Endophytic bacteria associated with bananas(*Musa* spp.) in Kenya and their potential as biological fertilizers

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DECLARATIONS

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

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To my husband,Prof. Kamau Ngamau and our daughters, Njoki Kamau and Muthoni Kamau for your friendship and love.

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This far the Almighty God has helped me.He has established the work of my hands. Glory, honour and praise to Him for ever and ever. Amen.

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LIST OF ABBREVIATIONS AND ACRONYMS

AASPP	Asia-Africa Scientific Platform Program
ACC	1-aminocyclopropane-1-carboxylate
ANOVA	Analysis of Variance
ARA	Acetylene Reduction Assay
asl	above sea level
BNF	Biological Nitrogen Fixation
BPB	Bromophenol blue
CAS	Chrome Azurol S
cfu	Colony Forming Units
CLSM	Confocal Laser Scanning Microscope
CNN	Competition for Nutrients and Niches
CRD	Complete Randomized Design
DAP	Days after planting
DNA	Deoxyribonucleic acid
DPI	Days Post Inoculation
GA	Gluconic acid
GDH	Glucose dehydrogenase
GFP	Green Fluorescent Protein
GPS	Global Positioning System
HDTMA	Hexadecyltrimethylammonium
IAA	Indole-3-acetic acid
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IPSR	Institute of Plant Science and Resources

ISR	Induced Systemic Resistance
JKUAT	Jomo Kenyatta University of Agriculture and Technology
JSPS	Japan Society for the Promotion of Science
LB	Luria Broth
LGI	Nitrogen free medium
MALDI-TOF/MS	Matrix-assisted laser desorption / ionization time of flight mass spectrometry
MPS	Mineral Phosphate Solubilisation
MoA	Ministry of Agriculture
NBRIP medium	National Botanical Research Institute's Phosphate medium
NCBI	National Center for Biotechnology Information
NFb	Nitrogen free broth
nifDK	Dinitrogenenase protein gene
nifH	Dinitrogenenase reductase protein gene
PCR	Polymerase Chain Reaction
PGP	Plant Growth Promoting
PIPES	Piperazine diethane sulfonic acid
ppb	parts per billion
ppm	parts per million
PQQ	Pyrrolquinoline quinone
PSM	Phosphate Solubilizing Microorganisms
PVK	Pikovskaya
RCBD	Randomized Complete Block Design
RDW	Root Dry Weight

RFW	Root Fresh Weight
rgr-dia	relative growth rate in diameter
rgr-ht	relative growth rate in height
rpm	resolution per minute
rRNA	Ribosomal ribonucleic acid
SAS	Statistical Analysis System
SDW	Shoot Dry Weight
SFW	Shoot Fresh Weight
SPAD	Chlorophyll meter
TC	Tissue Culture
UM	Upper Midland
YEM	Yeast Extract Mannitol
α-HCCA	α-cyano-4-hydroxy-cinnamic acid

ABSTRACT

Bananas and plantains are of special significance to human societies being the fourth most important food in the world today after rice, wheat, and maize. In Kenya, banana is the most popular fruit. In central and western Kenya, the cooking banana forms part of the staple food. Increased trade in local, regional and international markets has also made banana an important cash crop, and in some cases the only source of income for rural populations. Banana production in Kenya is however much below its potential with an estimated average yield of 19 tonnes per hectare as opposed to an average potential yield of 35-45 tonnes per hectare. Several factors including declining soil fertility contribute to this low productivity. The most obvious solution to declining soil fertility is application of mineral fertilizers. However, these are expensive and out of reach for most resource-poor farmers, who constitute the vast majority of banana farmers in Kenya. Additionally, use of chemical fertilizers is not environment friendly. An alternative approach is to improve nutrient uptake by plants through utilization of microbial inoculants. Endophytes, as microbial inoculants, are increasingly gaining scientific and commercial interest because of their potential to improve plant quality and growth and their close association with internal tissues of host plant. To the author's knowledge, endophytic bacteria of bananas in Kenya have not been isolated or identified and consequently there is limited information on their diversity and their functional potential in regard to banana growth and nutrition.

Four main studies were undertaken. In the first study bacteria were isolated from roots, corms and stems of two banana cultivars (*Musa* AAA – Cavendish &*Musa* AAB – plantain) collected from five different geographical regions (Juja,

Maragua, Embu, Meru and Kisii).With five different isolation media, a total of 2,717 isolates were obtained.Morphological characterization was done on the basis of colony color, appearance, motility and Gram staining; and based on the colony morphotypes 214 representative isolates were selected.Biochemical tests done included gelatinase test, starch hydrolysis, catalase production, nitrate reduction and citrate and sugar utilization. The isolates were profiled using the whole-cell matrix-assisted laser desorption / ionization time of flight mass spectrometry (MALDI-TOF/MS). Proteins from the living cells wereextracted using the ethanol/formic acid extraction procedure, and intact molecular weights of the ionized proteins and the pattern of the protein molecular weights were used as fingerprints.Forty three isolates were selected for partial 16S rRNA gene sequencing, whichgrouped them into three families *Enterobacteriaceae, Pseudomonadaceae* and *Bacillaceae.* The family *Enterobacteriaceae* was the most diverse with 8 genera namely *Serratia* (17 isolates), *Rahnella* (4 isolates), *Enterobacter* (2 isolates), *Yokenella* (2 isolates), *Raoultella* (2 isolates), *Klebsiella* (1 isolate), *Yersinia* (1 isolate) and *Ewingella* (1 isolate). Both the *Pseudomonadaceae* and *Bacillaceae* families were represented by only one genus, that is,*Pseudomonas* (12 isolates) and *Bacillus* (1 isolate), respectively.*Serratia* and *Pseudomonas* species were the most abundant with 17 isolates and 12 isolates, respectively. Of the 10 genera identified, *Rahnella, Yokenella*, *Raoultella, Yersinia andEwingella*had not been previously described as endophytic in banana plants. Full-length sequencing allowed identification of some five selected isolates as *Bacillus subtilis subsp. inaquosorum, Enterobacter ludwigii, Ewingella americana, Rahnella aquatilis* and *Flavimonas oryzihabitans* and the sequence data generated have been deposited with the NCBI GenBank under

accession numbers AB675632 to AB675636.Of significance is that isolate K22V1c is being proposed as novel having showed a similarity value of less than 97% (95.27%) with its closest relative *Klebsiella granulomatis*.

The second study characterized the 43 isolates on the basis of their *in-vitro* plant growth-promoting activities that included ability to fix free nitrogen, solubilize phosphates and produce siderophores. All the 12 *Pseudomonas* isolates showed potential for siderophore production with *Flavimonas oryzihabitans*isolates showing the highest potentialas determined on blue Chrome Azurol S (CAS) agar plates. Twenty seven isolates were observed to solubilize phosphates, with *Rahnella* isolates showing the highest potentialas determined on NBRIP growth medium. All the isolates grew on solid nitrogen-source free medium and had varied levels of acetylene reduction activity, suggesting their ability to fix atmospheric nitrogen.PCR amplification of the *nifH* gene, which codes for the enzyme nitrogenase reductase, was attempted without much success.

In the third study, tissue-cultured banana plantlets (*Musa* spp. cv. Grande Naine) were inoculated with some selected isolates and effects of inoculation on plant growth, physiology and mineral nutritionat different fertilizer regimes were investigated. *Ewingella americana*(K32V2c)showed a positive significant effect (P=0.05)on the relative change in plant height and number of green leaves while*Enterobacter ludwigii*(J1V1r)significantly (P=0.05) increased shoot dry weight. Inoculation of plants with *Bacillus subtilis subsp. Inaquosorum*(M9V1r), *Enterobacter ludwigii*(J1V1r)and *Ewingella americana*(K32V2c)significantly (P=0.05) increased chlorophyll content in plants while *Rahnella aquatilis*(ME19V2c) increased P concentration in plant shoots though not significantly.

The fourth study sought to confirm the endophytic habitat and colonization ability of the isolated bacteria. Endophytic habitat of *Flavimonas oryzihabitans*(K50V2s) was confirmed through microscopic evidence of "tagged" bacteria inside plant tissues.The green fluorescent protein reporter gene (mTn5gusA-pgfp21) from *E. coli* S17-1was used to tag the bacteria while fluorescence scanning was done under theConfocal Laser Scanning Microscope.Onlyconjugation of *Flavimonas oryzihabitans* (K50V2s) with *E. coli* S17-1 (mTn5gusA-pgfp21) resulted to successful transconjugants.

In conclusion, endophytic bacteria of bananas in Kenya were successfully isolated and identified as *Serratia, Pseudomonas, Rahnella, Enterobacter, Yokenella, Raoultella, Klebsiella, Yersinia, Ewingella* and *Bacillus* species.*Rahnella, Yokenella, Raoultella, Yersinia andEwingella*species having not been reported in association with bananas by the time of this study suggests a large richness of banana endophytic bacterial species than has so far been reported.It's now clear that many diazotrophic microbes inhabit the tissues of banana plants and there is the potential of exploiting them once conditions for their use is optimized. *Rahnella aquatilis* (ME19V2c and ME18V2c) and *Flavimonas oryzihabitans* (K50V2s)having showedability tosolubilize phosphate and produce siderophore, respectively and also ability to fix free nitrogen could be proposed as potential biofertilizers for sustainable banana production in Kenya.*Bacillus subtilis subsp. inaquosorum* (M9V1r), *Enterobacter ludwigii* (J1V1r), *Ewingella americana*(K32V2c), *Rahnella aquatilis* (ME19V2c) *and Flavimonas oryzihabitans* (K50V2s)have potential to promote plant growth. However, to facilitate their use in practical agronomic production, reliable and practical methods of inoculum delivery must be developed.

CHAPTER ONE

1.0 INTRODUCTION

1.1. Background Information

Bananas and plantains are of special significance to human societies being the fourth most important food in the world after rice, wheat, and maize (Scot *etal.*, 2006). Increased trade in local, regional and international markets has also made them an important cash crop, and in some cases the only source of income for rural populations (Frison and Sharrock, 2001). In Kenya, however, banana production is constrained by among others, declining soil fertility (Vanlauwe and Giller, 2006; Okumu, 2008). This is brought about by insufficient application of manure due to cost implications especially for the farmers without livestock, and limited use of inorganic fertilizers, which are expensive and therefore unaffordable for most banana farmers in Kenya.

A sustainable complementary approach would be to increase the biological inputs of nutrients by exploitation of microorganisms, which are largely untapped natural resources for plant growth promotion (Thomas and Soly, 2009). Notably, there is a renewed scientific and commercial interest in the use of microbes especially the endophytes because of their potential to improve plant quality and growth and their close association with internal tissues of host plant (Carroll, 1992; Schulz *et al.*, 1998; Schulz *et al.*, 1999). According to Azevedo*et al.*(2000), most researches on endophytes have been carried out using hosts from temperate countries, while data available from tropical regions remains scarce. This limited research has shown that tropical plant hosts contain a great diversity of endophytic microorganisms many of them not yet classified and possibly belonging to new

genera and species(Azevedo *et al.*, 2000). This is the case in Kenya where endophytic bacteria of bananas have not been isolated or identified and therefore there is limited information on their diversity and their functional potentiality in regard to banana growth and nutrition.

1.2. Statement of the Problem

In Kenya, banana production is much below its potential with an estimated average yield of 19 tonnes per hectare as opposed to an average potential yield of 35-45 tonnes per hectare. Several factors including declining soil fertility contribute to this low productivity. For example, phosphorus deficiency remains one of the reasons why sub-Saharan Africa is the only major region in the world where per-capita food production has declined in the past three decades. The most obvious solution to declining soil fertility is application of mineral fertilizers. However, these are expensive and out of reach for most resource-poor farmers, who constitute the vast majority of banana farmers in Kenya. Additionally, use of chemical fertilizers is not environment friendly. An alternative approach is to improve nutrient uptake by plants through utilization of microbial inoculants. In this regard, endophytes are increasingly gaining scientific and commercial interest because of their potential to improve plant quality and growth and their close association with internal tissues of host plant. However, to the author's knowledge, endophytic bacteria of bananas in Kenya have not been isolated or identified and consequently there is limited information on their diversity and their functional potentiality in regard to banana growth and nutrition.

1.3. Justification

In Kenya, banana is the most popular fruit and in central and western Kenya, the cooking banana forms part of the staple food. Increased trade in local, regional and international markets has also made banana an important cash crop, and in some cases the only source of income for rural populations. For increased banana production, tissue culture technology is highly recommended for banana propagation for several reasons that include pest- and disease-free planting material. By producing banana plants aseptically in the laboratory, through tissue culture, the plants do not only lose the pathogenic microbes but also the beneficial ones which include the endophytes. Endophytic bacteria are reported to enhance plant growth in non-leguminous crops and improve their nutrition through nitrogen fixation, phosphate solubilization or siderophore production (iron chelation). Reintroducing the endophytes into banana tissue culture plants would restore the natural equilibrium thus extending the benefits of clean planting material. In Kenya, where soils are largely depleted, a positive endophyte-banana association would contribute to the reduction of total fertilization costs while improving plant growth and productivity. Imported microbial inoculants could be used for banana production in Kenya but because of the fitness challenge, there is need to isolate locally occuring bacteria from bananas in practical farm fields and assess their functional potentiality as biological fertilizers.

1.4. Hypotheses

This study tested the following alternative hypotheses;

1. Bananas growing in Kenya have endophytic bacteria within their tissues.

2. Banana endophytic bacteria have capacity to fix free nitrogen, solubilize phosphates or produce siderophores.
3. Diazotrophic (nitrogen fixing)and non-diazotrophic endophytic bacteriaof bananas in Kenya have potential to promote plant growth and enhance uptake of mineral nutrients.
4. Bacteria isolated from surface-disinfected banana tissues are 'true' endophytes.

1.5. General Objective

To isolate and identify endophytic bacteria associated with bananas in Kenya and to explore theirfunctional potential as biological fertilizers.

1.6. Specific Objectives

1. To isolate and identify endophytic bacteria associated with bananas in Kenya.
2. To determine the isolates' capacity to fix free nitrogen, solubilize phosphates and produce siderophores.
3. To investigate the effects of the isolates on the growth, physiology and mineral nutrition of tissue cultured banana plants.
4. To determine the isolates' endophytic habitat and their capacity to colonize and re-infect bananas.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. Banana and Plantains

Bananas and plantains are herbaceous, perennial monocotyledonous plants, which grow in the tropics and subtropics. They belong to thegenus *Musa* in the family *Musaceae* (Rossel, 2001). The number of *Musa* cultivars is estimated at 100-300 (Frison and Sharrock, 2001). Almost all modern edible banana and plantain cultivars are hybrids and polyploids of the wild seeded bananas *Musa acuminata* and *Musa balbisiana*. The genotype of *Musa acuminata* is referred to as '**A'**, while that of *Musa balbisiana* is referred to as '**B'.** Polyploids of *Musa acuminata* (e.g. AA or AAA) are usually sweet and used as dessert while polyploids of *Musa balbisiana* (e.g. BB or BBB) and hybrids of the two (e.g. AB, ABB or AAB) are usually plantains or cooking bananas (Ortiz, 1995).

Bananas and plantains provide an important food source for over 100 million people in Sub-Saharan Africa (Sharrock and Frison, 1999). The region is also the world's leading consumer of bananas with an annual *per capita* consumption rate of 400-600 kg (Karamura *et al*., 1998). Majority of banana producers in Africa are small-scale farmers growing the crop either for home consumption or for local markets (Karamura *et al*., 1998; Sharrock and Frison, 1999). Bananas and plantains grow in a wide range of environments and produce fruit year-round, and are therefore a special source of energy when other crops are not available. They are especially suited to intercropping systems and to mixed farming with livestock and are also popular as a backyard crop in urban settings. Banana tolerates a wide range of soils, but well drained fertile loams are the most idealwith an optimal soil pH of

5.5 to 7.5 (Scot *et al.*, 2006; HortiNews, 2013). Bananas and plantains grow at elevations of 0 - 920 m or more above sea level, depending on latitude. In Africa, the crop is grown across diverse agro-ecological conditions ranging from lowlands at sea level to highlands above 2,000 meters above sea level. Bananas grow best where rainfall is distributed evenly throughout the year with an annual rainfall of 1,000 to 2,500 mm and a mean annual temperature of 20°C to 30°C, without long dry spells. Prolonged drought causes stunting and damage and since *Musabalbisiana* is considered more drought tolerant than*M. acuminata*, cooking varieties are more drought tolerant than dessert varieties. In commercial orchards, bananas yield up to 40 tonnes of fruit per hectare annually(HortiNews, 2013).

2.2. Banana Production in Kenya

In Kenya, area under banana production is about 63,290 ha with an estimated average yield of 19 tonnes per hectare as opposed to an average potential yield of 35-45 tonnes per hectare (FAOSTAT, 2011;HortiNews, 2013). Banana production in Kenya is constrained by among others, declining soil fertility (Vanlauwe and Giller, 2006; Okumu, 2008). Okumu (2008) identified soil fertility as the major problem in tissue cultured banana production in Central Province of Kenya, where yields depended more on soil fertility (67%) than either farm management (23%) or pests and diseases (10%). Declining soil fertility is brought about by insufficient application of manure due to cost implications especially for the farmers without livestock, and limited use of inorganic fertilizers, which are expensive and therefore unaffordable for most banana farmers in Kenya. This results in inadequate nutrients replenishment, which are mainly lost through crop harvest, heavy rains, widespread soil erosion and rapid organic matter decomposition. According to Lahav and

Lowengart(1998), nitrogen, phosphorus and potassium mining by bananas is estimated at 189 kg/ha, 29 kg/ha and 778 kg/ha, respectively for a 50 ton/ha/year yield. In the absence of replacement, this nutrient mining reduces yields and fruit quality (Kahangi, 2010).

In Kenya, farmyard manure and mulching are used to maintain or increase soil organic matter reserves in banana production. However, according to Vanlauwe and Giller (2006), organic inputs alone cannot sustain crop production in resource-poor farming systems due to limitations in their quality and availability. A sustainable alternative approach would be to improve nutrient uptake by plants through utilization of microbial inoculants.

2.3. Endophytic Bacteria

Plants are naturally associated with mutualistic microbes that include endophytes. Endophytes have been defined as diverse microbes, most commonly fungi and bacteria (Wilson, 1995; Strobel and Daisy, 2003), which spend the entire or part of their life cycle living in internal plant tissues causing no apparent or immediate disease symptoms (Hallmann *et al.*, 1997, Bacon and White, 2000; Long *et al.*, 2008). These non-pathogenic associations could be beneficial, neutral or detrimental (Sturz *et al.*, 2000).

According to Senthilkumar *et al.* (2011), sources of endophytic bacteria are several and they include rhizosphere soil, phylloplane, seeds and vegetative planting material. Rhizosphere soil is however the primary source for endophytic colonization. Almost all endophytic bacteria found in various plants are also found in the rhizosphere, supporting the hypothesis that there is a continuum of root-associated microorganisms from the rhizosphere to rhizoplane to epidermis and

cortex (Kloepper *et al.*, 1992). However, the endophytic population is usually less diverse than the rhizosphere population (Germida *et al*., 1998), suggesting that the root interior (endorhiza) is a distinct habitat from the rhizosphere.

Endophytic bacteria enter plant mainly through wounds naturally occurring as a result of plant growth or through root hairs and at epidermal conjunctions. Besides providing entry avenues, wounds also create favourable conditions for the bacteria by allowing leakage of plant exudates that serve as a nutrient source for the bacteria (Hallmann *et al*., 1997). Other entry sites for endophytic bacteria include flowers, stomataand stem lenticels (Ashbolt and Inkerman, 1990; Sharrock *et al*., 1991; Kluepfel, 1993). Except for bacteria transmitted through seeds, endophytic colonization of the root surface is critical before entry into the plant. Once in the plant, the endophytic bacteria have an ecological advantage over the epiphytic bacteria in that they are protected from external environmental conditions like temperature, osmotic potentials, and ultraviolet radiation, which are major factors limiting long-term bacterial survival (Senthilkumar *et al*., 2011).

