africa), 2.5mM of dNTPs (MBI Fermentas, USA), $MgCl_2$ (MBI Fermentas, USA) and 1 unit of Taq DNA polymerase (MBI Fermentas). The PCR for *rol*A was carried out by amplification under the following conditions: Initial denaturation at 94°C for 2 min, denaturation at 94°C for 20 sec, annealing at 50°C for 40 sec and extension at 72°C for 1 min for 40 cycles, and with a final extension at 72°C for 10 min using the thermal cycler PCR machine (MWG Biotech, Germany). The amplicons were analysed by electrophoresis on 1.2% Agarose gel along with 100bp molecular markers (Figure 4). The PCR products obtained were ran on 1.2% agarose gel, stained

with ethedium bromide, observed and documented using a transilluminator equipped with a gel documentation system (Herolab GMBH, Germany).

CHAPTER FOUR

RESULTS

4.1 Efficacy of infection and induction of hairy roots

Nine wild-type strains of *A. rhizogenes* were tested; 15835, A4, NCPPB4042, LMG150, LMG152, LBA9402, 5083, C58C1 (RiA4-PMP90) and C58C1 (RiA4-PGR2260) for ability to induce the hairy root trait in banana (*Musa* spp). Only 4 strains were capable of inducing transgenic roots on 10% of the explants. These were A4, 15835, LBA9402 and C58C1 (RiA4-PMP90) (Table 3). The other five strains caused no visible changes except profuse bacterial growth on both explants and media that were inoculated. The best root formation response (%Hairy Root Induction frequency) was achieved in the order of: LBA9402, 15835, A4 and PMP90 with the following % HRI, 18.75, 10.0, 7.5 and 3.75% respectively at 6 weeks of co-cultivation (Table 3).

Table 3: Performance of different strains of *Agrobacterium rhizogenes* in inducing of hairy roots in popular banana cultivars at OD_{600nm}=0.8

Bacterial Strain	Days to induction of hairy roots	Frequency of root induction (%)	Number of hairy roots six weeks of culture
A4	25	7.5	18
15835	47	10.0	24
LBA9402	17	18.75	45
C58C1(RiA4-PMP90)	19	3.75	9
C58C1(RiA4-PGR2260)	-	-	-
LMG150	-	-	-
LMG152	-	-	-
NCPPB4042	-	-	-
5083	-	-	-

A minimum of 20 explants (corms, apical meristems and leaves) was used for the experiment.

The top four *A. rhizogenes* strains, namely, A4, 15835, LBA9402 and C58C1 (RiA4-PMP90) were selected for subsequent optimizations of procedures (optical density and inoculation time for transformation of banana). Hairy roots developed on the explants and did not necessarily follow the laws of geotropism but grew horizontally and also upwards, that is, against geotropism (Figure.2). This appears to be a distinct characteristic that separates them from the other roots not induced by *A. rhizogenes*. The infection sites that served as source of infection and thus from which hairy roots were induced as shown in Figure 2. The points of infection turned black or dark and this was probably due to the phenolic compounds that were produced by the explant as they formed a defense mechanism against the bacterial infection.

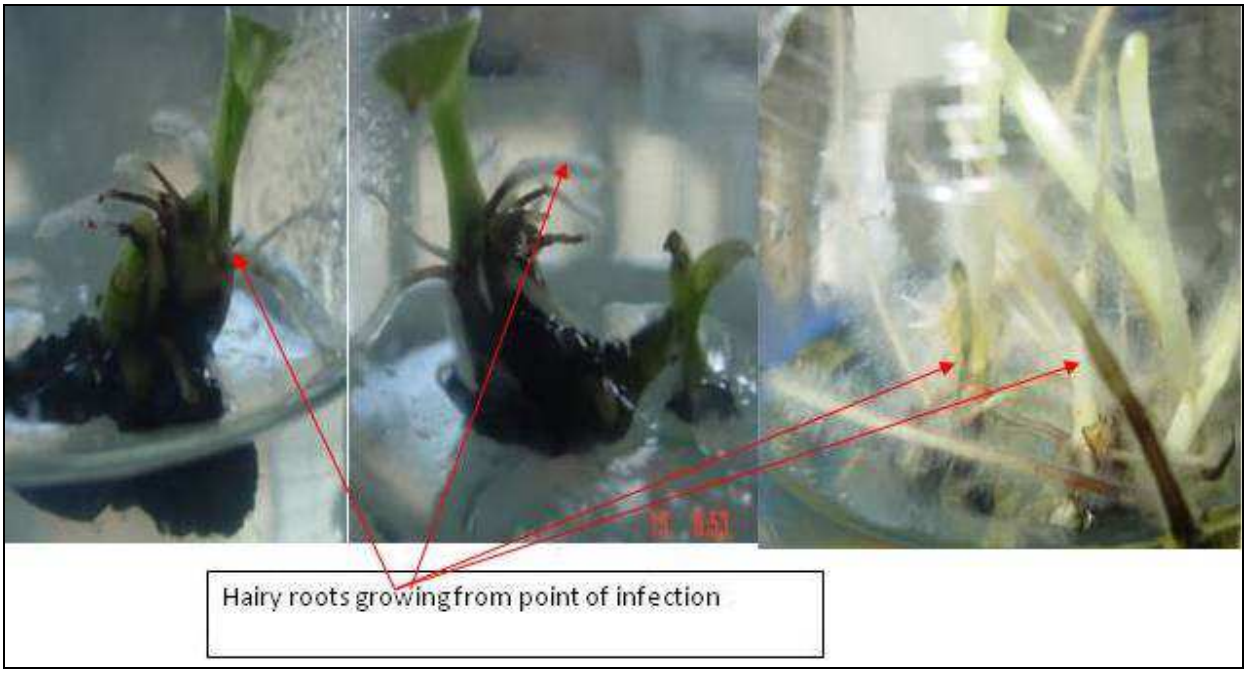

Figure 2: Hairy roots development on explants (corm) cut surface of banana genotype *M. balbisiana* at 42 DAI, on hormone-free MS media.

To improve the efficacy to induce root hairs, a range of bacterial concentrations (OD_{600nm}=0.4, 0.6, 0.8 and 1.0) and three different inoculation times (10, 20 and 30 minutes) were tested. The results showed that the best hairy root induction (%HRI) occurred at concentration of 0.8, OD_{600nm} and when inoculated for 30 minutes. No hairy roots were induced at an OD of 0.4 regardless of the inoculation time. Explants inoculated with bacterial culture of OD600nm 0.4

did not produce hairy roots at all levels (10, 20 and 30 minutes) of infection, even after 60 days of co-cultivation. With OD_{600nm} 0.6, there was generally no hairy roots induction observed except only with 30minutes of inoculation. This suggested limited cell population at infection site to initiate and sustain infection and induce hairy roots. Similar results were also observed at concentrations below 0.4. While at 0.8, hairy root induction was observed regardless of the inoculation time used (Table 4). In fact, higher % HRI was achieved as inoculation time increased. For example, at inoculation times of 10, 20 and 30 minutes % HRI achieved was 3.75, 8.33 and 25.0% respectively (Table 7). However, at the inoculation time of 1.0, a decreased induction of hairy roots was observed which was slightly lower than that recorded at 0.8 (Table 4).

No varietal differences were observe in the genoptypes evaluated (Pr.<.001) (Table 5). Thus co-cultivation of plant tissue and bacterial strain for over 10 days has been found optimum. This appears to allow enough interaction of bacterial cells with the explant cells for infection to effectively happen before change of media. A bacterial concentration of 0.8 was also shown to induce an average of HRI of 12.36% at the optimum inoculation time of 30 minutes. These, therefore, were the most efficient conditions to attain hairy root induction (Table 4) in the *Musa* spp across the majority of the cultivars/genotypes and explants used.

Table 4. Effect of optical density and inoculation time on induction of hairy roots in various *Musa* spp genotypes on hormone-free MS media

Concentration of *A. rhizogenes* (OD_{600nm})	Inoculation Time (mins)	Frequency of root induction (%)	Number of hairy roots 6 weeks of culture
0.4	10	0	0
	20	0	0
	30	0	0
0.6	10	0	0
	20	0	0
	30	2.5	6
0.8	10	3.75	9
	20	8.33	20
	30	25.0	60
1.0	10	0	0
	20	0	0
	30	0.42	1

A minimum of 20 explants (corms, apical meristems and leaves) was used for the experiment.

In general, the hairy roots started to form on the cut surfaces of explants of the various genotypes earliest at 10-47 days after inoculation with *A. rhizogenes*. Hairy root formation (% Hairy Root Induction frequency) on inoculated explants was between 3.75 % and 18.75 % (Table 3). For all the strains used, diploids, triploids and tetraploids were used as sources of the explants (corms, apical meristems and leaves) for the experiments performed and of the explants, some explants, both non-inoculated and inoculated, did not produce any roots. Some kanamycin and rifampicin-resistant root clones were highly branched and grew rapidly in hormone-free liquid medium; characteristic of hairy roots.