The population density of endophytic bacteria is highly variable, depending mainly on the bacterial species and host genotypes but also on the host developmental stage, inoculum density, and environmental conditions (Pillay and Nowak, 1997; Tan *et al.*, 2003). For instance, the presence of different endophytic species in soybean depended on plant genotype, plant age, the tissue sampled and the season of isolation (Kuklinsky-Sobral *et al.*, 2004). Soil type also to a large extent, determined endophytic populations in wheat (Conn and Franco, 2004). Molecular analysis has shown that plant defense responses also limit bacterial populations inside plants (Rosenblueth and Martínez-Romero, 2006). Senthilkumar *et al*. (2011)

alluded to the fact that organisms occupying the endosphere have most likely been selected for this niche by the plant because of the beneficial effects they offer their host and their abilities to resist the effects of plant defence products.

Endophytic bacteria have been isolated from roots, stems, leaves, seeds, fruits, tubers, ovules, and also inside legume nodules (Hallmann *et al.*, 1997; Sturz *et al.*, 1997; Sturz *et al.*, 2000; Benhizia *et al.*, 2004) and in most cases, bacterial populations are larger in roots than in above ground tissues (Rosenblueth *et al.*, 2004). Endophytic bacteria in a single plant host are not restricted to a single species but comprise several genera and species. Of the roughly 300,000 plant species found on earth, each plant is a host to one or more endophytes, however only a few of these plants have been completely studied in regard to their endophytic biology. As a result, the opportunity to find novel and unique endophytic microbes amid numerous plants in different ecosystems is considerable (Senthilkumar *et al*., 2011).

In bananas, genera and species of endophytic bacteria identified have included: *Azospirillum brasilense* and *A. amazonense* (Weber *et al*., 1999), *Bacillus* spp. (Harish, 2008), *Burkholderia* spp. (Weber *et al*., 1999; Ting *et. al*., 2008), *Citrobacter* spp. (Martínez *et al*., 2003), *Enterobacter* spp. (Martínez *et al*., 2003), *Herbaspirillum* spp. (Weber *et al*., 1999; Weber *et al.*, 2001), *Klebsiella* spp. (Martínez *et al*., 2003 and Rosenblueth *et al*., 2004), *Pseudomonas* spp. (Harish, 2008; Ting *et. al*., 2008), *Rhizobium* spp. (Martínez *et al*., 2003) and *Serratia* spp. (Ting *et al*., 2008).

2.4. Endophytic BacteriaPotential as Biofertilizers

Despite the fact that the interaction between endophytic bacteria and host plants has not been fully understood, it is well established that some of these interactions are beneficial to the plant (Rosenblueth and Martinez-Romero, 2006; Long *et al.*, 2008). Endophytes are increasingly gaining scientific and commercial interest because of their potential to improve plant quality and growth and their close association with internal tissues of host plant (Carroll, 1992; Schulz *et al.*, 1999).Endophytic bacteria are reported to enhance plant growth in non-leguminous crops and improve their nutrition through nitrogen fixation, phosphate solubilization or siderophore production (Dobereiner and Baldani, 1998;Sturz *et al*., 2000; Sevilla *et al*., 2001; Hurek *et al*., 2002; Boddey *et al*., 2003; Iniguez *et al*., 2004; Ryan et al., 2008; Uribe *et al*., 2010).

Besides biofertilization, endophytic bacteria are also reported to promote plant growth and yield through production of phytostimulators such as phytohormones, the cofactor pyrrolquinoline quinone (PQQ) and the volatile acetoin; or by producing stress controllers like the enzyme 1-aminocyclopropane- 1-carboxylate (ACC) deaminase, which facilitate plant growth and development by lowering plant ethylene levels; or indirectly through biological control of plant diseases or induced resistance response (Long *et al.*, 2008; Lugtenberg and Kamilova, 2009). In return, the plant protects endophytes and provides them with nutrients in form of photosynthates.

2.4.1. Nitrogen fixation

Nitrogen fixation is the reduction of atmospheric nitrogen (N_2) to ammonia (NH_3), a product that is subsequently assimilated into biomass (Staal *et al*., 2001). Organisms

that are capable of fixing atmospheric nitrogen possess the enzyme nitrogenase and are called diazotrophs. Nitrogenase is only found in prokaryotic organisms. Nitrogenase is a highly conserved enzyme complex consisting of two proteins, dinitrogenase (MoFe-protein) and dinitrogenase reductase (Fe-protein), which are encoded by the *nifDK* and *nifH* genes, respectively (Fedorov *et al.*, 2008; Rajeswari and Mangai, 2009). The most convenient method of assessing the nitrogen-fixing ability in different prokaryotes is amplification of nitrogenase structural genes (*nif* genes) using degenerate oligonucleotide primers in polymerase chain reaction. Other methods of assessing nitrogen fixation including acetylene reduction assay, hybridization of genome DNA with labelled probes, or methods using ^{15}N require expensive equipment and reagents, and their results are not always reliable.

The element nitrogen is highly abundant in the atmosphere and it is a major component of dietary proteins (Muthukumarasamy *et al*., 2002). However, the availability of fixed N is the most significant yield-limiting factor in many agricultural production systems making it necessary to apply fertilizers. Nitrogenous chemicals account for approximately 30% of the total fertilizers needed for agricultural crops. With the increasing cost of chemical fertilizers especially in tropical countries where subsidies are not available, the high cost of transportation, and the environmental pollution concern, the role of biological nitrogen fixation (BNF) in supplying plants with needed N has to be harnessed efficiently (Matiru and Dakora, 2004). This is because it can make agriculture more productive and sustainable without harming the environment. BNF accounts for 65% of the nitrogen currently utilized in agriculture and will be increasingly important in future crop productivity especially for sustainable systems (Dakora and Keya, 1997). Since BNF

is not limited to legumes only, for sustainable agriculture it becomes necessary to increase the amount of biologically fixed N in non-legume crops as well.

BNF microbial inoculants may be symbiotic or non-symbiotic. According to Ando *et al*. (2008), symbiotic nitrogen fixation between leguminous plant and root-nodule bacteria is well known and has been studied extensively, but non-symbiotic nitrogen fixation between non-leguminous plant and nitrogen-fixing bacteria has not received sufficient recognition. Non-symbiotic (associative) N-fixing bacteria can be rhizosphere-based or endophytic. It has been suggested that endophytic N-fixing bacteria may be more important than rhizospheric bacteria in promoting plant growth because they escape competition with rhizosphere microorganisms and achieve close contact with the plant tissues (Döbereiner, 1992; Assmus *et al.*, 1995). As such, new research efforts have focused on identification of endophytic diazotrophs that are able to supply biologically fixed nitrogen directly to their host ensuring a highly efficient nitrogen uptake by the plant. For the endophyte the advantages of such an association would be reduced competition from rhizosphere microorganisms, a more reliable supply of metabolic substrates, adequate reducing conditions, and protection against too-high oxygen concentrations (Quispel, 1991).

Nitrogen-fixing endophytic bacteria have been isolated from several groups of plants (Ladha and Reddy, 2000) since the isolation of the endophytic diazotrophic bacterium *Gluconacetobacter diazotrophicus* (previously known as *Acetobacter diazotrophicus*) from a Brazilian variety of sugarcane (James and Olivares, 1997). They include species of the genera *Azoarcus, Herbaspirillum, Azospirillum, Gluconacetobacter, Klebsiella, Serratia* and *Burkholderia,* which have been isolated from important crop plants such as rice, maize and wheat (Reinhold-Hurek and

Hurek, 1998; Gyaneshwar *et al.*, 2002a; Potrich *et al.*, 2003; Iniguez *et al.*, 2004; Muthukumarasamy *et al.*, 2005). In bananas, endophytic diazotrophic bacteria isolated have included *Azospirillum, Burkholderia, Citrobacter, Enterobacter, Herbaspirillum, Klebsiella* and *Rhizobium* species (Weber *et al.*, 1999; Weber *et al.*, 2001; Martinez *et al.*, 2003; Weber *et al.*, 2007). However,endophytic diazotrophs seem to constitute only a small proportion of total endophytic bacteria (Ladha *et al.*, 1983; Barraquio *et al.*, 1997; Martínez *et al.*, 2003) and increasing N_2-fixing populations in plants has been considered as a means of increasing nitrogen fixation.

Although a number of diazotrophs are known to enrich fertility of soil by releasing fixed nitrogen, associative/endophytic diazotrophic bacteria can also trigger plant growth via production of phytohormones, phosphate solubilization, siderophores production and inhibition of or resistance to, certain pathogens (Hallmann *et al.*, 1997; Conn and Franco, 2004). Endophytic diazotrophs may have a considerable potential for raising the productivity of non-legumes including bananas in view of their wide occurrence and inherent biological properties.

2.4.2. Phosphate solubilization

Phosphorus is second only to N among mineral nutrients that limit plant growth(Vessey, 2003). This is despite the fact that soils have large reserves of total P but amounts available to plants are usually a tiny proportion of this total as a result of high phosphorus fixation. Phosphorus fixation is worse in the tropics and subtropics because of the high soil acidity (Norman *et al.*, 1995). Whereas most mineral nutrients in soil solutions are present in millimolar amounts, soluble phosphorus is present only in micromolar or lesser quantities. The biggest reserves of phosphorus are rocks and other mineral deposits formed during the geological age (Rodriguez

and Fraga, 1999). Mineral phosphate can also be found fixed on the surface of hydrated oxides of iron, aluminium and manganese, which are poorly soluble and assimilable.

In most agricultural ecosystems, a considerable part of the phosphorus reserves is what has accumulated as a result of regular applications of P fertilizers (Richardson, 1994). This is because a large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is quickly immobilized after application becoming unavailable to plants (Dey, 1988). In Kenya and most countries of the sub-Saharan Africa, phosphorus constraints are much more severe because phosphorus in the harvested crops is removed from the system, with only limited quantities being returned in crop residues and animal manures. Phosphorus replenishment, especially with small scale farmers, remains a challenge because of the prohibitive high cost and limited availability of phosphate fertilizers at farm level (Brady and Weil, 2002).

Fixation and precipitation of P in soil is highly dependent on pH and soil type. In acid soils, phosphorus is fixed by free oxides and hydroxides of aluminium and iron, while in alkaline soils it is fixed by calcium and magnesium, causing a low efficiency of soluble P fertilizers (Goldstein, 1986; Gyaneshwar *et al*., 2002b). Consequently, in the tropics, acidity and toxicity of aluminium and iron are normally major constrains to soil fertility (Place *et al*., 2003). The other major component of soil P is organic P, which may constitute 30-50% of the total phosphorus in most soils, although it may range as low as 5% to as high as 95% (Paul and Clark, 1988). Organic phosphate is also immobilized by organic soil matter or the formation of complexes with aluminium and iron (Gyaneshwar *et al*., 2002b). Releasing of

insoluble and fixed forms of phosphorus is therefore an important aspect of increasing soil phosphorus availability.

Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate and rock phosphate (Goldstein, 1986). Bacterial genera with this capacity include *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aerobacter, Flavobacterium* and *Erwinia*. Phosphate-solubilizing micro-organisms (PSM) dissociate the phosphorus from soil complexes through several mechanisms, such as the production of organic acids, which dissolve or chelate inorganic phosphate, or the production of phosphatases and phytases, which dissociate phosphorus from organic sources (Rodriguez and Fraga, 1999). PSM enhance availability of soil phosphate, promoting its uptake by plants (Gyaneshwar *et al.*, 2002b). Besides phosphorus solubilisation, other mechanisms involved in plant growth promotion by PSM include nitrogen fixation, production of phytohormones and other plant growth-promoting substances, enhancement of availability of other trace elements like iron and zinc, and enhanced availability of microbially immobilized phosphorus (Rodriguez and Fraga, 1999; Gyaneshwar *et al*., 2002b). Phosphate solubilizing bacteria including some rhizobia species have been shown to enhance P and K uptake in cereal plants (Matiru and Dakora, 2004). Phosphate-solubilizing bacteria play an important role in plant nutrition through increase in P uptake by the plant, and their use as plant growth promoting bacteria is an important contribution to biofertilization of agricultural crops.

Despite the importance of phosphorus for agriculture, the high cost of this element, and the anticipated depletion of sources of high quality phosphate rocks

(Richardson, 2001), PSM inoculants are not in widespread use in the tropics. This could be as a result of the lack of consistent and conclusive field results that would demonstrate the benefits of PSM inoculants. Reasons for this inconsistency have included limited knowledge of the plant-microbe interactions, the uncontrolled effect of plant varieties and soil types on microbial colonization and functional capacity, and inaccurate strategies for screening of potential PSM strains (Rodriguez and Fraga, 1999; Gyaneshwar *et al*., 2002b).

2.4.3. Siderophores production

Although iron is the fourth most abundant element in the earth's crust, many microorganisms and plants have difficulty obtaining enough of it in nonacidic, oxygenated environments as it is usually found in the trivalent form Fe^{3+}, which forms hydrated hydroxides (such as rust) that are insoluble (Drechsel and Winkelmann, 1997). By contrast, the Fe^{2+} ion is soluble but is invariably oxidized by hydrogen peroxide in aerobic conditions. Most microorganisms can increase the concentration of extracellular soluble iron by releasing small molecules that scavenge ferric ion from ferric hydroxides and iron transport proteins. These small molecule iron scavengers are known as siderophores. Sharma and Johri, (2003) have defined siderophores (Greek: "iron carriers") as low-molecular-weight compounds with high iron (III) chelating affinity. They are responsible for the dissolution, chelation and transport of iron (III) into microbial cells. Chen *et al.*, (1998) have also shown that Fe, chelated by microbial siderophores, can also be utilized by plants. Siderophore-producing bacteria would therefore improve the iron nutrition of plants. *Pseudomonas* species have been reported as high siderophore producers (Gangwar and Kaur, 2009, Ngamau *et al.*, 2012).

2.4.4. Phytostimulatorsproduction

According to Long *et al.* (2008) plant growth promoting (PGP) mechanisms of endophytic bacteria are thought to be similar to those of PGP rhizobacteria, that is they affect plant growth by producing phytohormones, such as cytokinins or auxins, or by degrading hormone precursors, such as 1-aminocyclopropane-1-carboxylate (ACC) by ACC deaminase consequently lowering plant ethylene levels. Ethylene and IAA are implicated in virtually all aspects of plant growth and development, ranging from seed germination to shoot growth and leaf abscission (Woodward and Bartel, 2005). Therefore, through the production of ACC deaminase and IAA, endophytes can efficiently manipulate their plant hosts in regard to growth and development. IAA is produced by root associated bacteria such as *Enterobacter* spp., *Pseudomonas* spp., and *Azospirillium* spp. (Spaepen *et al*., 2007). Lowering ethylene in plant roots also relieves the suppression of auxin response factor synthesis, and indirectly increases plant growth (Glick *et al.*, 2007). In addition, bacteria could promote plant growth by releasing certain volatiles (potential signal molecules) like the acetoin and the cofactor pyrrolquinoline quinone (PQQ), which promotes plant growth because of its antioxidant activity (Choi *et al.*, 2008; Lugtenberg and Kamilova, 2009).

2.4.5. Microplant biotization

Biotization has been defined as the metabolic response of *in-vitro* grown plant material to microbial inoculants, which promote developmental and physiological changes that enhance biotic and abiotic stress resistance in subsequent plant progeny (Sturz *et al.*, 2000, Senthilkumar *et al*., 2011). Microplant biotization is an emerging biotechnology aimed at reducing chemical input in plant production whilst

increasingplant fitness and productivity in the context of sustainable horticulture. Induction of stress resistance in plant propagules produced *in-vitro* before transplanting is a primary target of several researchers attempting utilization of microbial inoculants in micro-propagation (Nowak, 1998). There is enough experimental evidence with bacteria (bacterization) and vesicular arbuscular mycorrhiza (mycorrhization) inoculations to recommend utilization of this technology in commercial micro-propagation (Nowak, 1998). For example, biotization of potato plantlets enhanced the transplant stress tolerance and eliminated an expensive greenhouse hardening step (Herman, 1996). Greenhouse experiments have also shown that plants derived from potato bacterized with pseudomonad bacteria had larger root system, set stolons, tuberized earlier, and gave better tuber yield than non-bacterized control. Pillay and Nowak (1997) however, noted that both *in-vitro* and *ex-vitro* benefits of biotization depended on plant species, cultivar, growth conditions, and degree of endophytic colonization and that in addition, a certain threshold of bacteria concentration is required to trigger beneficial responses.

2.4.6. Crop adaptation to abiotic stress environment

One aim of plant production technology is to induce stress resistance in crops grown under adverse environments like water stress, which is common in the tropics. Microorganisms have been reported to improve water uptake and/or protect plants against water stress or aid plants in withstanding water stress.Experiments with cowpea inoculated with *Bradyrhizobim* showed that infected plants withstood water stress better than non-infected ones (Figueiredo *et al.*, 1999). Joseph and Phillips (2003) also reported that metabolites from soil bacteria affect plant water relations. The physiological responses include water use efficiency, relative water content and

osmotic potential. In addition, ACC deaminase producers are also reported to relieve plants of drought stress (Glick *et al*., 2007). Since water deficit is a major constraint to banana production in Kenya, any endophytic bacteria that can confer some protection against the effects of water stress would go a long way towards improving the growth of these crops.

2.4.7. Endophytes as biological control agents

Increased environmental awareness has prompted the development of biological alternatives to chemical crop protection agents (Dimock *et al.,* 1989). In addition, the use of agrochemicals is negatively perceived by consumers especially in the European Union (Lugtenberg and Kamilova, 2009). In contrast, the use of microbes to control plant diseases is an environment-friendly approach and the intimate association of endophytic bacteria with their host plants offers a unique opportunity for their potential application as biological control agents (Senthilkumar *et al*., 2011). The mechanisms by which endophytes act as biocontrol agents include the production of antifungal or antibacterial agents, siderophore production, competition for nutrients and niches (CNN), and indirectly through induced systemic resistance (Sturz *et al.,* 2000; Sessitsch *et al*., 2002; Lugtenberg and Kamilova, 2009).

Majority of bacterial biological control agents have been selected from among the rhizobacteria (Weller, 1988; Kloepper, 1992; Beauchamp, 1993). Unfortunately, most of these biocontrol agents have not fulfilled their initial promise; their failure usually being attributed to poor rhizosphere competence and the difficulties associated with the instability of bacterial biocontrol agents in long-term culture (Schroth *et al.,* 1984; Weller, 1988).However, the intimate relationship between endophytic bacteria and their hosts make them natural candidates for

selection as biocontrol agents (Van Buren *et al.*, 1993; Chen *et al.*, 1995) and would preclude the need for selecting bacterial types with high levels of rhizosphere competence often considered necessary for successful seed or root bacterization treatments before or at planting. However, the effectiveness of endophytic bacteria as biological control agents is dependent on many factors including the host specificity, the population dynamics and pattern of host colonization, the ability to move within host tissues, and the ability to induce systemic resistance (Backman *et al.*, 1997).

Endophytes potential as biological control agents is nonethelessunderutilized, especially in Africa, due to limited fundamental information on them and their ecology and the high cost of product development and required regulatory approvals (Cook *et al.*, 1996; Lugtenberg and Kamilova, 2009).

CHAPTER THREE

3.0 GENERAL MATERIALS AND METHODS

3.1. Introduction

In Kenya, bananas are grown across diverse agro-ecological zones ranging from Coastal Lands at sea level to Lower Highlands at about 2,000 meters above sea level (HortiNews, 2013).However, the most suitable banana growing regions includeKisii, Kakamega, Bungoma, Meru, Murang'a, Embu, Nyeri, Kerio Valley, Baringo, Kirinyaga and the coastal region. Bananas can also be grown in drier areas like Kitui and Machakos but under irrigation. Murang'a South (Maragua), Embu (Embu Central &Manyata), Meru (Imenti North - Miriga Mieru), Kisii Central (Kiogoro) Distictswere selected for sampling(**Figure 3.1**). Financial and other logisticalconstraints did not allow sampling from the other banana growing regions. JujaDistrict, though not a main banana growing region, was also selected because of its proximity to Jomo Kenyatta University of Agriculture and Technology where the study was hosted. The University banana orchard was one of the farms selected in the Juja District. With the guidance of the local agricultural officers, five farms per District were randomly selected within a radius of about 20 km from the Ministry of Agriculture local office. In Juja District, however, only three farms were available for sampling within the 20 km radius from the University.

A data collection sheet (questionnaire) adopted from previous study done on banana fungal endophytes was used to solicit basic information onselected farms,which included;topographic details, farmer's personal details, farm description, farming activities, soil management, weatheramong others (**Appendix 1**).

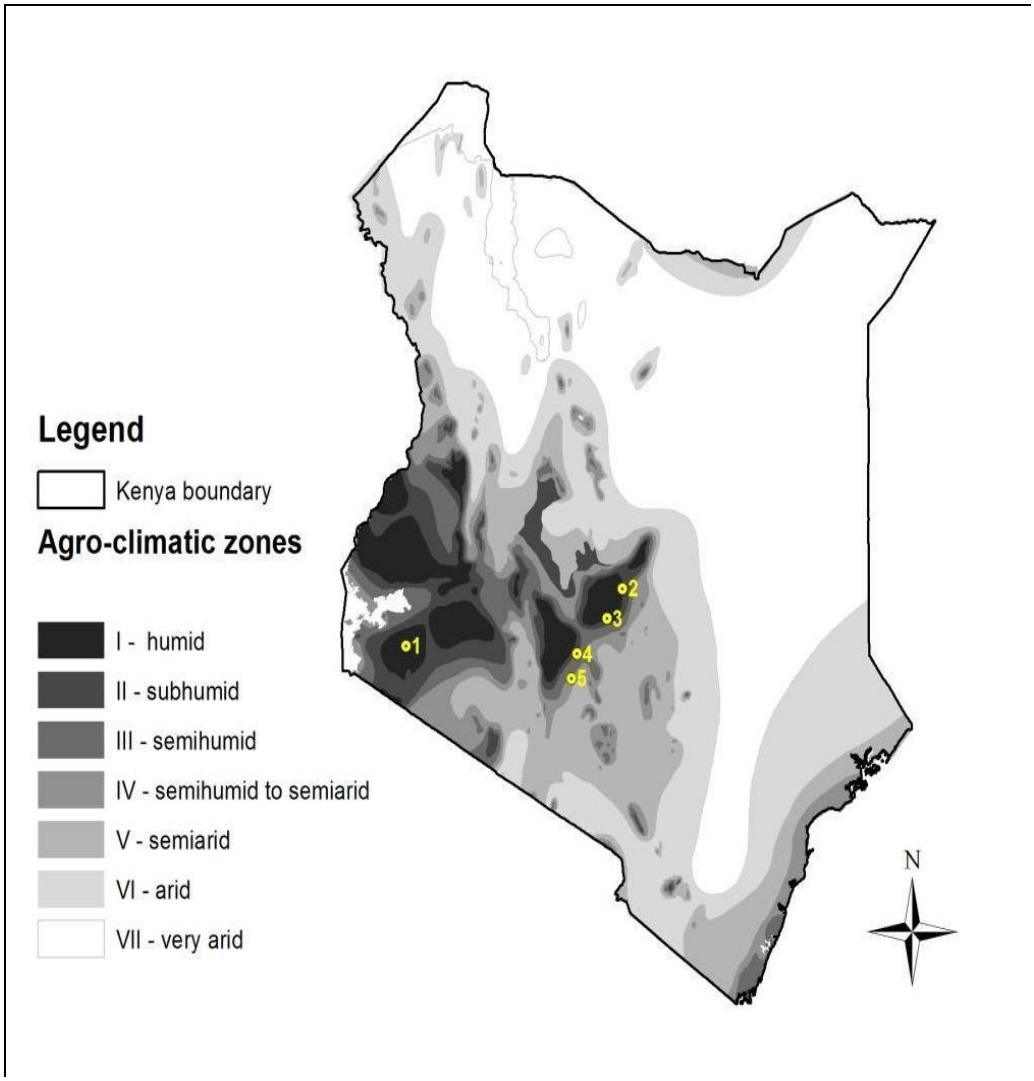


Figure 3.1: Location map of the five study sites. Key: 1-Kisii Central (Kiogoro); 2-Meru (Imenti North - Miriga Mieru); 3-Embu (Embu Central & Manyata); 4-Murang'a South (Maragua); 5-Juja.

3.2. Sample collection

Study samples were collected from Juja and Maraguain February 2009; Embu (Embu Central& Manyata)and Meru (Imenti North - Miriga Mieru) in March 2009 and Kisii Central (Kiogoro)in January 2010. In every farm, with the consent of the farmer, one

healthy dessert banana plant (*Musa* AAA – Cavendish)and one healthy cooking banana plant(*Musa* AAB – plantain)both in fruit were identified for sampling. From each of the selected banana plant, three samples namely roots, corm and stem were collected. Soil samples were also collected around the mats of selected banana plants at a depth of 0-20 cm and 20-40 cm.Study material was collected from five farms per District except in Juja where the farms were three. Topographic details of the selected farms are as shown in **Table 3.1**. Agro-ecological zones and agro-climatic classification of the five studysitesare described in **Table 3.2**. Collected samples were transported in cool boxes and stored in refrigerator at 4°C for further processing. Soil pH and EC were determined based on a 1:2.5 soil to water ratio; N percentage using the Kjeldahl method, basic cations (K, Ca and Mg) using ammonium acetate method and P percentage using calorimetric method.

Table 3.1: Topographical details of study sites

Study site	*Site no.*	*Site ID*	*Latitude (S)*	*Longitude (E)*	*Altitude asl (m)*
KisiiCentral (Kiogoro)	1	A	00°42′00.7″	034°45′46.1″	1824
		B	00°43′47.0″	034°46′46.8″	1878
		C	00°44′14.3″	034°46′27.3″	1870
		D	00°43′21.1″	034°48′38.4″	1827
		E	00°42′31.8″	034°49′47.9″	1795
Imenti North (Miriga Mieru)	2	A	00°01′17.9″	037°41′19.7″	1381
		B	00°03′16.3″	037°37′52.0″	1755
		C	00°03′10.6″	037°37′54.3″	1764
		D	00°03′12.5″	037°37′48.1″	1759
		E	00°02′46.7″	037°38′11.9″	1772
Embu (Embu Central & Manyata)	3	A	00°30′07.7″	037°27′08.5″	1497
		B	00°25′35.5″	037°29′17.1″	1722

Study site	*Site no.*	*Site ID*	*Latitude (S)*	*Longitude (E)*	*Altitude asl (m)*
		C	00°26′32.1″	037°29′21.9″	1652
		D	00°24′35.1″	037°28′12.9″	1760
		E	00°24′33.1″	037°28′20.6″	1758
Murang'a South (Maragua)	4	A	00°48′19.1″	037°07′15.9″	1374
		B	00°49′17.8″	037°08′42.9″	1402
		C	00°50′01.1″	037°10′09.5″	1315
		D	00°49′52.8″	037°09′58.1″	1333
		E	00°50′18.0″	037°09′49.0″	1328
Juja	5	A	01°05′12.0″	037°00′33.1″	1542
		B	01°08′13.2″	037°00′57.7″	1530
		C	01°07′47.8″	037°01′25.1″	1530

Table 3.2: Agro-ecological zones and agro-climatic classification of study sites

Study site	*Zone belt*	*Agro-climatic classification*	*Elevation (m asl)**	*Soil type*	*Soil pH**	*Annual mean rainfall (mm)*	*Annual mean temperature (°C)*
Kisii Central (Kiogoro)	UM1	I - Humid	1780-1880	ando-humic NITISOLS	5.1 – 6.9	1605	19.4
Imenti North (Miriga Mieru)	UM2	II - Sub-humid	1370-1770	humic NITISOLS	5.9 - 7.37	1083	20.7
Embu (Embu Central & Manyata)	UM1	I - Humid	1430-1760	ando-humic NITISOLS	4.46 - 6.6	1091	21.4
Murang'a South (Maragua)	UM3	III - Semi-humid	1300-1400	eutric NITISOLS	4.79 - 7.5	1074	20.5
Juja	UM4	IV - Semi-humid to semi-arid	1530-1542	rhodic FERRALSOLS	6.6 - 7.65	1074	20.5

Adopted and modified from World Bank Climate Change Knowledge Portal 2.0 and Niels H. Batjes, (2006). Key: UM – Upper midland. Numbers 1 to 4 in the second column correspond to the agro-climatic classification Ito IV in the third column. *From baseline data collected in the present study.

CHAPTER FOUR

4.0 ISOLATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA ASSOCIATED WITH BANANAS IN KENYA

4.1. Introduction

According to Senthilkumar *et al.* (2011), of the roughly 300,000 plant species found on earth, each plant is a host to one or more endophytes, however only few plants have been completely studied in regard to their endophytic biology. As a result, the opportunity to find novel and unique endophytic microbes amongnumerous plants in different ecosystems is considerable. In bananas, genera and species of endophytic bacteria identified have included: *Azospirillum brasilense* and *A. amazonense* (Weber *et al*., 1999), *Bacillus* spp. (Harish, 2008), *Burkholderia* spp. (Weber *et al*., 1999 and Ting *et. al*., 2008), *Citrobacter* spp. (Martínez *et al*., 2003), *Enterobacter* spp. (Martínez *et al*., 2003), *Herbaspirillum* spp. (Weber *et al*., 1999 and Weber *et al.*, 2001), *Klebsiella* spp. (Martínez *et al*., 2003 and Rosenblueth *et al*., 2004), *Pseudomonas* spp. (Harish, 2008 and Ting *et. al*., 2008), *Rhizobium* spp. (Martínez *et al.*, 2003) and *Serratia* spp. (Ting *et. al.*, 2008). However, to the author's knowledge, endophytic bacteria of bananas in Kenya have not been isolated or identified. Thus, there is limited information on their diversity and their functional potentiality in regard to banana growth and nutrition.

Isolation of bacteria from surface sterilized plant material usually allows recovery of large numbers of putative endophytic bacteria. Identification and classification of such large numbers of microorganisms using 16S rRNA gene sequencing is expensive and time consuming. Recent advances in mass spectrometry have however shed light on a rapid and precise identification and classification of

microorganisms. Whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) profiling of whole cell proteins is such an emerging technology for rapid microorganism profiling and identification of large numbers of microorganisms. The intact molecular weights of the ionized proteins are directly measured and the pattern of the protein molecular weights used as fingerprints. The fingerprints can be used to construct a hierarchical dendrogram (cluster analysis) based on the spectral similarity (Tani *et al.*, 2012).

Imported microbial inoculants could be used for banana production in Kenya but because of the fitness challenge, there is need to isolate domestic bacteria from bananas in practical farm fields and assess their functional potentiality as biological fertilizers. The objective of this study was therefore to isolate and identify endophytic bacteria associated with bananas in Kenya,in view of their functional potentiality as biofertilizers.

4.2. Materials and Methods

4.2.1. Surface sterilization

The banana samples namely roots, corm and stem were thoroughly washed in running tap water. They were then surface-sterilized using 70% ethanol for 2 minutes and immersed in 150 ml of 1.5% sodium hypochlorite plus a few drops of Tween 20 for 5 minutes with shaking. The samples were then rinsed thoroughly in five changes of sterile distilled water and dried in sterile paper towels.

4.2.2. Isolation and characterization

Surface sterilized samples were macerated with a sterile mortar and pestle and then serially diluted in 12.5 mM potassium phosphate buffer at pH 7. To target a wide range of bacterial endophytes, five different isolation media were used; LGI solid

medium(0.2 g K_2HPO_4, 0.6 g KH_2PO_4, 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 0.002 g $NaMoO_4$0.01 g $FeCl_3$, 0.2 g BT_B, 0.02 g yeast extract, 100 g sucrose, per litre, and 1.5% agar at pH 7.0)(Cavalcante and Dobereiner, 1988), nitrogen-free media (0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.2 g yeast extract, 15 mg $FeCl_3.6H_2O$, 6.6 g DL-Na-Malate, per litre, and 1.5% agar at pH 7.0)(Dobereiner*et al.*, 1976), MacConkey and Congo Red as on package (Rodriguez Caceres, 1982), YEM agar (10 g Mannitol, 0.5 g K_2HPO_4, 0.8 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.01 g $FeCl_3$, 1 g yeast extract, per litre, and 1.5% agar at pH 7.0) (Vincent, 1970) and nutrient agar (HIMEDIA, HiMedia Laboratories Pvt. Ltd.). 0.75 g/l antifungal (Nystatin) was added to the isolating media after autoclaving. Cultivation of the isolates was done at 30°C, which is within the optimum range of temperatures (20°C - 40°C)for the growth of mesophilic environmental microbes.Extensive colony purification was done to attain single strain cultures. Morphological characterization was done on the basis of colony color, appearance, motility and Gram staining and based on the colony morphotypes selection of representative isolates was done.Biochemical characterization was based on tests that includedgelatin hydrolysis, starch hydrolysis, catalase production, nitrate reduction, citrate and sugar utilization, growth in NaCl, Methyl red test and urease test according to the Bergey's Manual of Determinative Bacteriology9thEdition (Williams and Wilkins, 1994).

4.2.3. Microorganism profiling with MALDI-TOF/MS

Profiling of isolated bacteria was done using matrix-assisted laser desorption / ionization time of flight mass spectrometry (MALDI-TOF/MS, Bruker Daltonics). MALDI-TOF/MSprofiling of whole cell proteins is an emerging technology for rapid microorganism profiling and identification of large numbers of microorganisms.The

bacterial cells (five colonies) grown on nutrient agar medium were placed into an eppendorf tube and 100 µl water added and mixed carefully. Three hundred microliter ethanol was added and mixed well. The mixture was then centrifuged at 15,000 rpm for 2 minutes at 4℃ and supernatant was removed. The pellet was vacuum dried for 10 minutes to completely remove ethanol. Five microliter 70% formic acid was added to the pellet and mixed well using a vortex. Five microliter acetonitrile was then added and the mixture centrifuged at 15,000 rpm for 2 min at 4°C.The supernatant of 1 µl was transferred to MALDI-plate, allowed to dry and then overlaid with 2 µl matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid (α-HCCA) in 50% acetonitrile and 2.5% tri-fluoro-acetic acid). The samples were analyzed with MALDI-TOF/MS equipped with a 50 Hz nitrogen laser (Ultraflex, Bruker Daltonics). The intact molecular weights of the ionized proteins were directly measured and the pattern of the protein molecular weights used as fingerprints. Mass spectra were obtained using a positive linear mode in the range of mass to charge ratio (m/z) 2,000 to 20,000 (Tani *et al.*, 2012).The fingerprints werethen used to construct a hierarchical dendrogram (cluster analysis) based on the spectral similarity (Tani *et al*., 2012).

4.2.4. Identificationof bacterial isolates

Approximately 1.5-kb 16S rRNA genes of the bacterial isolates were amplified by polymerase chain reaction using Eu8f (5’-AGAGTTTGATCCTGGCTCAG-3’) and Eu1492r (5’-GGCTACCTTGTTACGACTT-3’) primers(Weisburg *et al.*, 1991). The PCR mixture composed of 0.1 µl Blend Taq Plus DNA polymerase (TOYOBO, Japan), 2 µl dNTP (2 mM), 2.5 µl Blend Taq Plus DNA polymerase buffer, 1 µl of each primer (12.5 µM), 17.4 µl sterile MilliQ water and 1 µl sample DNA. The

sample DNA was extracted by putting cells of a single bacterial colony in 40 µl BL buffer plus 10 µl Proteinase K solution followed by incubation at 60°C for 20 min and then at 95°C for 5 min.The thermal program was 2 min at 96°C and then 30 sec at 95°C, 30 sec at 52°C, 1.5 min at 72°C for 25 cycles, followed by final polymerization for 5 min at 72°C. Five microliter of the reaction mixture was used for agarose gel electrophoresis. Lambda EcoT14l was used as the standard marker with 0.7% agarose gel.The DNA bandswere visualized by staining with ethidium bromide. The amplified DNA (amplicons) were purified with the DNA Fragment Purification kit (MagExtractor, TOYOBO, Japan) and subjected to DNA sequencing according to the manufacture's protocol (BigDye Ready Reaction Mix, Applied Biosystems) using EU8f and EU518r (5'-GTATTACCGCGGCTGCTGG-3') primers. Some selected isolates were subjected to full-length sequencing using EU8f, Eu1492r, Eu518r, Eu1093r (5'-TTGCGCTCGTTGCGGGACT-3'), Eu803r (5'-CATCGTTTACGGCGTGGAC-3'), Eu1389r (5'-ACGGGCGGTGTGTACAAG-3') and Eu1092f (5'-AAGTCCCGCAACGAGCGCA-3') primers. Sequencing was carried out using an automated DNA sequencer (model 3130; Applied Biosystems) and ca. 1.5 kb sequence was determined.Obtained sequences were processed using the Staden Package software (Bonfield *et. al*., 1995) and BIOEDIT sequence alignment editor. Assembled sequences were then analyzed at EzTaxon-e database (Kim *et al*., 2012).

4.3. Results

4.3.1. Isolation and characterization

With five different isolation media, a total of 2,717 isolates were obtained and 214 representative isolates selected on the basis of their colony morphotypes. Most of the cells exhibited formation of creamy coloured colonies and the cells were motile, Gram negative rods as observed under the microscope. A few were motile Gram positive cells while a few others were non-motile Gram negative cells. Most of the isolates exhibited catalase positive reaction, negative starch hydrolysis reaction with a few isolates from Kisii Central District exhibiting high starch hydrolysis reaction. Majority of the isolates also showed positive citrate utilization reation except for Maragua District isolates whose citrate utilization reaction was mostly negative.

4.3.2. Microorganism profiling with MALDI-TOF/MS

The selected isolates were profiled using MALDI-TOF/MS.Isolates that showed similarity higher than 60% were regarded as same species due to high mass spectrometric pattern similarity, and those showing less than that were regarded as different species. The profiling resulted into 53 clusters, which based on partial 16S rRNA gene sequencing could be grouped into three families *Bacillaceae*, *Pseudomonadaceae* and *Enterobacteriaceae* (**Figure 4.1**).From the 53 clusters 43 representative isolates were selected for 16S rRNA partial gene sequencing.

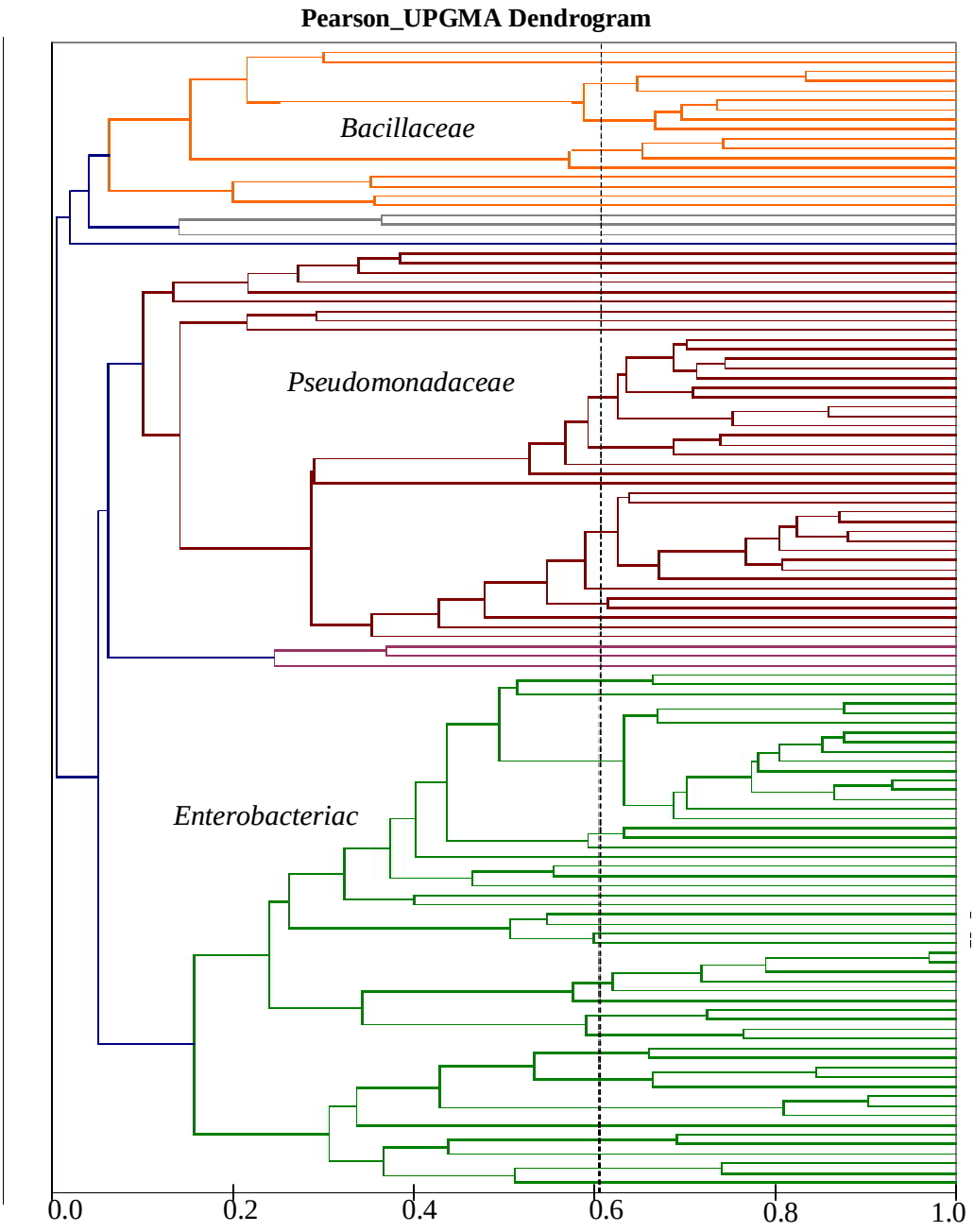


Figure 4.1: Microorganism profiling using MALDI-TOF/MS. The profiling resulted into 53 clusters, which could be grouped into three main families namely *Bacillaceae* (orange colour), *Pseudomonadaceae* (brown colour) and *Enterobacteriaceae* (green colour).