The results showed (Table 5), that there was a significant difference ($P<0.05$) amongst the selected *A. rhizogenes* strains. The results also indicated a significant difference amongst the *Musa* cultivars. With strain 15835, outgrowths of hairy roots on explants were observed to emerge from the sites of infection 47 DAI which indicated longest time to induce hairy roots production. There was no prolific bacterial growth on the media instead infection resulted into hairy roots induction and the % HRI was 10.0%In strain LBA9402, inoculation was done by dipping the wounded explants into the culture for 30 minutes and this strain produced mass of cells first from which the hairy roots emerged and most hairy roots produced, thus had the

highest % HRI (Table 3) hence transformation frequency. Strain LBA9402 produced hairy roots in the shortest time (17 DAI) and it had the highest transformation efficiency (18.75%). While strain PMP90, the explants were inoculated with PMP90 on the apical meristems but subsequent leaves (along the shoot) had hairy roots developing on the infection site of the leaf and inoculation done by syringe. This confirmed that hairy roots only develop(ed) at the specific sites of infection. However this strain showed the least transformation frequency (Table 3). It was noted that once the hairy roots got into contact with bacterial mass, they would die since the bacterial growth overwhelmed the hairy roots. Finally, with strain A4, inoculation was done by dipping the wounded explants into the culture for 30 minutes. In this case, *M.balbisiana* (a diploid) produced hairy roots 25 DAI but performs better than PMP90 (Table 3).

Table 5: Analysis of variance of DAI

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
+ Musa_Cultivars	5	1582.4011	316.4802	1513.95	<.001
+ *A_rhizogenes*_Strain	3	3598.4984	1199.4995	5738.04	<.001
Residual	14	2.9266	0.2090		
Total	22	5183.8261	235.6285		