4.3.3. Identificationof bacterial isolates

PCR amplification of the 43 bacterial isolates selected for sequencing yielded approximately 1.5 kb 16S rRNA genes (**Plate 4.1**). Partial sequencing of the 16S rRNA genes grouped them into three families namely*Enterobacteriaceae, Pseudomonadaceae* and *Bacillaceae* (**Table 4.1**). The family *Enterobacteriaceae* was the most diverse with 8 genera namely *Serratia* (17 isolates), *Rahnella* (4 isolates), *Enterobacter* (2 isolates), *Yokenella* (2 isolates), *Raoultella* (2 isolates), *Klebsiella* (1 isolate), *Yersinia* (1 isolate) and *Ewingella* (1 isolate). Both the *Pseudomonadaceae* and *Bacillaceae* families were represented by only one genus, that is,*Pseudomonas* (12 isolates) and *Bacillus* (1 isolate), respectively. *Serratia* and *Pseudomonas* species were the most abundant with 17 isolates and 12 isolates,respectively. The isolates' similarity values with their 16S rRNA closest relatives ranged between 95.27% and 100%. Isolate K22V1c (2) identified as *Klebsiella granulomatis*had the lowest similarity value at 95.27%.

Full-length sequencing allowed identification of isolates M9V1r, J1V1r, K32V2c, ME19V2c and K50V2s as *Bacillus subtilis subsp. inaquosorum*, *Enterobacter ludwigii*, *Ewingella americana*, *Rahnella aquatilis* and *Flavimonas oryzihabitans* respectively. The sequence data generated have been deposited with the NCBI GenBank under accession numbers AB675632 to AB675636.

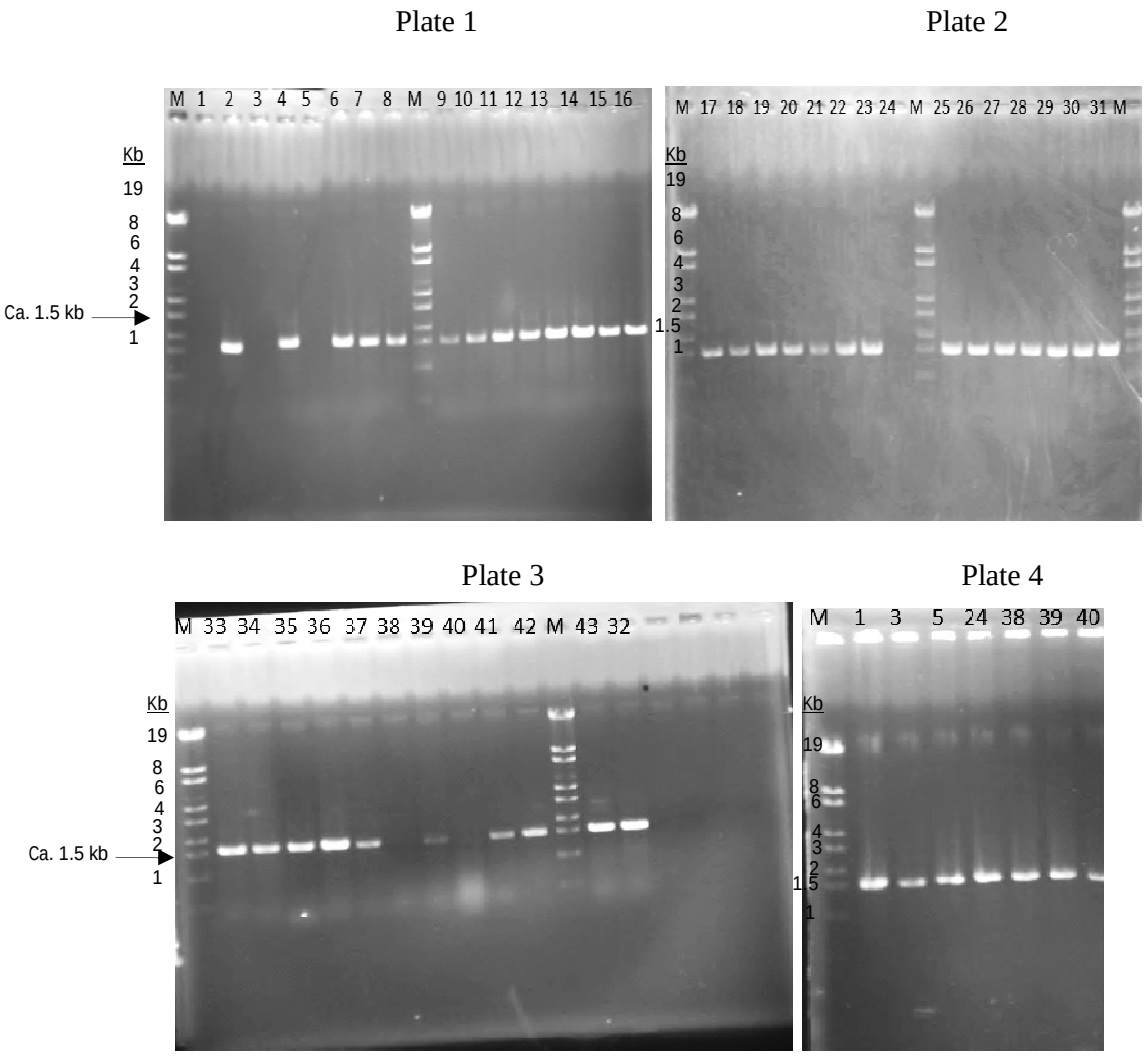


Plate 4.1: Gel electrophoresis of amplified ca. 1.5 kb 16S rRNA gene of the 43 bacterial isolates. Numbers 1- 43 corresponds to the serial numbers of the 43 bacterial isolates indicated in brackets on table 3.1 in the 1st column. Lambda EcoT14l was used as the standard marker (M) with 0.7% agarose gel.

Table 4.1: Probable identification of 43 endophytic bacteria isolates from banana plants in Kenya in 2009/2010 based on partial sequencing of 16S rRNA gene. Similarity values with their 16S rRNA closest relatives ranged between 95.27% and 100%.

Isolate ID	16S rRNA closest relative	Accession no.	Similarity
M9V1r (30)	*Bacillus subtilis subsp. subtilis* NCIB 3610(T)	ABQL01000001	99.74
K34V2c (23)	*Pseudomonas protegens* CHA0(T)	AJ278812	97.24
J1V1r (31)	*Enterobacter asburiae* JCM 6051(T)	AB004744	100.00
M28V2s (28)	*Pseudomonas koreensis* Ps 9-14(T)	AF468452	97.87
ME10V1r (35)	*Serratia glossinae* C1(T)	FJ790328	99.30
K50V2s (43)	*Flavimonas oryzihabitans* IAM 1568(T)	D84004	99.03
K49V2s (21)	*Pseudomonas palleroniana* CFBP 4389(T)	AY091527	99.87
K49V2s (22)	*Pseudomonas palleroniana* CFBP 4389(T)	AY091527	99.87
E29V2c (3)	*Pseudomonas psychrophila* E-3(T)	AB041885	97.81
E18V1c (16)	*Pseudomonas graminis* DSM 11363(T)	Y11150	99.02
K50V2s (27)	*Flavimonas oryzihabitans* IAM 1568(T)	D84004	98.91

Isolate ID	16S rRNA closest relative	Accession no.	Similarity
K23V1c (4)	*Pseudomonas protegens* CHA0(T)	AJ278812	98.78
K10V1r (12)	*Pseudomonas moraviensis* CCM 7280(T)	AY970952	99.74
K39V1s (10)	*Pseudomonas protegens* CHA0(T)	AJ278812	99.23
K36V2c (37)	*Pseudomonas protegens* CHA0(T)	AJ278812	99.50
E35V1s (18)	*Serratia fonticola* DSM 4576(T)	AJ233429	99.54
E17V1c (8)	*Serratia fonticola* DSM 4576(T)	AJ233429	99.44
E17V1c (19)	*Serratia glossinae* C1(T)	FJ790328	99.58
J22V1c (41)	*Serratia glossinae* C1(T)	FJ790328	100.00
ME7V1r (32)	*Serratia glossinae* C1(T)	FJ790328	100.00
E2V1r (13)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.61
E15V1c (15)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.10
E10V1r (14)	*Serratia fonticola* DSM 4576(T)	AJ233429	99.86
E13V2r (26)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.62

Isolate ID	16S rRNA closest relative	Accession no.	Similarity
K30V2c (34)	*Serratia proteamaculans* DSM 4543(T)	AJ233434	99.46
E13V2r (20)	*Serratia plymuthica* DSM 4540(T)	AJ233433	99.39
M20V2c (25)	*Serratia proteamaculans* DSM 4543(T)	AJ233434	99.23
M20V2c (40)	*Serratia plymuthica* DSM 4540(T)	AJ233433	100.00
ME19V2c (42)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.87
E25V2c (7)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.39
ME18V2c (36)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.24
ME19V2c (24)	*Rahnella aquatilis* DSM 4594(T)	AJ233426	99.86
E43V2 (1)	*Yersinia kristensenii* ATCC 33638(T)	ACCA01000078	98.40
K32V2c (39)	*Ewingella americana* GTC 1277(T)	AB273745	99.50
ME18V2c (6)	*Serratia glossinae* C1(T)	FJ790328	98.47
ME8V2r (11)	*Serratia glossinae* C1(T)	FJ790328	98.83
K29V1c (5)	*Raoultella terrigena* ATCC 33257(T)	Y17658	99.82

Isolate ID	16S rRNA closest relative	Accession no.	Similarity
K22V1c (2)	*Klebsiella granulomatis* KH 22	AF010251	95.27
M32V1s (33)	*Yokenella regensburgei* GTC 1377(T)	AB273739	100.00
K32V2c (29)	*Raoultella terrigena* ATCC 33257(T)	Y17658	99.47
K24V1c (9)	*Serratia ureilytica* NiVa 51(T)	AJ854062	98.05
E41V2 (17)	*Enterobacter amnigenus* JCM 1237(T)	AB004749	99.43
J4V1c (38)	*Yokenella regensburgei* GTC 1377(T)	AB273739	99.62

4.3.4. Bacterial isolates distribution

Serratia isolates were found in all the five study sites, in both the banana cultivars and in all the three tissues. *Pseudomonas* isolates were found in three of the five study sites with majority of them (9 isolates) isolated from Kisii Central District. They were however found in both the banana cultivars and in all the three plant tissues. On the other hand, *Rahnella* isolates were found only in Embu and Imenti North (Meru)Districts and only in the corm tissues of the cooking variety of banana. For the other isolates it was difficult to conclude on their host and area specificity because they were represented by only one or two isolates. Kisii Central and Embu Districts, both belonging to the agroecological zone UM1, humid with ando-humic NITISOLS, exhibited the highest bacterial isolates diversity with a total of ten and eight different species respectively. Maragua District of agroecological zone UM3, semi-humid with eutricNITISOLS and Juja District of agroecological zone UM4, semi-humid to semi-arid with rhodic FERRALSOLS had five and three different species respectively while Imenti North (Meru)District of agroecological zone UM2, sub-humid with humic NITISOLS displayed the lowest diversity with only two species (**Table 4.2**).

Table 4.2: Distribution of endophytic bacteria associated with bananas in Kenya in 2009/2010.

Study site	Zone belt	Soil type	Banana cultivar	Tissue type	Isolates' 16S rRNA closest relative
KisiiCentral (Kiogoro)	UM1	ando-humic NITISOLS	V1	root	*Pseudomonas moraviensis* CCM 7280(T)
				corm	*Klebsiella granulomatis* KH 22
				corm/stem	*Pseudomonas protegens* CHA0(T)
				corm	*Serratia ureilytica* NiVa 51(T)
				corm	*Raoultella terrigena* ATCC 33257(T)
			V2	corm	*Serratia proteamaculans* DSM 4543(T)
				corm	*Raoultella terrigena* ATCC 33257(T)
				corm	*Ewingella americana*GTC 1277(T)
				corm	*Pseudomonas protegens* CHA0(T)
				stem	*Pseudomonas palleroniana* CFBP 4389(T)
				stem	*Flavimonas oryzihabitans* IAM 1568(T)
Embu (Embu Central & Manyata)	UM1	ando-humic NITISOLS	V1	stem/corm/root	*Serratia fonticola* DSM 4576(T)
				root/corm	*Serratia plymuthica* DSM 4540(T)
				corm	*Serratia glossinae* C1(T)
				corm	*Pseudomonas graminis* DSM 11363(T)
			V2	root	*Serratia plymuthica* DSM 4540(T)
				corm	*Rahnella aquatilis* DSM 4594(T)
				corm	*Pseudomonas psychrophila* E-3(T)
				-	*Enterobacter amnigenus* JCM 1237(T)
				-	*Yersinia kristensenii* ATCC 33638(T)

Study site	Zone belt	Soil type	Banana cultivar	Tissue type	Isolates' 16S rRNA closest relative
Imenti North (Miriga Mieru)	UM2	humic NITISOLS	V1	root	*Serratia glossinae* C1(T)
			V2	corm	*Rahnella aquatilis* DSM 4594(T)
				corm/root	*Serratia glossinae* C1(T)
Murang'a South (Maragua)	UM3	eutric NITISOLS	V1	stem	*Yokenella regensburgei* GTC 1377(T)
				root	*Bacillus subtilis subsp. subtilis* NCIB 3610(T)
			V2	corm	*Serratia proteamaculans* DSM 4543(T)
				corm	*Serratia plymuthica* DSM 4540(T)
				stem	*Pseudomonas koreensis* Ps 9-14(T)
Juja	UM4	rhodic FERRALSOLS	V1	root	*Enterobacter asburiae* JCM 6051(T)
				corm	*Serratia glossinae* C1(T)
				corm	*Yokenella regensburgei* GTC 1377(T)

Key: UM1 – Upper midland (humid); UM2 – Upper midland (sub-humid); UM3 – Upper midland (semi-humid); UM4 – Upper midland (semi-humid to semi-arid); V1 – dessert banana cultivar; V2 – cooking (plantain) banana cultivar.

4.4. Discussion

Endophytic bacteria of bananas in Kenya have previously not been isolated or identified and consequently there has beenlimited information on their diversity and their functional potential in regard to banana growth and nutrition. In this study, 10 genera of endophytic bacteria of bananas were isolated, profiled and identified as*Serratia, Pseudomonas, Rahnella, Enterobacter, Yokenella, Raoultella, Klebsiella, Yersinia,Ewingella* and *Bacillus.* This endophytic bacteria population differed from that of previously reported endophytic bacteria associated with bananas, which include *Azospirillum brasilense* and *A. amazonense* (Weber *et al*., 1999), *Bacillus* spp. (Harish, 2008), *Burkholderia* spp. (Weber *et al*., 1999; Ting *et. al*., 2008), *Citrobacter* spp. (Martínez *et al*., 2003), *Enterobacter* spp. (Martínez *et al*., 2003), *Herbaspirillum* spp. (Weber *et al*., 1999; Weber *et al*., 2001), *Klebsiella* spp. (Martínez *et al*., 2003; Rosenblueth *et al*., 2004), *Pseudomonas* spp. (Harish, 2008; Ting *et. al*., 2008), *Rhizobium* spp. (Martínez *et al*., 2003) and *Serratia* spp. (Ting *et. al*., 2008). Of the 10 genera identified; *Rahnella, Yokenella, Raoultella, Yersinia* and*Ewingella*had not been previously described as endophytic in banana plants.This suggests richness of endophytic bacterial species associated with bananas than has so far been reported. The diversity of endophytic bacteria identified in this study could be explained by the different agroecological conditions of study sites and the unique East African highland banana cultivars especially the cooking banana variety (*Musa* AAB) whose endophytic biology has not been widely studied. It is worth noting that*Rahnella, Raoultella, Yersinia* and*Ewingella* were isolated from the corm of the cooking banana variety(*Musa* AAB).These findings agree with those of Azevedo *et al.*(2000) who showed that tropical plant hosts contain a great diversity of endophytic

microorganisms, many of them not yet classified and possibly belonging to new genera and species. In addition, isolate K22V1c is being proposed as a novel species havingshowed a similarity value of less than 97% (95.27%) with its closest relative*Klebsiella granulomatis*.

In this study, *Serratia*species were found to benon-host and non-area specific.Ting *et al*. (2008) also found *Serratia*species to be non-host specific. *Pseudomonas* species were non-host specific but area specific while*Rahnella*species were found to be host and area specific. For the other species it was difficult to conclude on their host and area specificity because they were represented by atmost two species. Humid conditions and ando-humic NITISOLS soil type typical of Kisii Central and Embu Districts seemed to favour bacteriadiversity as compared to sub-humid conditions with humic NITISOLS soil type found in Imenti North District in Meru.

In general, the distribution of bacterial species in the banana plants sampled depended on plant variety, tissue sampled, soil and agro-climatic conditions. Similarly, Zinnel *et al*.(2002) attributed variations in the populations of both indigenous and introduced endophytes to plant source, plant age, tissue type, time of sampling, and environment.

4.5. Conclusions

The composition of endophytic bacteria associated with bananas in Kenya in 2009/2010 was uniquein that 5 of the 10 genera identified had previously not been described as endophytic in banana plants. In regard to host and area specificity, *Serratia*species were found to be non-specific while *Rahnella*species were specific. *Pseudomonas* species were non-host specific but area specific. Kisii Central and

Embu Districts, both of agroecological zone UM1 (humid), exhibited the highest bacterial isolates diversity while Imenti North (Meru) Districtof agroecological zone UM2 (sub-humid) displayed the lowest diversity.

CHAPTER FIVE

5.0 PLANT GROWTH PROMOTING POTENTIAL OF BANANA ENDOPHYTIC BACTERIA IN KENYA

5.1. Introduction

In Kenya, banana production is constrained by among others, declining soil fertility (Vanlauwe and Giller, 2006; Okumu, 2008). This is brought about by insufficient application of manure due to cost implications especially for the farmers without livestock and limited use of inorganic fertilizers, which are expensive and therefore unaffordable for most banana farmers in Kenya. This means that the nutrientsare not adequately replenished.The most obvious solution to nutrient replenishment is increased use of chemical fertilizers. However, these are expensive and out of reach for most resource-poor farmers, who constitute the vast majority of banana farmers in Kenya. Additionally, use of chemical fertilizers is not environment friendly.

A sustainable complementary approach would be to increase the biological inputs of nutrients by exploitation of microorganisms, which are largely untapped natural resources for plant growth promotion (Thomas and Soly, 2009; Uribe *et al.*, 2010). Microbial inoculants based on Arbuscular mycorrhizal fungi have successfully been used by small scale farmers on bananas in some tropical countries like Colombia, Malaysia and Cuba (Uribe *et al.*, 2010). Endophytes are also increasingly gaining scientific and commercial interest because of their potential to improve plant quality and growth and their close association with internal tissues of host plants (Carroll, 1992; Schulz *et al.*, 1998; Schulz *et al.*, 1999).Endophytes are of agronomic interest in that they can enhance plant growth in non-leguminous crops and improve their nutrition through nitrogen fixation, phosphate solubilization or

iron chelation (Dobereiner and Baldani, 1998; Sturz *et al*., 2000; Boddey *et al*., 2003; Iniguez *et al*., 2004; Ryan et al., 2008; Uribe *et al*., 2010). Imported microbial inoculants could be used for banana production in Kenya but because of the fitness challenge, there is need to isolate locally occuring bacteria from bananas in our practical farm fields and assess their functional potentiality as biological fertilizers.

The objective of this study was to determine the isolates' capacity to fix free nitrogen, solubilize phosphates and produce siderophores*in vitro*.

5.2. Materials and Methods

5.2.1. Bacterial isolates tested

The 43 bacterial isolates used in this study belonged to the genera*Serratia* (17 isolates), *Pseudomonas* (12 isolates), *Rahnella* (4 isolates), *Enterobacter* (2 isolates), *Raoultella* (2 isolates), *Yokenella* (2 isolates), *Bacillus* (1 isolate), *Klebsiella* (1 isolate), *Yersinia* (1 isolate) and *Ewingella* (1 isolate) as detailed in Chapter 3.

5.2.2. Screening for nitrogen fixation ability

To determine the isolates' ability to fix atmospheric nitrogen, qualitative screening of growth was done on solid N-free medium while nitrogenase activity was determined using the acetylene reduction assay. A molecularapproach based on PCR amplification of the *nifH* gene was also used.

5.2.2.1. Growth on solid N-free medium

Bacterial isolates were culturedon solid N-free medium (1 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 1 g $CaCO_3$, 0.2 g NaCl, 5 mg $FeSO_4.7H_2O$, 10 g glucose, 5 mg $NaMoO_4$ per litre, and 1.5% agar at pH 7.0) and their growth observed at four and ten days post inoculation. Growth on the N-free medium was used as an indication of isolates' ability to fix free nitrogen.

5.2.2.2. Acetylene Reduction Assay (ARA)

Organisms that are capable of fixing atmospheric dinitrogen (N_2) possess the enzyme nitrogenase, which reduces N_2 to ammonia (NH_3). The enzyme nitrogenase catalyzes not only the reduction of dinitrogen, but also a variety of other substrates like acetylene (C_2H_2). The reduction of acetylene (C_2H_2) to ethylene (C_2H_4) is widely used as a method of measuring nitrogenase activity in natural samples, isolates and cell-free extracts (Rogel *et al*., 2001). The isolates were grown for 3 days in N-free medium. Individual colonies were placed in 5 ml of semi-solid N-free medium containing 2.3 g of agar per litre in 10 ml vials. Acetylene gas was then added to attain a concentration of 12% v/vand ethylene production was determined after 12 hours on a Shimadzu Gas Chromatograph (**Plate 5.1**) as described by Rogel *et al.*, (2001). The GC column initial temperature was 120°C while injection and flame ionization detector temperatures were both at 220°C.

Plate 5.1: Shimadzu Gas Chromatograph (GC-9A) at the Food Science and Technology Department, JKUAT.

5.2.2.3. *nifH*-gene analysis

Organisms' N fixation ability was also determined using a molecular approach, which is based on PCR amplification of a marker gene known as *nifH* gene. The gene codes for the enzyme nitrogenase reductase. For the *nifH*-gene amplification the organisms' genomic DNA was extracted and then purified using the genomic DNA purification kit. The purified genomic DNA from the isolates was subjected to nested PCR. This allowed an extra level of specificity using two different sets of primers, one set internal to the other. The PCR primers were used as described by Burgmann *et al*. (2004). The first set of degenerate primers (*nifH* – universal for A site and reverse site) were forward primer 5'-GCIWTITAYGGNAARGGNGG-3' and reverse primer 5'-GCRTAIABNGCCATCATYTC-3'. The nested primers used were specific for the gammaproteobacteria namely forward primer (*nifH*-g1B) 5'-GGTTGTGACCCGAAAGCTGA-3' and reverse primer (*nifH*-g1R) 5'-GCGTACATGGCCATCATCTC-3'.

The thermocycler program was 5 min at 95°C (initial denaturation) and then 11 sec at 94°C (denaturation), 8 sec at 48°C (annealing), and 10 sec at 72°C (elongation) for 40 cycles, followed by a final polymerization step for 10 min at 72°C. One micro-liter of the 25 µl first reaction sample was used in the nested reactions. Two percent agarose gel was used for gel electrophoresis with 100bp ladder as the standard marker. For sequencing, the DNA Fragment Purification kit (MagExtractor – YOYOBO, Japan) was used for purifying the PCR products. Partial DNA sequencing was done using the BigDye Ready Reaction Mix (BigDye Terminator version 1.1) and the forward primer (*nifH*-g1B) 5'-GGTTGTGACCCGAAAGCTGA-3' and reverse primer (*nifH*-g1R) 5'-

GCGTACATGGCCATCATCTC-3'. The PCR conditions were 1 min at 96°C and then 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C for 25 cycles.

5.2.3. Screening for phosphate solubilization ability

Qualitative screening for phosphate solubilizing isolates was done using the National Botanical Research Institute's phosphate (NBRIP) growth medium (Nautiyal, 1999). The medium composed of 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2$, 0.25 g $MgSO_4$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ per litre and 1.5% agar at pH 7. Screening of phosphate solubilizers was based on formation of visible halo zones on agar plates, which is as a result of organism's production of organic acids into the surrounding medium that dissolves inorganic phosphate resulting to a clear zone around them. The halo size was used as a measure of relative efficiency of the isolates. The halo and colony diameters were measured at 13 and 21 days post inoculation. Halo size (mm) was calculated by subtracting colony diameter from the total diameter.

5.2.4. Screening for siderophore production ability

A universal method to detect and determine siderophores has been developed based on their high affinity for iron (III) (Schwyn and Neiland, 1987). A highly coloured complex of chrome azurol S, iron (III), and hexadecyltrimethylammonium bromide is used as the indicator. When a strong chelator like siderophore removes the iron from the highly coloured iron dye complex, its colour turns from blue to orange. This method is used for determination of siderophores in solution and paper electrophoresis, and its exceptionally high sensitivity allows its use also on agar plates, where orange halos around the colonies on blue agar are indicative of siderophore excretion.

Siderophore medium used composed of three solutions. Solution A composed of 750 ml distilled water, 100 ml MM9 (x10) salts, 15 g agar, 30.24 g PIPES, 10 g glucose and 50% NaOH solution to raise pH to the pKa of PIPES (6.8). 100 ml MM9 (x10) was made up of 0.3 g KH_2PO_4, 1.0 g NH_4Cl, 0.5 g NaCl, 0.2 mM (0.05 g) $MgSO_4$ and 0.1 mM (0.015 g) $CaCl_2$. Solution B was made up of 60.5 mg CAS in 50 ml water, 10 ml 1 mM $FeCl_3.6H_2O$ in 10 mM HCl and 72.9 mg hexadecyltrimethylammonium bromide (HDTMA) in 40 ml water whilesolution C was 30 ml 10% Casamino acid. The three solutions were autoclaved separately and mixed at the clean bench. Solution A was allowed to cool to 50°C, after which solution C was added. Solution B was then added to solutions A and C along the glass wall, with enough agitation to achieve mixing without generation of foam. Each plate received about 30 ml of the blue agar. Orange halos around colonies on blue agar indicated siderophore excretion. Data were taken four and seven days post inoculation.

5.3. Results

5.3.1. Nitrogen fixation ability of the isolates

All the 43 isolates showed growth on solid N-free medium (**Table5.1**); colony sizes ranged between 1 and 13 mm (**Figure 5.1**). However, the colony size may have also been a factor of the isolates ability to utilize glucose as a carbon source. The isolates also showed varied levels of nitrogenase activity with production of ethylene gas ranging between 0.0001 to 0.0514 µl/ml (**Figure 5.2**). Ethylene gas production was highest with strains from the Kisii Central District. **Plate 5.2**shows strainsfrom the Kisii Central District with higherGCethylene peak areas as compared to those of strains from Juja District.The higher the ethylene peak areas the higher the microbe's

nitrogenase activity. For the *nifH* gene analysis, purified genomic DNA was subjected to nested PCR that allows an additional level of specificity utilizing two different sets of primers, one set internal to the other.However, the first set of degenerate primers (*nifH*–universal for A site and reverse site) as well as the nested primers, which were specific for the gammaproteobacteria (*nifH*-g1B and *nifH*-g1R) did not result to any visible bands (**Plate 5.3**).

Table 5.1: Qualitative screening for bacterial isolates' ability to fix free nitrogen, solubilize phosphates and produce siderophores *in vitro*.

Isolate ID	**16S rRNA closest relative**	**N-fixation**	**Phosphates solubilization**	**Siderophore production**
M9V1r (30)	*Bacillus subtilis subsp. subtilis* NCIB 3610(T)	+	-	-
K34V2c (23)	*Pseudomonas protegens* CHA0(T)	+	+	+
J1V1r (31)	*Enterobacter asburiae* JCM 6051(T)	+	+	+
M28V2s (28)	*Pseudomonas koreensis* Ps 9-14(T)	+	+	+
ME10V1r (35)	*Serratia glossinae* C1(T)	+	+	-
K50V2s (43)	*Flavimonas oryzihabitans* IAM 1568(T)	+	+	++
K49V2s (21)	*Pseudomonas palleroniana* CFBP 4389(T)	+	+	+
K49V2s (22)	*Pseudomonas palleroniana* CFBP 4389(T)	+	+	+
E29V2c (3)	*Pseudomonas psychrophila* E-3(T)	+	+	+
E18V1c (16)	*Pseudomonas graminis* DSM 11363(T)	+	+	+

Isolate ID	**16S rRNA closest relative**	**N-fixation**	**Phosphates solubilization**	**Siderophore production**
K50V2s (27)	*Flavimonas oryzihabitans* IAM 1568(T)	+	-	++
K23V1c (4)	*Pseudomonas protegens* CHA0(T)	+	+	+
K10V1r (12)	*Pseudomonas moraviensis* CCM 7280(T)	+	+	+
K39V1s (10)	*Pseudomonas protegens* CHA0(T)	+	+	+
K36V2c (37)	*Pseudomonas protegens* CHA0(T)	+	+	+
E35V1s (18)	*Serratia fonticola* DSM 4576(T)	+	+	-
E17V1c (8)	*Serratia fonticola* DSM 4576(T)	+	+	-
E17V1c (19)	*Serratia glossinae* C1(T)	+	+	-
J22V1c (41)	*Serratia glossinae* C1(T)	+	+	-
ME7V1r (32)	*Serratia glossinae* C1(T)	+	+	-
E2V1r (13)	*Serratia plymuthica* DSM 4540(T)	+	-	-
E15V1c (15)	*Serratia plymuthica* DSM 4540(T)	+	-	-

Isolate ID	16S rRNA closest relative	N-fixation	Phosphates solubilization	Siderophore production
E10V1r (14)	*Serratia fonticola* DSM 4576(T)	+	+	-
E13V2r (26)	*Serratia plymuthica* DSM 4540(T)	+	-	-
K30V2c (34)	*Serratia proteamaculans* DSM 4543(T)	+	-	+
E13V2r (20)	*Serratia plymuthica* DSM 4540(T)	+	-	-
M20V2c (25)	*Serratia proteamaculans* DSM 4543(T)	+	-	-
M20V2c (40)	*Serratia plymuthica* DSM 4540(T)	+	-	-
ME19V2c (42)	*Rahnella aquatilis* DSM 4594(T)	+	++	-
E25V2c (7)	*Rahnella aquatilis* DSM 4594(T)	+	-	-
ME18V2c (36)	*Rahnella aquatilis* DSM 4594(T)	+	++	-
ME19V2c (24)	*Rahnella aquatilis* DSM 4594(T)	+	++	-
E43V2 (1)	*Yersinia kristensenii* ATCC 33638(T)	+	+	-
K32V2c (39)	*Ewingella americana* GTC 1277(T)	+	+	-

Isolate ID	16S rRNA closest relative	N-fixation	Phosphates solubilization	Siderophore production
ME18V2c (6)	*Serratia glossinae* C1(T)	+	+	-
ME8V2r (11)	*Serratia glossinae* C1(T)	+	+	-
K29V1c (5)	*Raoultella terrigena* ATCC 33257(T)	+	-	-
K22V1c (2)	*Klebsiella granulomatis* KH 22	+	-	-
M32V1s (33)	*Yokenella regensburgei* GTC 1377(T)	+	-	-
K32V2c (29)	*Raoultella terrigena* ATCC 33257(T)	+	-	-
K24V1c (9)	*Serratia ureilytica* NiVa 51(T)	+	-	-
E41V2 (17)	*Enterobacter amnigenus* JCM 1237(T)	+	-	-
J4V1c (38)	*Yokenella regensburgei* GTC 1377(T)	+	+	-

Key: positive (+); strongly positive (++); negative (-).

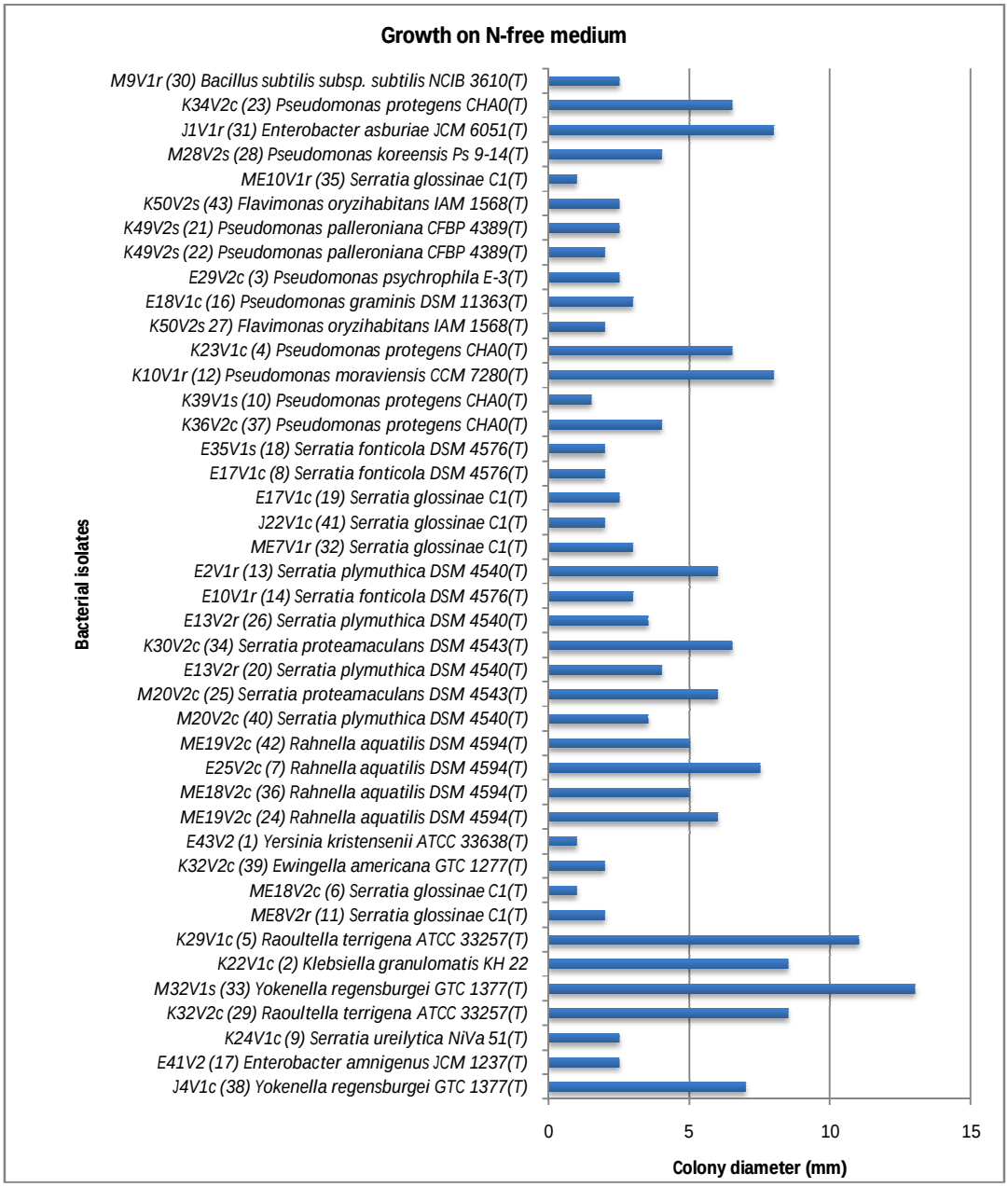


Figure 5.1: Growth of banana endophytic bacterial isolates on solid N-free medium 10 days after inoculation. All the 43 strains grew on N-free medium and colony sizes ranged between 1 and 13 mm.

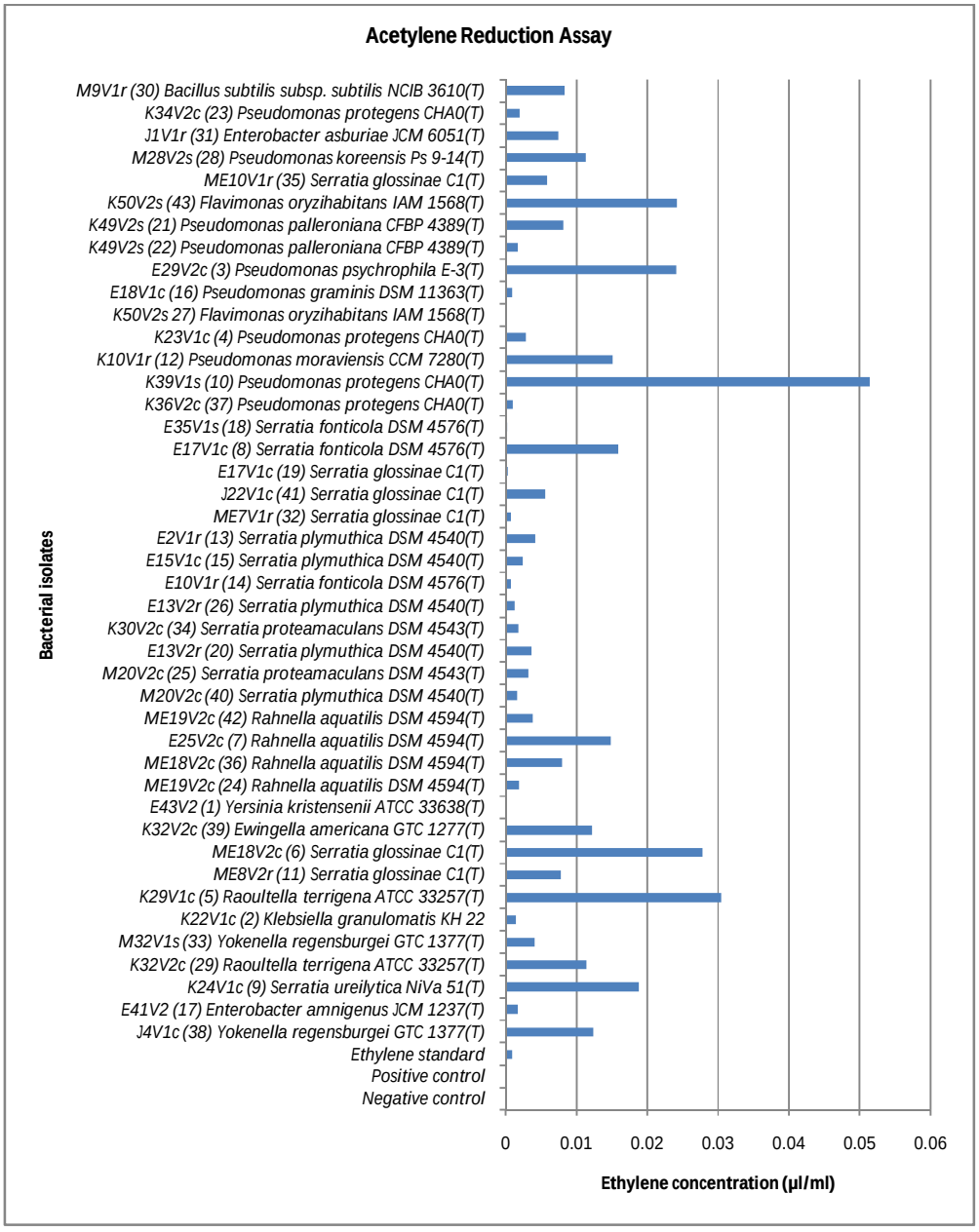


Figure 5.2: Qualitative screening of acetylene reduction activity on 43 bacterial isolates associated with bananas in Kenya. Positive control sample constituted acetylene gas without bacterial inoculation while negative control sample was without acetylene gas and without inoculation. Ethylene standard concentration was 0.000948 µl/ml.