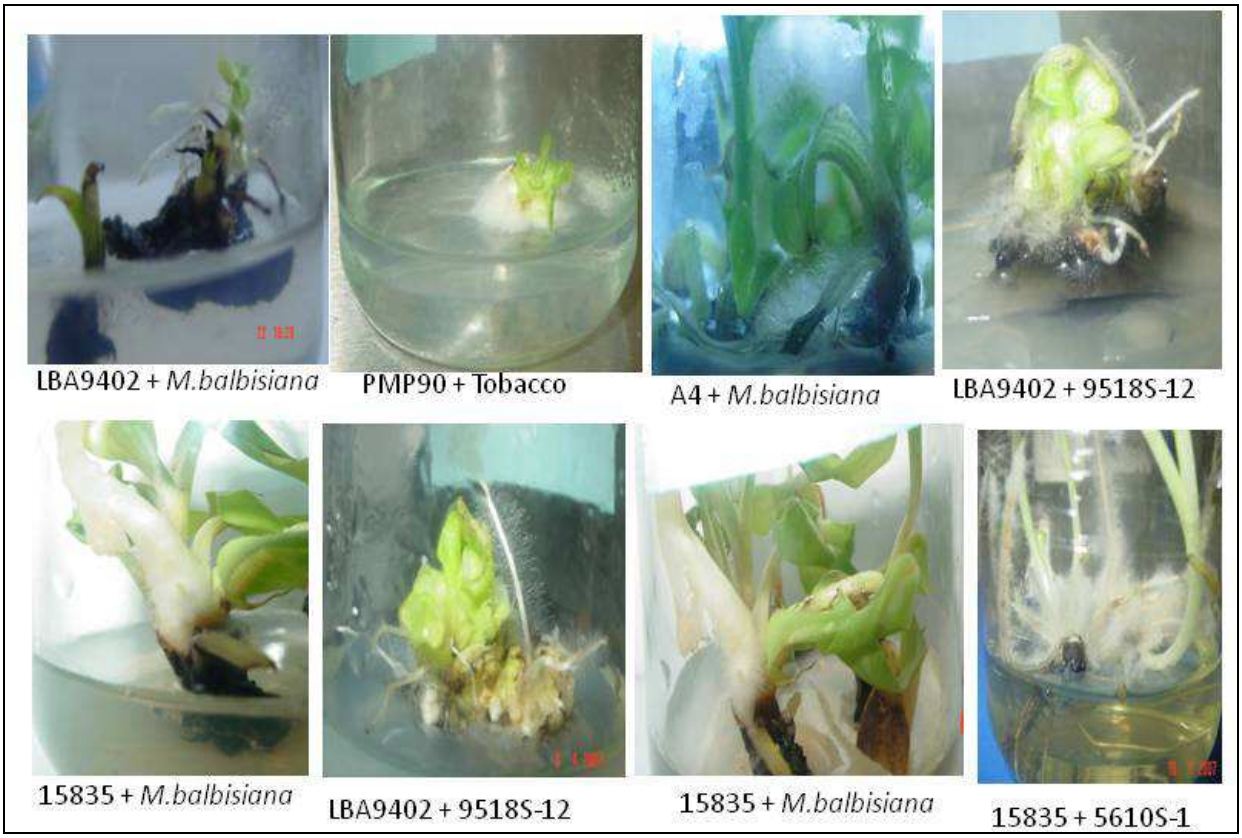

Figure 3: Hairy roots induced by selected strains LBA9402, PMP90, A4 and 15835 of *Agrobacterium rhizogenes* on apical meristems of specific *Musa* spp i.e. *M. Balbisiana*, 9518S-12, and 5610S-1 and Tobacco after 42DAI on hormone-free MS media

These roots differed from normal roots in the following ways: - (1) Roots were initiated in the presence of little or no auxin in the culture medium, (2) Hairy roots arose on the cut surface from cells that were transformed with *A. rhizogenes*, and (3) Hairy roots developed a large number of root hairs and lateral branches (Figures 2 and 3).

The results showed that all the five selected strains infected the explants and induced hairy roots at the various sites of infection on all the diploid explants which was not the same in the triploids and tetraploids. Explants from diploids produced % HRI that ranged between 2.92-12.5%; that is, 2.92, 4.17, 7.08, 7.5 and 12.5% for *Pisang lilin*, 5610S-1, Calcutta 4, 9518S-12 and *Musa balbisiana* genotypes respectively; while triploids produced roots in Kisansa with 3.75% and none in KM5 and tetraploids came in last, 3162K-5 with 2.08% (Table 6). The high % HRI

obtained in *Musa balbisiana* is probably attributed to the high levels of polyphenols produced by the injured sites which are used as infection sites too and since the acetosyringone which is added to the media for inoculation and co-cultivation is a phenol and increases infection efficiency of a given strain, then the phenols in the diploids help increase the infection rates too.

Table 6: Effect of different banana genotypes on induction of hairy roots after co-cultivation of *A. rhizogenes*

Banana Genotype	Ploidy Level	Frequency of root induction (%)	Number of hairy roots 6 weeks of culture
Musa balbisiana	2X (BB)	12.50	30
Pisang lilin	2X (AA)	2.92	7
KM5	3X (AAA)	-	-
9518S-12	2X	7.50	18
Calcutta 4	2X (AA)	7.08	17
Kisansa	3X (AAA-EA)	3.75	9
5610S-1	2X	4.17	10
3162K-5	4X	2.08	5

A minimum of 20 explants (apical meristems) was used for the experiment

In this study, leaves, apical meristems and corm segments, were used for transformation but they performed in this order; apical meristems, corms and leaves respectively (Table 7). It was discovered that all the explants were infected by the selected strains. The % HRI ranged from 0.83% for leaves through corm (8.33%) to 30.83% for apical meristems which produced the highest % HRI. Despite the results showing that leaves had the least %HRI while the apical mersitems produced the highest %HRI after 6 weeks of culture of the explants on the hormone-free MS media (Table 7), there was no significant difference ($P<0.05$) amongst the *Musa* cultivars when apical mersitems were used as explants.

Table 7: Effect of different explants on induction of hairy roots in different banana cultivars

Banana Explants	%Frequency of HRI	Number of hairy roots after 6 weeks of culture
Corms	8.33	20
Leaves	0.83	2
Apical meristems	30.83	74

A minimum of 20 explants was used for the transformation experiment

There was also no significant difference ($P<0.05$) amongst the *A. rhizogenes* strains when apical mersitems were used (Table 8). This therefore, means that between the various *Musa* cultivars and *A. rhizogenes* strains there was no significance difference when apical meristems were used as explants to induce hairy roots. The results showed that leaf explants had the least response and further analysis indicated there was no significant difference amongst the *Musa* cultivars and *A. rhizogenes* strains (Table 8). The results also showed that corms ranked second to apical meristems in % HRI, however, further analysis showed that there was no significant difference amongst *Musa* cultivars ($P<0.05$) in addition to no significant difference amongst *A. rhizogenes* strains ($P<0.05$) when corms where used as explants (Table 8).