Plate A Ethylene peaks Plate B

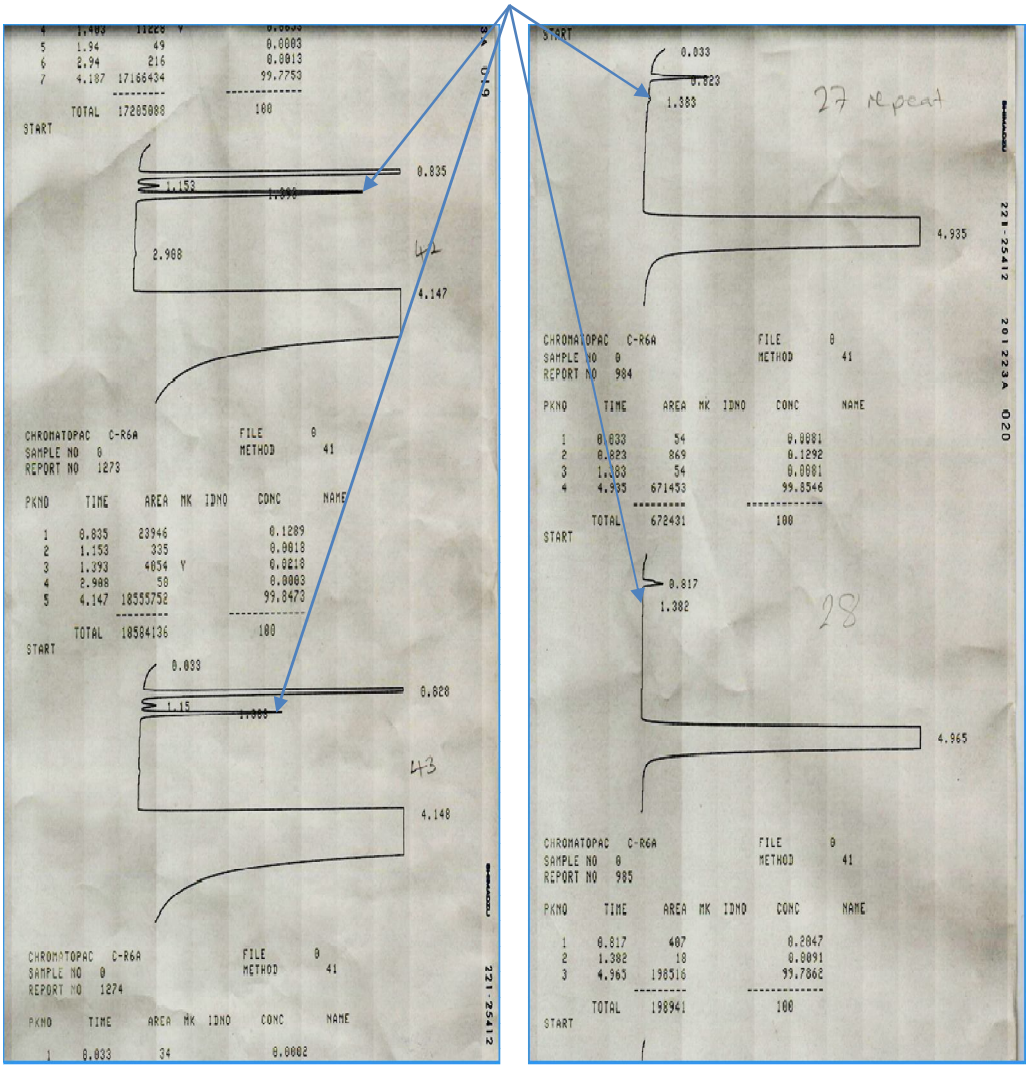


Plate 5.2: Acetylene Reduction Assay: Gas Chromatograph ethylene peak areas of some strains from Kisii Central District (plate A) and Juja District (plate B). The ethylene gas detection time was at 1.388.

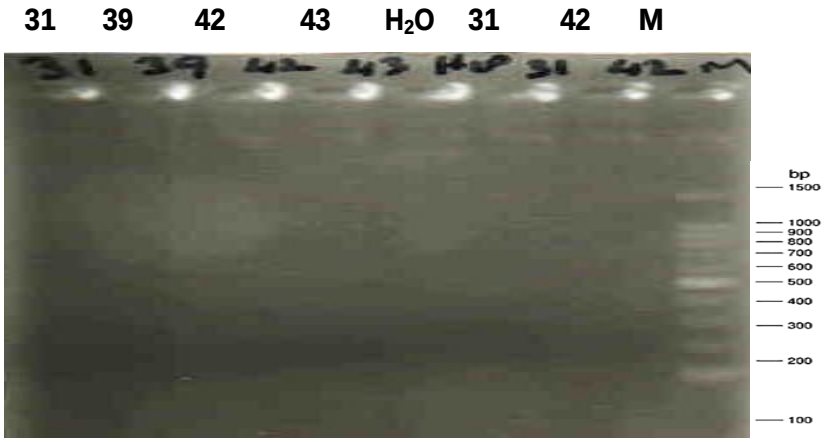


Plate 5.3: *nifH* gene amplification subjected to nested PCRfor *Enterobacter ludwigii* (31), *Ewingella americana* (39), *Rahnella aquatilis* (42) and *Flavimonas oryzihabitans* (43). H_2O was used as template in the control and 100-bp DNA ladder as the standard marker (M).

5.3.2. Phosphate solubilization ability of the isolates

All the 43 isolates grew on NBRIP medium albeit some colonies being very small (1.5 mm diameter). However, not all the isolates were observed to form visible dissolution halos on the NBRIP medium agar plates. Screening of isolates' phosphate solubilizing abilityis based on formation of visible halo zones on agar plates.At 13 days post inoculation (DPI) some strains had started forming visible dissolution halosbut most (63%) started forming visible dissolution halosat 21 DPI(**Figure 5.3**). The halo sizes ranged between 1.5 and 17 mm. The strainsthat showed positive activity included *Pseudomonas* (11 isolates), *Serratia* (9 isolates), *Rahnella* (3 isolates), *Enterobacter* (1 isolate), *Yersinia* (1 isolate), *Yokenella* (1 isolate) and *Ewingella* (1 isolate). Isolates ME19V2c (42), ME19V2c (24), and ME18V2c (36)

all *Rahnella aquatilis* had the largest halo size of 17 mm, 16 mm and 12 mm, respectively (**Plate 5.4**).A qualitative summary of the isolates' phosphate solubilization ability is shown on **Table 5.1**.

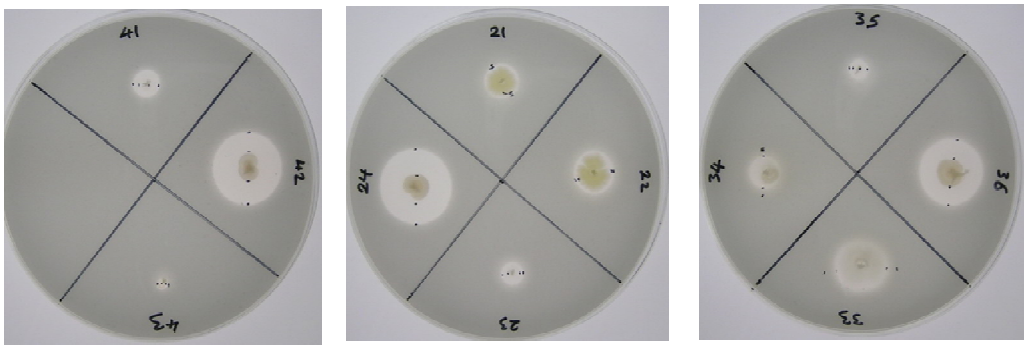


Plate 5.4: Qualitative screening for phosphate solubilizing isolates on National Botanical Research Institute's phosphate (NBRIP) growth medium agar plates (Nautiyal, 1999), 21 days post inoculation.

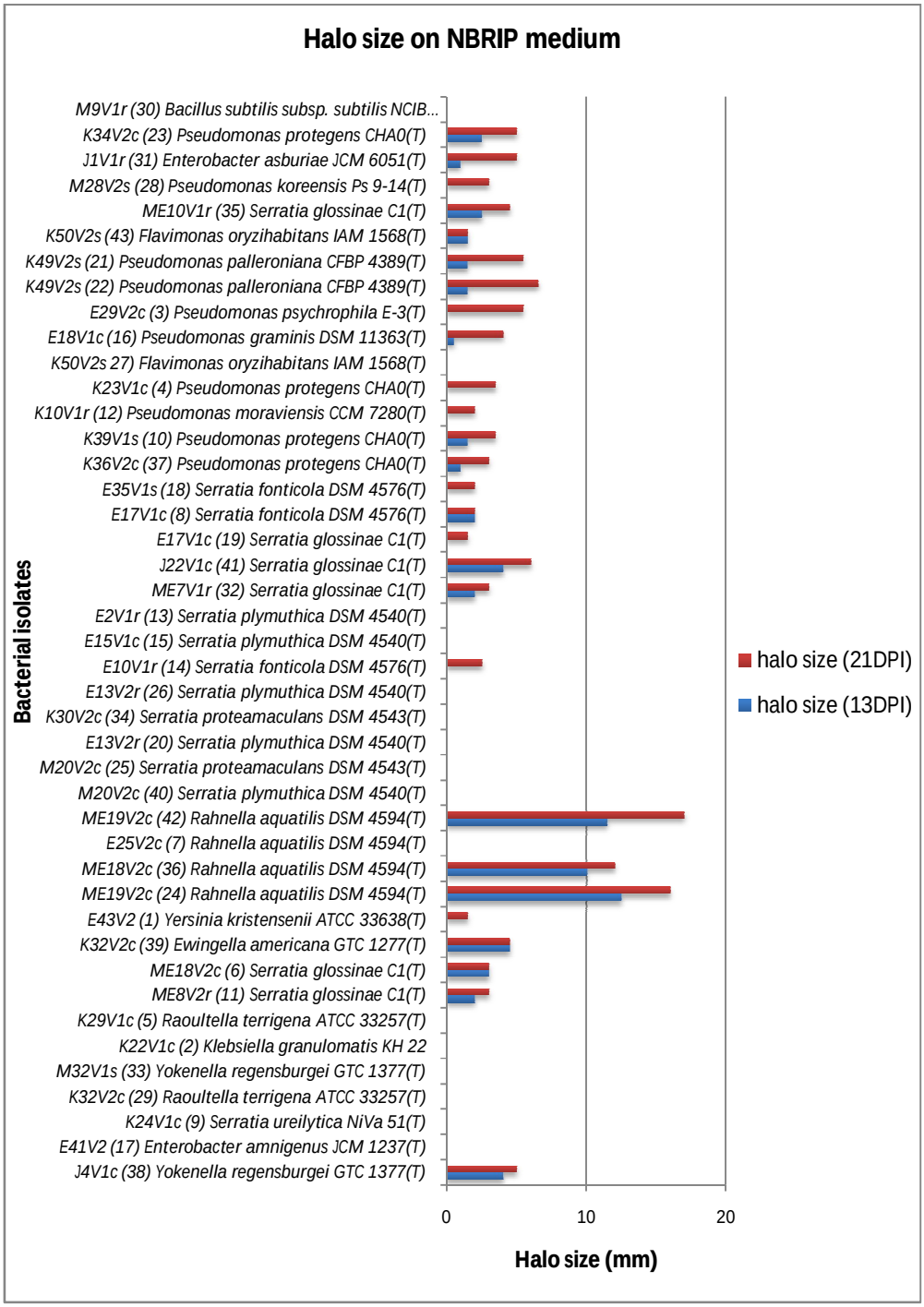


Figure 5.3: Qualitative screening of 43 isolates for phosphate solubilization on NBRIP medium agar plates (Nautiyal, 1999). Halo and colony diameters were measured at 13 &21 days post inoculation (DPI).

5.3.3. Siderophore production ability of the isolates

All the 43 isolates except M9V1r (30) - *Bacillus subtilis subsp. subtilis*grew on CAS agar plates, however not all had orange halos around their colonies(**Plate5.5**). Distinct orange halos were observed with all the 12 *Pseudomonas* isolates with isolates K50V2s (43) and K50V2s (27) both identified as*Flavimonas oryzihabitans* having the largestorange halos, that is strongly positive for siderophore production (**Plate 5.5&Table 5.1**). In addition *Enterobacter asburiae* (J1V1r) and *Serratia proteamaculans* (K30V2c) showed positive siderophore production activity (**Table 5.1**).

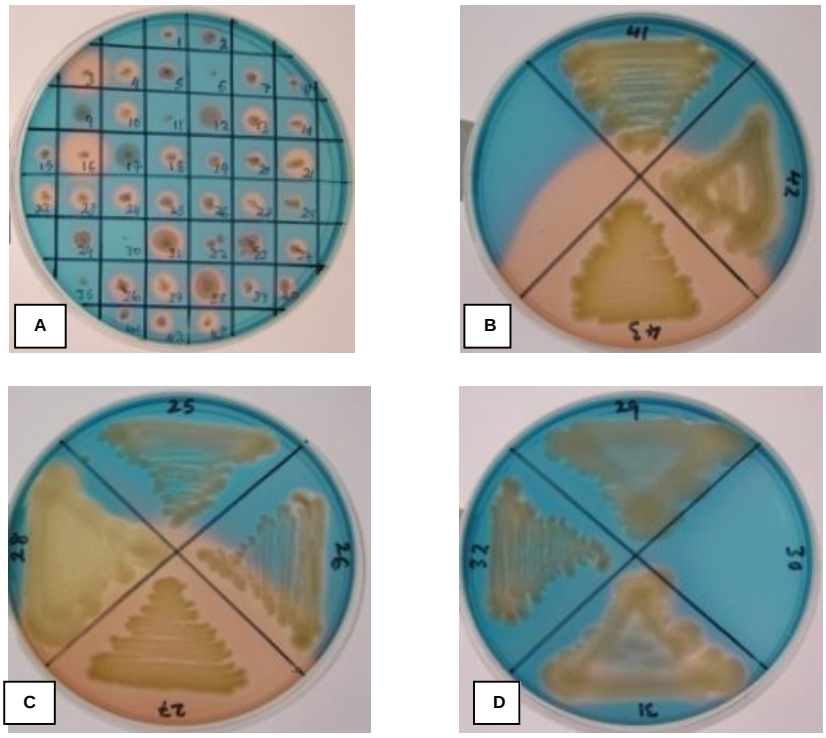


Plate 5.5: Qualitative screening for siderophore production on Chrome Azurol S (CAS) agar plates. A: All the 43 strains on one plate at one DPI. B and C: Isolates K50V2s (43) and K50V2s (27) with the largest orange halos at 7 DPI, respectively. D: Isolate M9V1r (30) - *Bacillus* spp., which did not grow on CAS agar plate.

5.4. Discussion

Screening for nitrogen fixation ability was qualitatively done on solid N-free medium and all the isolates tested showed growth on the medium. This may be explained by the fact that two of the isolation media used were nitrogen free (LGI and NFb). Growth of the bacterial isolates on N-free medium was suggestive of the isolates ability to fix atmospheric nitrogen.In addition, the isolates showed varied levels of nitrogenase activity. The highest nitrogenase activity was observed with Kisii Central District isolates, implying that the isolates had high capacity to fix nitrogen. Since plate tests for nitrogen fixation ability are consideredunreliable, the molecular approach based on PCR amplification of the *nifH* gene, which codes for the enzyme nitrogenase reductase (Burgmann *et al.*, 2004) is recommended for a more precise determination of organisms' N fixation ability. In the present study, the nested PCR amplification of the *nifH* gene for *Enterobacter ludwigii* (J1V1r), *Ewingella americana* (K32V2c), *Rahnella aquatilis* (ME19V2c) and *Flavimonas oryzihabitans* (K50V2s) yielded no DNA bands despite the fact that they had grown on N-free medium and showed nitrogenase activity. The inconsistency may have been as a result of sub-optimal PCR conditions. A repeat of the *nifH* gene PCR amplification is therefore recommended.Nitrogen-fixing endophytic bacteria have been isolated from several groups of plants (Ladha and Reddy, 2000) since the isolation of the endophytic diazotrophic bacterium *Gluconacetobacter diazotrophicus* from a Brazilian variety of sugarcane (James and Olivares, 1997). The commonly reported endophytic diazotrophic bacteria associated with bananas include *Azospirillum, Burkholderia, Citrobacter, Enterobacter, Herbaspirillum, Klebsiella* and *Rhizobium* species (Weber *et al.*, 1999; Weber *et al.*, 2001; Martinez *et al.*, 2003; Weber *et al.*,

2007). Biological nitrogen fixation (BNF) accounts for 65% of the nitrogen utilized in agriculture (Dakora and Keya, 1997). Since BNF is not limited to legumes only, increasing the amount of biologically fixed N in non-legume crops like bananas is of paramount importance for sustainable production.

Rahnella aquatilis (ME19V2c and ME18V2c)formed the largest visible dissolution halos and were therefore considered the most efficient phosphate solubilizers. Screening of phosphate solubilizers on NBRIP medium agar plates is based on formation of visible halo zones on the agar plates as a result of organism's production of organic acids into the surrounding medium (Nautiyal, 1999). The organic acids dissolve inorganic phosphate resulting to clear zones around them. The halo size is used as a measure of relative efficiency of the isolates. The findings of this study are consistent with those of Kim *et al.* (1998) who reported *Rahnella aquatilis* having genes (pyrroloquinoline quinone) that are necessary for mineral phosphate solubilization. Vyas *et al.* (2010) also identified a phosphate-solubilizing bacterial strain from *Hippophaerhamnoides* rhizosphere as *Rahnella* spp. The use of phosphate solubilizing bacteria as inoculants increases P uptake by the plant and the crop yield as well (Rodriguez and Fraga, 1999).Utilization of identified *Rahnella* species as microbial inoculantsin banana production in Kenya would therefore enhance P uptake and hence banana productivity.

Distinct orange halos were observed with all the 12 *Pseudomonas* isolates with *Flavimonas oryzihabitans*(K50V2s) having the largest orange halos. Orange halos around bacterial colonies on blue Chrome Azurol S (CAS) agar are indicative of siderophore excretion(Schwyn and Neiland, 1987).When a strong chelator like siderophore removes iron from the highly coloured iron dye complexof chrome

azurol S, iron (III), its colour turns from blue to orange. The *Pseudomonas* isolates and especially*Flavimonas oryzihabitans* (K50V2s) could therefore be considered high siderophore producers. These findings are similar to those of Gangwar and Kaur (2009) who reported *Pseudomonas* spp. isolated from ryegrass as high siderophore producer. Siderophores are responsible for the dissolution, chelation and transport of iron (III) into microbial cells (Sharma and Johri, 2003). It has also been shown that Fe chelated by microbial siderophores can also be utilized by plants (Chen *et al.*, 1998). Siderophore-producing bacteria would therefore improve the iron nutrition of plants. Siderophores can also promote plant growth indirectly by reducing or preventing harm caused by plant-pathogenic microorganisms (Leong, 1986).

Isolate M9V1r (30), identified as *Bacillus subtilis subsp. subtilis*did not show growth on CAS agar plates. This could be explained by the fact that*Bacillus subtilis subsp. subtilis*is Gram-positive and Gram-positive bacteria are reported to be sensitive to HDTMA detergent used in the siderophore medium (Schwyn and Neilands, 1987). HDTMA may therefore have become toxic to *Bacillus subtilis subsp. subtilis*(M9V1r)causing it not to grow.

5.5. Conclusions

From the current study, it is apparent that many diazotrophic microbes inhabit the tissues of banana plants and there is the potential of exploiting them once conditions for their use is optimized. *Rahnella aquatilis*(ME19V2c and ME18V2c)and*Flavimonas oryzihabitans*(K50V2s)having showed ability to solubilize phosphate and produce siderophore, respectively and also ability to fix free nitrogen could be proposed as potential biofertilizers for sustainable banana production in Kenya.

CHAPTER SIX

6.0 EFFECTS OF INOCULATION WITH SELECTED BANANA ENDOPHYTIC BACTERIA ISOLATES ON GROWTH, PHYSIOLOGY AND MINERAL NUTRITION OF TISSUE CULTURED BANANA PLANTS

6.1. Introduction

Endophytes are of agronomic interest as they can enhance plant growth in non-leguminous crops and improve their nutrition through nitrogen fixation, phosphate solubilization or iron chelation (Dobereiner and Baldani, 1998; Sturz *et al*., 2000; Boddey *et al*., 2003; Iniguez *et al*., 2004; Ryan *et al*., 2008; Uribe *et al*., 2010).Endophytic bacteria have the potential of being incorporated into propagation materials such as tissue cultured bananas, and since they live inside the plant, they would be able to provide the nutrients directly to the host plant.

Inoculation experiments have been performed to reveal the effects of endophytes although elimination of resident or indigenous endophytes to achieve bacteria-free plants or seeds remains a challenge (Rosenblueth and Martínez-Romero, 2006). Tissue culture (TC) has however been used to eliminate or reduce indigenous endophytes (Holland and Polacco, 1994; Leifert *et al*., 1994). Colonization might therefore be more successful with tissue cultured plants, as there are few or no other microorganisms with which to compete.

The objective of this study was to investigate the effects of the isolates on the growth, physiology and mineral nutrition of tissue cultured banana plants. To achieve this three greenhouse experiments were conducted. The 1stexperiment was carried out during the months of May to July 2011 (cool and cloudy weather conditions); the

2nd experiment, August to October 2011 (sunny and dry weather conditions) and the 3rd experiment,March to May 2012 (main rainy season).

6.2. Materials and Methods

6.2.1 Inoculation and planting

Fourty three isolates, representing 24 species, werescreened*in-vitro*for their plant growth promoting potential that included ability to fix atmospheric nitrogen, solubilize phosphates and produce siderophores. Based on their potential to promote plant growth and their uniqueness, 5 bacterial strainswere selected for investigation in the 1st and 2nd greenhouse experiments. The selected strains were *Bacillus subtilis subsp. inaquosorum*(M9V1r), *Enterobacter ludwigii*(J1V1r), *Ewingella americana*(K32V2c), *Rahnella aquatilis*(ME19V2c) and *Flavimonas oryzihabitans* (K50V2s).Based on the results of the 1st and 2nd experiment, a 3rd greenhouse experiment was conducted where 24 bacterial strains, representingall the species, were tested.

A tissue-cultured Cavendish banana variety (*Musa* spp. cv. Grande Naine) from IBR, JKUAT was used as the test plant and sterile cocopeat (Kocos Kenya limited, Kilifi) as the inert support medium. Agroblen controlled release fertilizer (Scotts International B.V.) with mineral composition of 13% Nitrogen (5% nitrate N and 8% ammoniacal N), 13% Phosphorus pentoxide (P_2O_5), 13% Potassium oxide (K_2O) and 3% total Magnesium oxide (MgO) was used as the source of nutrients. The controlled release fertilizer has a longevity period of 3-4 months at 21°C average soil temperature.

6.2.1.1 Microbial inoculum preparation

For the 1st and 2nd greenhouse experiment, individual bacterial isolates were inoculated in mineral medium (5g $(NH_4)_2SO_4$, 5g K_2HPO_4, 1g NaCl, 0.2g $MgSO_4.7H_2O$, 10g glucose and 2g yeast extract per liter at pH 7) on a rotary shaker (100rpm) for 48 hours at 30°C. A final concentration of about 10^8 cfu per ml of bacterial suspension was used.For the 3rd greenhouse experiment, individual bacterial isolates were inoculated in 40 ml nutrient broth (HIMEDIA, HiMedia Laboratories Pvt. Ltd.) in 50 ml falcon tubes and placed on rotary shaker (100 rpm) for 24 hours at 30°C. A final concentration of about 10^8 cfu per ml of bacterial suspension was used. The cells were then washed in sterile distilled water and the clean cells re-suspended in 20 ml sterile distilled water.

6.2.1.2 Plant inoculation

For the 1st and 2nd greenhouse experiment, the five bacterial endophytes tested were individually inoculated onto two-month old rooted and hardened TC banana plantlets (8 to 10 cm height, 3 to 4 leaves) by dipping 40 plantlets in 300ml of respective bacteria inoculum (10^8 cfu / ml) for about 24 hours (**Plate 6.1**). Sterile mineral medium was used in place of bacteria inoculum in the control.

Plate 6.1: Inoculation of 2-month old rooted and hardened TC banana plantlets with individual bacterial inoculums.

For the 3rd greenhouse experiment, one month old rooted *in-vitro* banana plantlets (10 plantlets per rooting jar) were inoculated by aseptically adding, into each rooting jar, respective washed bacterial cells (10^8 cfu / ml) suspended in 20 ml sterile distilled water and immediately resealed using parafilm. For the control jar (10 plantlets), 20 ml sterile distilled water was aseptically added and immediately resealed. The inoculated and un-inoculated jars were left on a lab bench for 48 hours.

6.2.1.3 Plant potting

Inoculated and uninoculated plantlets were planted in individual plastic pots (2.5liters) with a bottom plate and maintained in the greenhouse with watering once a day with chlorinated tap water (**Plate 6.2**). The effect of inoculation with the selected isolates was tested at 4 levels of fertilization (2.5g, 5.0g, 7.5g and 10g) in the 1st experiment and 5 fertilization levels (0g, 2.5g, 5.0g, 7.5g and 10g) in the 2nd experiment. In the 3rd experiment, only one level (5 g per pot) of fertilization was used. This sub-optimal level of fertilization was chosen because plantgrowth-

promoting microorganisms have previouslybeen reported to often be effective under low-nutrient conditions and have little or no measurable effect on plant growth when the plants are grown in nutrient-rich soil under optimal conditions (Penrose and Glick, 2003).

The plastic pots were filled with coco peat and watered to maximum water holding capacity a day before planting. Various amounts of fertilizer were appropriately added to the pots as described in section 5.2.1.3 and mixed well before planting.

Plate 6.2: Greenhouse experiments at JKUAT in year 2011 and 2012.

6.2.2 Growth Measurements

6.2.2.1 Non-destructive growth measurements

Non-destructive growth measurements included the plant height, basal trunk diameter and total number of leaves. They were measured weekly starting at 14 days after planting (DAP) until70DAP for experiment 1 and 2. In the 3rd experiment, the measurements were also taken weekly starting at 28DAPuntil 70DAP (non-hardened

plantlets used in the 3rd experiment needed about a month to stabilize). The height from ground level to the tip of the youngest leaf was determined using a tape measure while basal trunk diameter was measured 1 cm above the soil surface using the vernier calipers. Relative change in plant height and diameter was determined as follows: Relative change = (1/W1)*(W2-W1)/(t2-t1), where W1 and W2 are the growth parameters at the beginning (t1) and end (t2) of the data taking intervalrespectively (South, 1995).The total number of leaves included the green, the yellow and the dead ones.

6.2.2.2 Destructive growth measurements

Destructive measurements included the shoot and root fresh weights and the shoot and root dry weights. The fresh weights were taken at the end of experimentthat is2 to 3 months after planting. The plants were pulled out of the pots and the coco peat carefully washed away with tap water. Shoot and root parts were separated by cutting at the trunk base and the number of leaves were recorded. The entire shoot (leaves and stem) and the roots were weighed separately and put in labeled paper bags. The samples were then oven-dried at 70°C for a week to determine their dry weights (Muthuri *et al*., 2005).

6.2.3 Mineral nutrient analysis

Plant mineral nutrient analysis was done using the ICP-MS (Agilent Technologies 7500 series) for the 1st experiment where 8 ml of 61% HNO_3 was added to 1-3 mg of oven-dried sample and digested using the START D microwave digestion system at 180°C. Digested samples were then diluted 12 times with MilliQ water since the ICP-MS can only manage 5% HNO_3. For the 2nd and 3rd experiment, plant nutrient analysis was done as described by Okalebo *et al.*(1993).

6.2.4 Chlorophyll content and SPAD calibration

Chlorophyll content was determined non-destructively using a SPAD-502 meter (Minolta, Japan). The instrument uses measurements of transmitted radiation in the red and near-infra red wavelengths to provide numerical values related to chlorophyll content (Lawson *et al*., 2001). SPAD measurements were made at four locations on two selected leaves at seven days interval. In order to derive estimates of chlorophyll content from SPAD values, a calibration curve was constructed. Leaves from selected banana plants were sampled to represent a range of color extending from most green, moderately green, moderately yellow and yellow.SPAD measurements were taken for the selected leaves, which were then analyzed immediately for chlorophyll content following extraction in 80% acetone (Leegood, 1993). Sub-samples from the sampled leaves (1 g fresh weight) were ground with a pestle using a mortar and pure quartz sand using 40 ml of 80% acetone as the extraction solvent. The resulting suspension was diluted to 100 ml using 80% acetone and centrifuged at 6000 rpm for three minutes. Optical density (absorbance) was taken using a 10 ml aliquot placed in a quartz cuvette using a spectrophotometer set at 645nm and 663 nm. The samples were read against an 80% acetone blank; three readings were taken for each sample and averaged. Chlorophyll a, b and total chlorophyll concentrations (mg g^{-1} tissue) were determined as follows:

Chlorophyll a = (12.7(D663) - 2.63(D645)) x V/1000xW

Chlorophyll b = (22.9(D645) - 4.68(D663)) x V/1000xW

Total Chlorophyll = Chlorophyll a + Chlorophyll b

Where D represents absorbance, V denotes volume and W is the measured tissue freshweight.

The values obtained were used to construct a calibration curve relating the greenness of the leaves as determined using the SPAD meter to their chlorophyll content expressed on a fresh weight basis. The calibration was used to convert SPAD values to the corresponding chlorophyll contents.

6.2.5 Experimental design and statistical analysis

The first greenhouse experiment carried out during the months of May to July 2011 was conducted in a complete randomized design (CRD) while the second and third greenhouse experiments carried out during the months of August to October 2011 and March to May 2012, respectively were conducted in randomized complete block design (RCBD).Data was subjected to analysis of variance (ANOVA) using SAS 9.1 statistical software for Windows (SAS institute Inc.) and means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

6.3. Results

6.3.1 Effect of bacterial isolates on banana growth

6.3.1.1 Non-destructive growth measurements

Non-destructive growth measurements included the plant height, basal trunk diameter and total number of leaves.

a. Relative change in plant height and diameter

In the 1st experiment,plant inoculation with*Bacillus subtilis subsp. inaquosorum*(M9V1r), *Enterobacter ludwigii*(J1V1r), *Ewingella americana*(K32V2c), *Rahnella aquatilis*(ME19V2c)and *Flavimonas oryzihabitans*(K50V2s)did not show significant effect on relative change in plant height and diameter throughout the growth period and across different fertilizer regimes(**Table 6.1and Table 6.2**).In addition, bacterial inoculation did not interact

significantly with eitherfertilizer application ordays after plantingin regard to relative change in height and diameter.However, there was a significant interaction between fertilizer application and days after planting (DAP). The relative change in plant height and diameter decreased significantly 56days after planting except at 2.5 g fertilizer regime where the relative change in plant height decreasedsignificantly at 42 DAP (**Table 6.3**).

Table 6.1: Relative change in plant height (rgr-ht) and diameter (rgr-dia) of tissue cultured banana plants as affected by inoculation with 5 selected bacterial strains at different days after planting (DAP) in experiment 1.

Bacterial isolates	**28 DAP**		**42 DAP**		**56 DAP**		**70 DAP**	
	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia
***Bacillus subtilis subsp. Inaquosorum*(M9V1r)**	0.286 a	0.384 a	0.269 a	0.351 a	0.124 a	0.246 a	0.053 ab	0.123 a
***Enterobacter ludwigii*(J1V1r)**	0.317 a	0.347 a	0.248 a	0.354 a	0.095 a	0.216 a	0.033 b	0.089 a
***Ewingella americana*(K32V2c)**	0.296 a	0.331 a	0.274 a	0.376 a	0.122 a	0.258 a	0.071 a	0.105 a
***Rahnella aquatilis*(ME19V2c)**	0.286 a	0.354 a	0.276 a	0.348 a	0.133 a	0.272 a	0.056 ab	0.106 a
***Flavimonas oryzihabitans*(K50V2s)**	0.300 a	0.337 a	0.263 a	0.323 a	0.106 a	0.235 a	0.055 ab	0.113 a
Control	0.265 a	0.344 a	0.277 a	0.372 a	0.120 a	0.242 a	0.061 a	0.107 a

n=32

Values are means of 8 replicates and the means with the same letter in a column are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

Table 6.2: Relative change in plant height (rgr-ht) and diameter (rgr-dia) of tissue cultured banana plants as affected by inoculation with 5 selected bacterial strains at different fertilizer regimes in experiment 1.

Bacterial isolates	**2.5 g fertilizer**		**5 g fertilizer**		**7.5 g fertilizer**		**10 g fertilizer**	
	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia
***Bacillus subtilis subsp. inaquosorum*(M9V1r)**	0.149 a	0.228 a	0.175 a	0.260 a	0.188 a	0.285 a	0.221 a	0.332 a
***Enterobacter ludwigii*(J1V1r)**	0.153 a	0.221 a	0.162 a	0.237 a	0.178 a	0.257 a	0.200 a	0.292 a
***Ewingella americana*(K32V2c)**	0.127 a	0.216 a	0.176 a	0.252 a	0.224 a	0.292 a	0.236 a	0.309 a
***Rahnella aquatilis*(ME19V2c)**	0.147 a	0.209 a	0.180 a	0.259 a	0.208 a	0.315 a	0.215 a	0.296 a
***Flavimonas oryzihabitans*(K50V2s)**	0.129 a	0.203 a	0.168 a	0.239 a	0.211 a	0.273 a	0.216 a	0.294 a
Control	0.132 a	0.225 a	0.170 a	0.247 a	0.209 a	0.293 a	0.212 a	0.301a

n=32

Values are means of 8 replicates and the means with the same letter in a column are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

Table 6.3: Relative change in plant height (rgr-ht) and diameter (rgr-dia) of tissue cultured banana plants as affected by number of days after planting at different fertilizer regimesin experiment 1.

Days after planting	2.5 g fertilizer		5 g fertilizer		7.5 g fertilizer		10 g fertilizer	
	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia
28	0.287 a	0.317 a	0.289 a	0.351 a	0.317 a	0.371 a	0.274 a	0.359 ab
42	0.216 b	0.295 a	0.278 a	0.334 a	0.290 a	0.398 a	0.288 a	0.389 a
56	0.036 c	0.185 b	0.095 b	0.218 b	0.138 b	0.259 b	0.198 b	0.318 b
70	0.020 c	0.072 c	0.025 c	0.094 c	0.066 c	0.115 c	0.108 c	0.148 c

n=48

Values are means of 8 replicates and the means with the same letter in a column are not significantly different. Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

In experiment 2, as in experiment 1, plant inoculation with bacterial strains did not show significant effect on relative change in plant height and diameter across different fertilizer regimes (**Table 6.4**). There was also no significant interaction between bacterial inoculation and fertilizer application in regard to relative change in plant height and diameter. However, there was a significant interaction between bacterial inoculation and days after planting in regard to relative change in plant height but not in regard to relative change in plant diameter. At 28and 70 DAP,inoculation of plants with bacterial isolates showed no significant effect on the relative change in plant height (**Table 6.5**). However, at 42DAP and 56DAP, plants inoculated with *Ewingella americana* (K32V2c)showed a significantly higher relative change in plant height than the uninoculated ones.The interaction between fertilizer application and days after planting wasalso significant.At 0 g fertilizer regime, the relative change in plant height was negative across the growth period, while the relativechange in plant diameter was minimally positive except at 42DAP where it was negative.At 2.5 g fertilizer regime, the relative change in plant height and diameter decreased with increasing number of days after planting. At 5 g, 7.5 g and 10 g fertilizer regimes, the relative change in plant height and diameter increasedwith increasing number of days after planting up to 42 DAP but reduced significantly thereafter(**Table 6.6**).

Table 6.4: Effect of inoculation with 5 selected bacterial strains on the relative change in height (rgr-ht) and diameter (rgr-dia) of tissue cultured banana plants at different fertilizer regimes (g)in experiment 2.

Bacterial isolates	**0 g**		**2.5 g**		**5 g**		**7.5 g**		**10 g**	
	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia
***Bacillus subtilis subsp. inaquosorum*(M9V1r)**	-0.040 a	0.026 a	0.054 a	0.114 a	0.130 a	0.188 a	0.163 ab	0.238 a	0.180 a	0.264 a
***Enterobacter ludwigii*(J1V1r)**	-0.042 a	0.017 a	0.079 a	0.147 a	0.119 a	0.162 a	0.139 ab	0.184 a	0.172 a	0.240 a
***Ewingella americana*(K32V2c)**	-0.047 a	0.013 a	0.085 a	0.136 a	0.121 a	0.212 a	0.188 a	0.245 a	0.193 a	0.278 a
***Rahnella aquatilis*(ME19V2c)**	-0.036 a	0.013 a	0.076 a	0.169 a	0.126 a	0.177 a	0.171 ab	0.211 a	0.172 a	0.247 a
***Flavimonas oryzihabitans*(K50V2s)**	-0.034 a	0.024 a	0.099 a	0.160 a	0.104 a	0.165 a	0.134 b	0.201 a	0.172 a	0.236 a
Control	-0.052 a	0.007 a	0.068 a	0.125 a	0.112 a	0.174 a	0.145 ab	0.215 a	0.170 a	0.240 a

n=20

Values are means of 5 replicates and means with the same letter in a column are not significantly different. Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

Table 6.5: Effect of inoculation with 5 selected bacterial strains on the relative change in height (rgr-ht) and diameter (rgr-dia) of tissue cultured banana plants at different days after planting (DAP)in experiment 2.

Bacterial isolate	**28DAP**		**42DAP**		**56DAP**		**70DAP**	
	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia
***Bacillus subtilis subsp. inaquosorum*(M9V1r)**	0.115ab	0.233a	0.181ab	0.240a	0.056b	0.141 ab	0.038 a	0.050 a
***Enterobacter ludwigii*(J1V1r)**	0.149a	0.217 a	0.150ab	0.216a	0.056b	0.118ab	0.017a	0.049a
***Ewingella americana*(K32V2c)**	0.086 b	0.219a	0.199a	0.237a	0.107a	0.167a	0.040a	0.085 a
***Rahnella aquatilis*(ME19V2c)**	0.132 ab	0.209 a	0.166 ab	0.234 a	0.081 ab	0.145ab	0.029 a	0.065 a
***Flavimonas oryzihabitans*(K50V2s)**	0.142 ab	0.250 a	0.154ab	0.200 a	0.063b	0.124ab	0.021a	0.055a
Control	0.128ab	0.240a	0.136b	0.212 a	0.069b	0.117ab	0.020a	0.040 a

n=25

Values are means of 5 replicates and means with the same letter in a column are not significantly different. Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

Table 6.6: Relative change in plant height (rgr-ht) and diameter (rgr-dia) of tissue cultured banana plants as affected by days after planting at different fertilizer regimesin experiment 2.

Days after planting	0 g		2.5 g		5 g		7.5 g		10 g	
	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia	rgr-ht	rgr-dia
28	-0.063 b	0.011 a	0.131 a	0.238 a	0.181 a	0.258 a	0.199 b	0.289 a	0.193 b	0.326 a
42	-0.079 b	-0.004 a	0.112 a	0.199 a	0.203 a	0.259 a	0.266 a	0.298 a	0.293 a	0.355 a
56	-0.018 a	0.040 a	0.037 b	0.083 b	0.056 b	0.144 b	0.111 c	0.167 b	0.159 b	0.212 b
70	-0.0004 a	0.008 a	0.015 b	0.047 b	0.029 b	0.054 c	0.037 d	0.087 c	0.060 c	0.105 c

n=35

Values are means of 5 replicates and means with the same letter in a column are not significantly different. Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

In the 3rd experiment, inoculation with bacterial isolates had no significant effect on both plant height and plant diameter (**Table 6.7**). However, plants inoculated with *Klebsiella granulomatis* (K22V1c), *Raoultella terrigena*(K29V1c), *Rahnella aquatilis* (E25V2c), *Pseudomonas protegens* (K39V1s), *Pseudomonas graminis* (E18V1c) and *Serratia proteamaculans* (M20V2c) performed slightly better than the control. From 42 DAP, relative change in plant height and diameter decreased significantly with increasing number of days after planting(**Figure 6.1**).