Table 8: Pooled Analysis of variance of Apical_meristem, leaf and corm explants

	Explants			
Source of variation	DF	Apical meristem	Leaf	Corm
		Mean square		
Musa cultivars	5	2843.7	0.5520	1.343
A. rhizogenes strains	3	1905.2	1.6297	3.443
Residual	14	941.7	0.6276	1.441
		Variance component		
Musa cultivars	5	3.02	0.88	0.93
A. rhizogenes strains	3	2.02	2.60	2.39

4.2 Effect of optical density and inoculation time on induction of hairy roots

Optimal optical density (OD_{600nm}) 0.8 and inoculation time of 30 minutes produced the highest %HRI. %HRI ranged from 3.75 to 18.75% and hairy roots formed at infection sites at 10-47 DAI.

4.3 Effect of different banana genotypes on induction of hairy roots after co-cultivation of *A. rhizogenes*

Hairy roots induced in all diploids which was not the same in triploids and tetraploids. Diploids produced %HRI (2.92, 4.17, 7.08, 7.5 and 12.5%) for *Pisang lilin,* 5610S-1, Calcutta 4, 9518S-12 and *M.balbisiana* respectively. Triploids (Kisansa with 3.75% and none in KM5) and tetraploids came in last, 3162K-5 at 2.08%.

4.4 Characterization of transformed tissues

PCR analysis was performed on five lines of roots transformed with *A. rhizogenes* LBA9402, A4, PMP90 and 15835 resulted into amplification of the 300bp band size, which matches a fragment size of the Ri-plasmid (Figures 4) and these tests confirmed the transgenic nature of the hairy roots. The results showed that some hairy roots were transgenic with Ri-genes (Figure 4).

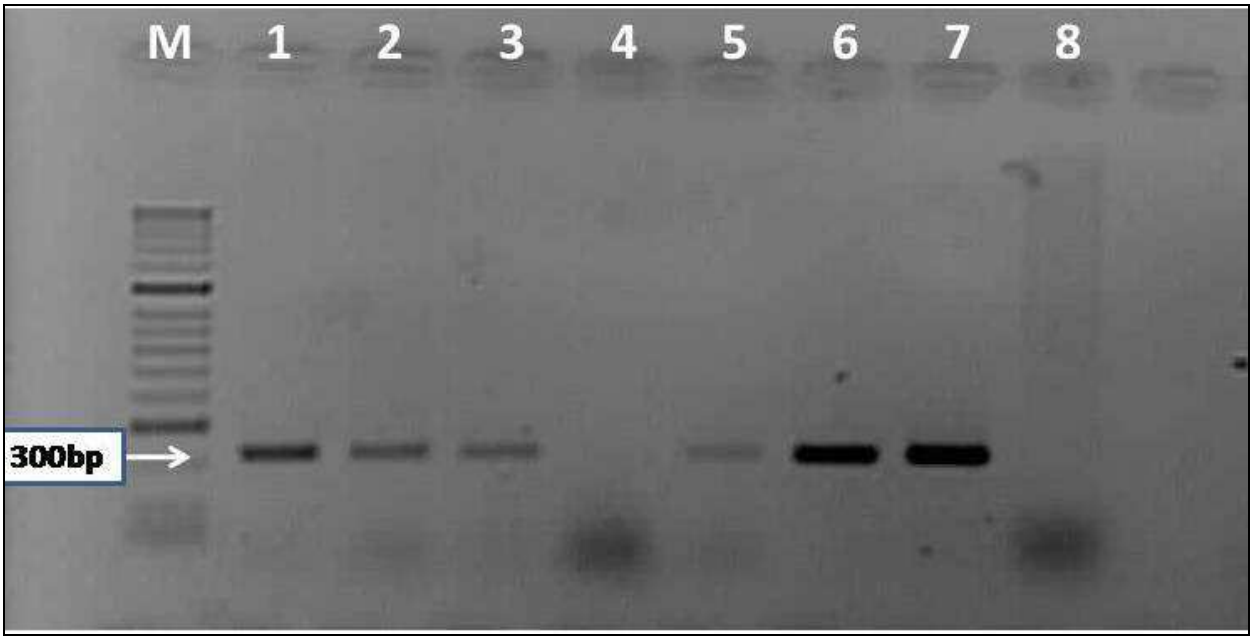

Figure 4. PCR analysis for confirmation of presence of Ri-genes in DNA extracted from explants from *A. rhizogenes*-transformed banana (*Musa* spp). M=HyperLadderI; **1**= Strain 15835 + 5610S-1; **2**= Strain A4 + 5610S-1; **3**= Strain 15835 + 9518S-12; **4**= Untransformed root DNA (*M. balbisiana*); **5**= Strain LBA9402 + Calcutta 4; **6**= Positive Control (A4); **7**= Positive Control (15835); **8**=Water control.

CHAPTER FIVE

DISCUSSION

We have established a convenient transformation protocol that allows the production of transformed *Musa* spp hairy roots at the explants-*A. rhizogenes* infection/interaction sites. This report shows this study to be the first one where *Musa* cultivars and genotypes are transformed by four *A. rhizogenes* and this transformation was confirmed by PCR amplification of the *rol*A gene in the hairy root DNA indicating that the Ri-TDNA was incorporated into the plant genome. The method described in this work is rapid and not laborious as compared to *A. tumefaciens*-mediated method. It resulted in the production of hairy roots in one month, making it directly usable for rapid validation and functional study of gene expression in the roots. In comparison, using an *A. tumefaciens* mediated transformation with selective pressure usually takes up to 6 months to produce similar well-developed transformed plantlets starting from primary explants, that is, embryogenic cell suspensions which also take close to 6-9 months to come up with viable cell suspensions. *Musa* explants (apical meristems, leaves and corms) were infected in culture to induce hairy roots which have plagiotropic characteristics when compared to the non-transformed roots.

By combining the optimal conditions determined above, we established a rapid and efficient *A. rhizogenes*-mediated root transformation system of *Musa* spp as highlighted in Figures 2 and 3. The protocol can be best illustrated as follows: apical meristems are inoculated at the cut surfaces with appropriate *A. rhizogenes* strain (A4, 15835, LBA9402 and C58C1 (RiA4-PMP90) (Fig.3 and Table 3) suspension (OD_{600nm}= 0.8) and the inoculated explants are kept in 12 hour light/12 hour dark at room temperature in growth chambers for 14 days until hairy roots appear. The explants are transferred onto new and fresh hormone-free MS media and kept at room temperature for more hairy root induction and growth. At day 17 after inoculation, the number of hairy roots per plant was counted and the highest number was scored by strain LBA9402 and this was correlated by its highest transformation efficiency, 18.75% (Table 3). For preliminary screening of hairy roots, we did PCR analysis of *rol*A gene from the hairy root tissues from each of the samples from each of the four strain induced hairy roots. The results showed that hairy

roots from all PCR-positive plants carried the *rol*A gene (Figures 4 and 5). In summary, previous studies were conducted to investigate the conditions affecting *A. rhizogenes*-mediated transformation in some plant species, but no assessments have been done in *Musa* spp. Here, we assessed the conditions affecting the hairy root transformation process based on the previously published work (Runo *et al*., 2010; Zhi-Bi and Du, 2006; Tzfira *et a*l., 1996).

Three diploid *Musa* spp cultivars/ genotypes, *Musa balbisiana*, 9518S-12 and Calcutta 4, were found to be superior for genetic transformation mediated by *A. rhizogenes* strains A4, 15835, LBA9402 and C58C1 (RiA4-PMP90. Other factors that influence the hairy root induction and the transformation efficiency were also studied. The protocol described in this study is rapid and simple. Since this study demonstrated that diploids had the best response to infection of *A. rhizogenes* with the highest transformation frequency and hence most hairy roots obtained. Among the diploids, both *Musa balbisiana* and Calcutta 4 produced hairy roots but *M. balbisiana* had higher transformation frequency than Calcutta 4. Thus, from the results, it was noted that the diploids, which are wild type *Musa* cultivars produced the more hairy roots and this is caused by the presence of high polyphenolic levels that act just like acetosyringone which increases or enhances interaction between Agrobacterium cells and plant cells as a resulting increasing Agrobacterium-plant host interaction leading to higher transformation efficiency and/ rates.

The gap to reaching this objective has been the lack of reproducible and speedy transformation protocols reflecting the recalcitrance of *Musa* spp to *in vitro* regeneration. *A. tumefaciens*-assisted transformation is a standard protocol successfully applied to generate transgenic plants of some *Musa* spp, although the frequency of transformation for *Musa* spp varies across cultivars (Bosque Perez *et al*., 2000). This protocol (Centrifugation assisted Agrobacterium Transformation, modified by Khanna Haarjet). Although this novel and cheap transformation method looks quite promising, the transformation efficiency (2 to 12%) must be improved. A fast, reproducible, and efficient transformation procedure is crucial not only for gene function studies but also to allow crop improvement. In our hairy root induction protocol with the four *A. rhizogenes* strains, transformation frequencies up to 12.5% can be obtained for some *Musa* cultivars (Table 6). Significant differences were observed between the transformation ability of

different strains of *A. rhizogenes*. We tested four strains of *A. rhizogenes* to induce hairy roots on eight cultivars/genotypes of *Musa*, and the strain LBA9402 was the most effective in inducing hairy roots. Hairy root formation also depended on the host plant genotype ((Figure 3 and Table 6) and as earlier stated, *rol*A PCR amplification of DNA isolated from hairy roots was used to verify the presence of the Ri T-DNA.

It is important to ensure that explants are kept well apart to enable distinguishing independent root cultures. Hairy root cultures can be very fast growing and can quickly grow over each other and that is why baby jars are preferred since their height provides more space for the plagiotropic roots to growth into. All culture manipulations are conducted at room temperature, a 12 hour/day photoperiod, provided by white fluorescent lights. However, hairy roots also grow well in the dark which was made possible by sealing the baby jars and flasks with aluminum foil.

Transformed *Musa* hairy roots showed active production of lateral roots with vigorous elongation. In contrast, non transformed roots did not produce as many lateral roots. These alterations arise from the integration and expression in the plant cell of oncogenes such as the *rol* genes, *aux* genes involved in auxin synthesis or genes synthesizing opines, borne by the T-DNA of the Ri plasmid. For applications in root-pathogen interactions, it would be important to investigate whether there are significant alterations on root morphology and if these affect such interactions. The hairy root transformation protocol described here provides a key tool that was previously missing.

In principal then, *Musa* can be transformed with a binary vector harboring any gene of interest and this can be used as a rapid method to screen for phenotypes that are expressed in the roots. A good example where such a protocol can be applied would be analysis of candidate genes for plant parasitic nematodes resistance with genes such as Cry5B and Cry6A. Since nematodes attack their host roots, studies on host-parasite interactions can be efficiently addressed using the current protocol. By exploiting the hairy roots inducing properties of *A. rhizogenes*, it is possible to transform numerous roots and obtain gain of function mutants for genes that are expressed in the roots through activation tagging. Every hairy root is an individual event and can be infinitely replicated clonally. This makes screening for a large number of genes very feasible.

The results presented in this study indicate that the use of *A. rhizogenes* may be a successful approach to development of alternative system for transforming banana using *A. rhizogenes* although there were different responses to infection on different genotypes by different A. *rhizogenes* strains. In this study, *A. rhizogenes* strains LBA9402, PMP90, A4 and 15835 were able to induce hairy root production. The results showed that diploids were highly responsive in production of hairy roots. A combination of OD_{600nm} 0.8 and inoculation time of 30 minutes while the most responsive explants were apical meristems as the optimum factors for transformation of majority of the genotypes. The stable transfer of transgenes was shown by polymerase chain reaction (PCR) amplifications. The bacterial density or the number of cells/ml of the bacterial culture plays a significant role in transformation and thus hairy root induction.

The results showed % HRI ranging from 3.75-18.75% from the least to highest in the order of C58C1(RiA4-PGR2260), A4, 15835 and LBA9402 with 3.75, 7.5, 10.0 and 18.75% respectively (Table 3). This is due to the fact that their T-DNA provides high mobility which is largely attributed to their border sequences thus increasing efficiency and virulence of Ri-plasmid to infect and induce of hairy roots at the sites of infection. The functionally uncharacterized TL-DNA that confers to otherwise unresponsive cells the competence to respond to auxin by differentiating roots, that is, TL-DNA-transformed cells appear to be far more sensitive to auxin than their untransformed counterparts.

The results also showed that the number of days to hairy root induction (DAI) also differed among the strains; the fastest induction was by LBA9402 in 17, , followed by PMP90, A4 and lastly 15835 in 19, 25 and 47 days respectively (Table 3). Strain 15835 was a heavy feeder, requiring more nutrients that induced the rest to hairy roots. This strain required had % HRI of 10% despite taking 47 days to induce its first hairy roots. Strain LBA9402 took the shortest time to induction of hairy roots and still offered the highest % HRI. LBA9402 was, therefore, the most efficient strain used in this study.

The results showed that optical densities had an effect on the hairy root induction with low OD_{600} offering low or no hairy roots induction and the same applied to high OD_{600} 1.0 producing few

hairy roots and none at all in some *Musa* cultivars. This can probably be explained by the fact that too much optical density indicates too much bacterial cells which could have fed on the explant cells and this was evidenced by the rotting of explant material. The media supporting explant and bacterial strain was also consumed by the bacteria and this was evidenced by the decomposition and its change from solid to semi-liquid smelly mass of plant and media mixture.

Finally, Guivarc'h *et al.* (1996a, b) suggested the existence of diffuse factors associated with the expression of *rol*A and *rol*C, implying that the sites of action and expression of these genes are physically separated. Since these roots are easily transformed, the transgenic roots can then be used to evaluate effects of genes on biotic constraints which provides breakthrough to evaluate transgene function in *Musa* spp. These hairy roots provide an alternative transformation protocol for recalcitrant species and this can be done to solve the problem of preparation of somatic embryogenic cell suspensions and not only improve transformation efficiency in banana but make transformation to the important cultivars. The following important points were also noted in the infection experiment:-1) the susceptibility of plant species to *Agrobacterium* strains varies greatly. Significant differences were observed between the hairy root induction and transformation ability of different strains of *Agrobacterium*, (2) the age and differentiation status of plant tissue can affect the chances of successful transformation, and (3) the level of tissue differentiation determines the ability to give rise to transformed roots after *A. rhizogenes* inoculation. Further investigations are necessary to better understand the rooting and the transformation processes in banana (*Musa* spp) and to improve propagation protocols. Furthermore the field behaviour of plants rooted by localised infection must be investigated.
The PCR results showed that not all the hairy roots that were induced and grew were transgenic in nature. The hairy roots that were transgenic only showed to be positive with *rol*A Ri-gene specific primers (Figures 4 and 5). The observation made showed that majprity of the hairy roots that grew against the direction and force of geotropism were the transgenic ones and this was confirmed with PCR on those particular roots while the hairy roots that grew towards the direction and force of geotropism were non transgenic and PCR confirmed this too. This, therefore, clearly confirmed that transgenic hairy roots tend to grow against geotropism while the reverse is true for the non-transgenic hairy roots.

Detection of the presence of the Ri-TDNA in the hairy roots implies that *A. rhizogenes* strains can also carry binary vectors. Therefore, gene expression studies using *GUS* can be demonstrated in *A. rhizogenes*-mediated transformation in *Musa* spp., thus this making it a potent tool to produce transformed *Musa* spp roots using a binary vector. Visual selection with the *GUS* would allow for a rapid and efficient screening of transgenic roots avoiding the selection of co-transformed roots with antibiotics or herbicides. This histochemical assay would show GUS expression in the vascular cylinder and root tip of transformed hairy roots. However, in some cases, the GUS staining would vary non-uniformly probably because of the nature of the promoters used, that is, tissue specific or constitutive promoters. Gene expression studies using *GUS* demonstrate that *A. rhizogenes*-mediated transformation is a potent tool to produce transformed maize roots using a binary vector.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

As a new method in the study of plant genetic engineering, hairy root culture shows many useful functions. However, at the same time, it also has some potential problems that remain to be solved. It has been difficult to regenerate plants from the hairy roots except with *A. rhizogenes* strain LBA9402 on genotype 9518S-12 and 15835 on genotype 5610S-1. Poor regeneration systems can be complemented by increasing the efficiency of gene delivery and gene expression. The continued search for infective *Agrobacterium* strains can lead to development of new disarmed strains which can provide higher transformation efficiencies. Another major hurdle is of selection of transgenics *in vitro*. In most of the crops here, kanamycin has been the selective agent of choice. Thus, concerted efforts are needed on improving *in vitro* regeneration or developing non-tissue culture approaches to genetic transformation. Development of new disarmed *Agrobacterium* strains that are highly infective on specific grain legume crops could be another useful alternative. This transformation procedure is still dogged by poor regeneration and low frequency transformation protocols and this situation needs to be rectified if transgenics of these crops are to be developed.

This study demonstrated the susceptibility of banana to *A. rhizogenes* mediated transformation which presents an opportunity in the improvement of banana through genetic engineering. It was noted that that utilization of apical meristems/shoot bud explants produced highest transformation efficiency compared with ECS cultures. Use of *A. rhizogenes* harbouring a binary vector can be used in transgene expression studies. *A. rhizogenes*-mediated transformation can be enhanced by sonication-assisted transformation of shoot buds.

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APPENDIX

MEDIUM COMPOSITION

a) LB Media composition	
Compound	**Amount (g/1000mls)**
Bacto Trypton	10
Bacto Yeast Extract	5
NaCl	10
*Bacteriological Agar	15
pH 7.0	
b) YM Media composition	
Compound	**Amount (g/1000mls)**
Mannitol	10
Yeast Extract	0.4
K2HPO4	0.1
KH2PO4	0.4
NaCl	0.1
MgSO.7H20	0.2
*Bacteriological Agar	15
pH 6.8	