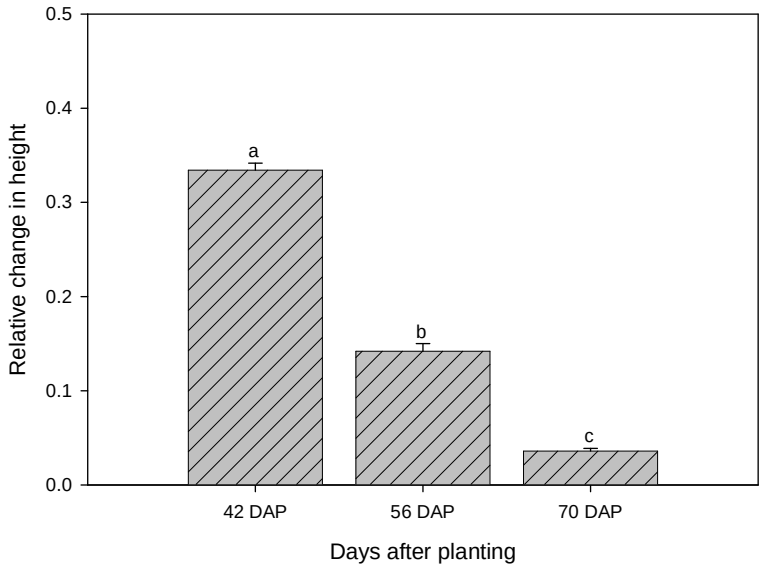
Table 6.7: Effect of bacterial strains on the relative change in height and diameter of tissue cultured banana plantsin experiment 3.

Bacterial isolates	**Relative change in height**	**Relative change in diameter**
***Yersinia kristensenii* (E43V2)**	0.178 abc	0.290 a
***Klebsiella granulomatis* (K22V1c)**	0.206 abc	0.305 a
***Pseudomonas psychrophila* (E29V2c)**	0.183 abc	0.262 a
***Pseudomonas protegens* (K23V1c)**	0.145 bc	0.261 a
***Raoultella terrigena* (K29V1c)**	0.240 a	0.290 a
***Rahnella aquatilis* (E25V2c)**	0.210 abc	0.304 a
***Serratia fonticola* (E17V1c)**	0.178 abc	0.267 a
***Serratia ureilytica* (K24V1c)**	0.141 bc	0.256 a
***Pseudomonas protegens* (K39V1s)**	0.210 abc	0.316 a
***Pseudomonas moraviensis* (K10V1r)**	0.137 c	0.247 a
***Serratia plymuthica* (E2V1r)**	0.152 bc	0.246 a
***Pseudomonas graminis* (E18V1c)**	0.241 a	0.348 a

Bacterial isolates	**Relative change in height**	**Relative change in diameter**
***Enterobacter amnigenus* (E41V2)**	0.170 abc	0.274 a
***Pseudomonas palleroniana* (K49V2s)**	0.149 bc	0.274 a
***Pseudomonas protegens* (K34V2c)**	0.166 abc	0.279 a
***Serratia proteamaculans* (M20V2c)**	0.225 abc	0.330 a
***Flavimonas oryzihabitans* (K50V2s)**	0.138 c	0.221 a
***Pseudomonas koreensis* (M28V2s)**	0.141 bc	0.233 a
***Bacillus subtilis subsp. inaquosorum* (M9V1r)**	0.148 bc	0.252 a
***Enterobacter ludwigii* (J1V1r)**	0.129 c	0.229 a
***Yokenella regensburgei* (M32V1s)**	0.141 bc	0.255 a
***Ewingella americana* (K32V2c)**	0.188 abc	0.296 a
***Serratia glossinae* (J22V1c)**	0.147 bc	0.286 a
***Rahnella aquatilis* (ME19V2c)**	0.137 c	0.278 a
Control	0.190abc	0.272a

n=12

Values are means of 4 replicates and means with the same letter in a column are not significantly different. Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.



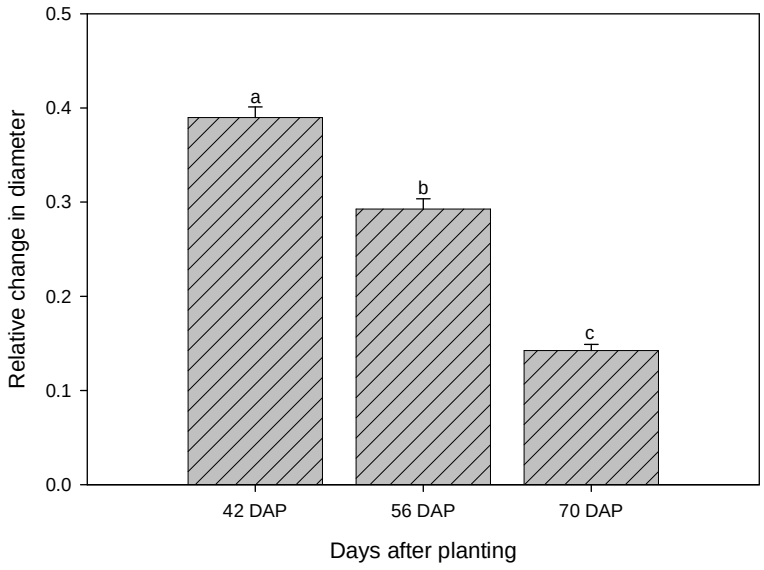


Figure 6.1: Relative change in plant height and diameter of tissue cultured banana plants as affected by number of days after plantingin experiment 3. Values are means of 4 replicates and means with different letters are significantly different. Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.Vertical bars indicate standard errors.n=104.

b. Number of leaves

In the 1st experiment, inoculation with *Bacillus subtilis subsp. inaquosorum*(M9V1r), *Enterobacter ludwigii*(J1V1r), *Ewingella americana*(K32V2c), *Rahnella aquatilis*(ME19V2c) and *Flavimonas oryzihabitans* (K50V2s) had no significant effect on the relative change in the number of green leaves(**Figure 6.2**).However, with increasing number of days after planting, the relative change in the number of green leaves decreased significantly (**Figure 6.3**). There was no significant interaction between bacterial inoculation, fertilizer application and days after planting.

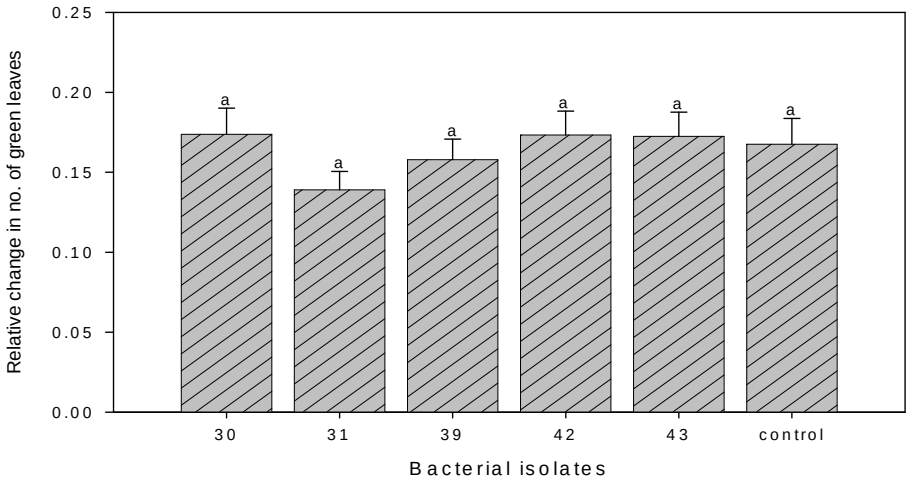


Figure 6.2: Relative change in the number of green leaves of tissue cultured banana plants as affected by inoculation with *Bacillus subtilis subsp. inaquosorum* (30), *Enterobacter ludwigii* (31), *Ewingella americana* (39), *Rahnella aquatilis* (42) and *Flavimonas oryzihabitans* (43)in the 1st experiment. Values are means of 8 replicates and means with the same letter are not significantly different. Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05. Vertical bars indicate standard errors. n=128.

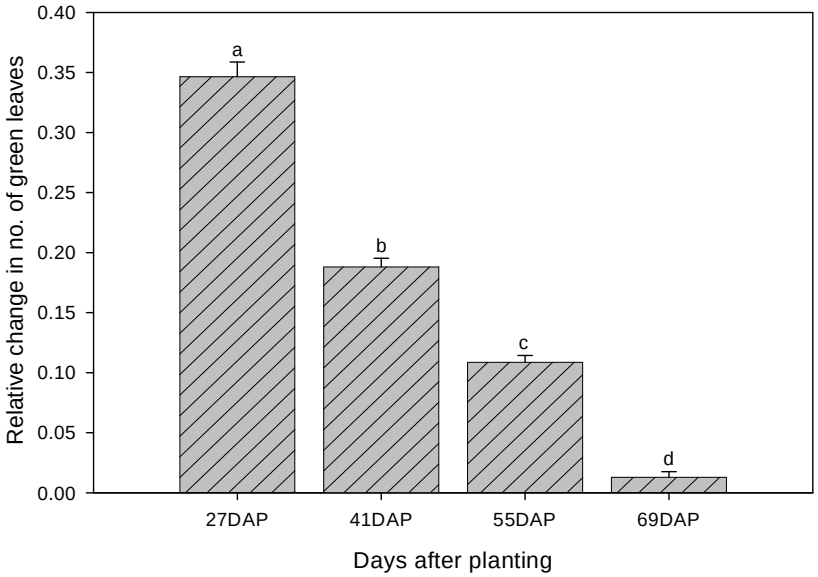


Figure 6.3: Relative change in the number of green leaves of tissue cultured banana plants as affected by days after planting (DAP)in the 1st experiment. Values are means of 8 replicates and means with the same letter are not significantly different. Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05. Vertical bars indicate standard errors. n=192.

In the 2nd experiment, days after planting interacted significantly with bacterial inoculation and fertilizer application. There was however no significant interaction between bacterial inoculation and fertilizer application. Bacterial inoculation did not significantly affect the relative change in the number of green leaves except at 42DAP where inoculation with *Ewingella americana* (K32V2c) had a positive significant effect on the relative change in the number of green leaves (**Table 6.8**). The relative change in the number of green leaves significantly reduced with increasing days after planting across the different fertilizer regimes (**Table 6.9**).

Table 6.8: Relative change in the number of green leaves of tissue cultured banana plants as affected by bacterial strains at different days after planting (DAP)in experiment 2.

Bacterial isolates	**28 DAP**	**42 DAP**	**56 DAP**	**70 DAP**
***Bacillus subtilis subsp. inaquosorum*(M9V1r)**	0.171 abc	0.178 ab	0.032 a	0.017 ab
***Enterobacter ludwigii*(J1V1r)**	0.152 bc	0.131 bc	0.046 a	-0.027 b
***Ewingella americana*(K32V2c)**	0.225 a	0.233 a	0.045 a	-0.002 ab
***Rahnella aquatilis*(ME19V2c)**	0.187 ab	0.145 bc	0.042 a	0.027 a
***Flavimonas oryzihabitans*(K50V2s)**	0.149 bc	0.148 bc	0.021 a	0.005 ab
Control	0.175 abc	0.140 bc	0.023 a	-0.013 ab

n=25

Values are means of 5 replicates and means with the same letter are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

Table 6.9: Relative change in the number of green leaves of tissue cultured banana plants as affected by different days after planting (DAP)at different fertilizer application regimes (g)in experiment 2.

DAP	0 g	2.5 g	5 g	7.5 g	10 g
28	0.02696 a	0.18664 a	0.19114 a	0.24787 a	0.23012 a
42	0.05119 a	0.17741 a	0.18807 a	0.18576 b	0.2113 a
56	-0.03977 b	0.02475 b	0.0374 b	0.07284 c	0.07743 b
70	0.01384 ab	-0.00342 b	0.00399 b	-0.00962 d	0.00054 c

n=30

Values are means of 5 replicates and means with the same letter are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

In the third experiment, the relative change in the number of green leaves was not significantly affected by bacterial inoculation (data not shown) but it significantly reduced with increasing days after planting (**Figure 6.4**).

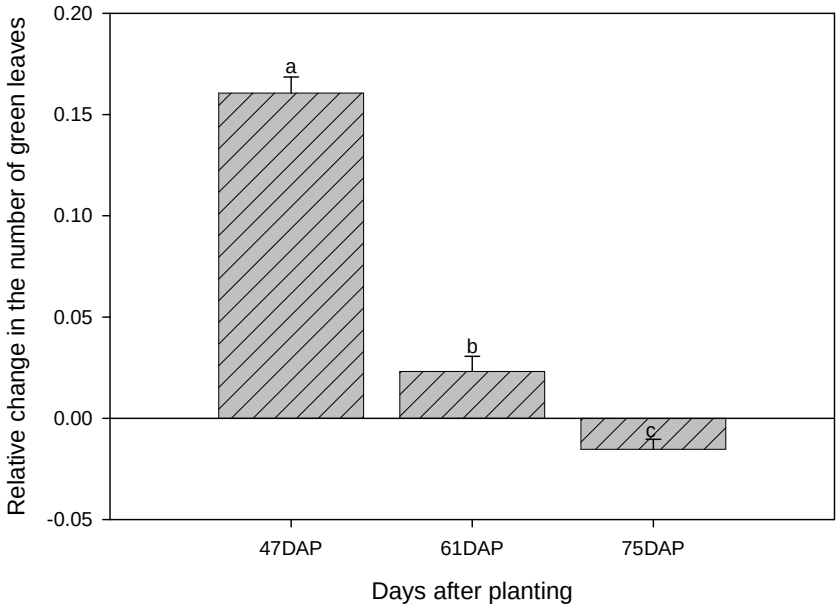


Figure 6.4: Relative change in the number of green leaves of tissue cultured banana plants as affected by days after plantingin experiment 3. Values are means of 4 replicates and means with different letters are significantly different. Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05. Vertical bars indicate standard errors. n=104.

6.3.1.2 Destructive growth measurements

Destructive growth measurements included the fresh and dry weights of both shoots and roots.In the 1st experiment, inoculation with5 selected isolates showed no significant effect on the shoot fresh and dry weights except*Enterobacter ludwigii* (J1V1r), which had a significant positive effect on shoot dry weight at 10 g fertilizer regime and*Bacillus subtilis subsp. inaquosorum* (M9V1r), which showed a significant negative effect on shoot fresh weight at 7.5 g fertilizer regime (**Table 6.10**).The root fresh and dry weights were not significantly affected by bacterial inoculationacross the different fertilizer regimes (**Table 6.11**).In the 2nd experiment,

inoculation with the 5 isolates had no significant effect on the shoot fresh and dry weights except *Ewingella americana* (K32V2c), which showed a significant negative effect at 0 g fertilizer regime (**Table 6.12**). The root fresh and dry weights were not significantly affected by bacterial inoculation (**Table 6.13**).In the 3rd experiment, inoculation with 24 bacterial strains also showed no significant effect on the above parameters (data not shown). The experiment was conducted at only one level of fertilizer application (5 g).

Table 6.10: Effect of inoculation with 5 selected bacterial strains on shoot fresh weight (SFW) and shoot dry weight (SDW) of tissue cultured banana plants at different fertilizer regimesin experiment 1.

Bacterial isolates	**2.5 g**		**5 g**		**7.5 g**		**10 g**	
	SFW	SDW	SFW	SDW	SFW	SDW	SFW	SDW
***Bacillus subtilis subsp. inaquosorum* (M9V1r)**	99.27 a	12.11 a	114.58 a	16.27 ab	166.56 b	20.54 a	231.06 b	24.56 b
***Enterobacter ludwigii* (J1V1r)**	83.47 a	12.38 a	139.86 a	17.81 a	207.01 a	24.40 a	285.57 a	31.05 a
***Ewingella americana* (K32V2c)**	85.64 a	11.06 a	119.15 a	16.26 ab	195.87 ab	22.24 a	253.67 ab	24.64 b
***Rahnella aquatilis* (ME19V2c)**	84.61 a	10.79 a	112.20 a	13.63 b	203.88 a	23.08 a	246.20 b	26.34 ab
***Flavimonas oryzihabitans* (K50V2s)**	80.43 a	11.07 a	143.47 a	17.89 a	204.01 a	23.58 a	240.41 b	25.41 b
Control	81.93 a	10.53 a	139.30 a	17.09 ab	201.91 a	23.18 a	250.91 ab	25.46 b

n=8

Values are means of 8 replicates and means with the same letter are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

Table 6.11: Effect of inoculation with 5 selected bacterial strainson root fresh weight (RFW) and root dry weight (RDW) of tissue cultured banana plants at different fertilizer regimesin experiment 1.

Bacterial isolates	**2.5 g**		**5 g**		**7.5 g**		**10 g**	
	RFW	RDW	RFW	RDW	RFW	RDW	RFW	RDW
***Bacillus subtilis subsp. inaquosorum* (M9V1r)**	74.24 a	4.78 a	83.64 a	5.50 a	85.08 a	5.27 a	86.77 a	6.09 a
***Enterobacter ludwigii* (J1V1r)**	70.24 a	4.49 a	88.98 a	5.53 a	90.18 a	5.47 a	82.68 a	5.00 a
***Ewingella americana* (K32V2c)**	81.84 a	5.06 a	79.73 a	5.19 a	83.05 a	5.25 a	74.17 a	4.30 a
***Rahnella aquatilis* (ME19V2c)**	71.43 a	4.15 a	75.73 a	4.84 a	88.85 a	5.58 a	81.79 a	4.91 a
***Flavimonas oryzihabitans* (K50V2s)**	71.43 a	4.38 a	92.48 a	6.54 a	84.08 a	5.28 a	85.09 a	6.12 a
Control	80.88 a	5.29 a	94.92 a	5.94 a	78.37 a	4.52 a	79.93 a	5.42 a

n=8

Values are means of 8 replicates and means with the same letter are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

Table 6.12: Effect of inoculation with 5 selected isolates on shoot fresh weight (SFW) and shoot dry weight (SDW) of tissue cultured banana plants at different fertilizer regimes (g)in experiment 2.

Bacterial isolate	**0 g**		**2.5 g**		**5 g**		**7.5 g**		**10 g**	
	SFW	SDW	SFW	SDW	SFW	SDW	SFW	SDW	SFW	SDW
***Bacillus subtilis subsp. inaquosorum* (M9V1r)**	5.10 abc	0.51 ab	47.39 a	6.01 a	116.34 a	13.19 a	220.45 ab	23.79 a	265.35 a	27.90 a
***Enterobacter ludwigii* (J1V1r)**	5.85 ab	0.66 ab	67.12 a	7.80 a	123.64 a	14.38 a	189.33 b	21.38 a	294.50 a	31.49 a
***Ewingella americana* (K32V2c)**	2.55 c	0.25 b	46.67 a	5.73 a	91.77 a	9.61 a	185.03 b	19.58 a	276.70 a	26.97 a
***Rahnella aquatilis* (ME19V2c)**	5.25 abc	0.52 ab	48.53 a	6.13 a	115.76 a	15.96 a	246.91 a	25.49 a	268.57 a	28.58 a
***Flavimonas oryzihabitans* (K50V2s)**	6.73 a	0.68 a	69.69 a	8.89 a	99.27 a	11.54 a	200.49 ab	22.49 a	268.48 a	28.44 a
Control	5.86 ab	0.67 a	58.45 a	7.49 a	112.44 a	13.10 a	195.93 ab	22.60 a	244.73 a	29.44 a

n=5

Values are means of 5 replicates and means with the same letter are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

Table 6.13: Effect of inoculation with 5 selected bacterial strains on root fresh weight (RFW) and root dry weight (RDW) of tissue cultured banana plantsat different fertilizer regimes (g)in experiment 2.

Bacterial isolate	**0 g**		**2.5 g**		**5 g**		**7.5 g**		**10 g**	
	RFW	RDW	RFW	RDW	RFW	RDW	RFW	RDW	RFW	RDW
***Bacillus subtilis subsp. inaquosorum* (30)**	4.14 abc	0.24 a	66.70 a	3.49 a	121.48 a	6.22 a	151.90 a	8.15 a	154.36 a	8.83 a
***Enterobacter ludwigii* (31)**	4.66 ab	0.30 a	85.80 a	4.08 a	119.29 a	6.31 a	148.40 a	8.49 a	165.35 a	8.70 a
***Ewingella americana* (39)**	1.98 c	0.18 a	61.73 a	3.08 a	92.44 a	5.84 a	142.12 a	7.53 a	140.21 a	7.70 a
***Rahnella aquatilis* (42)**	3.11 abc	0.15 a	65.02 a	3.33 a	116.68 a	6.27 a	153.20 a	8.37 a	148.81 a	8.23 a
***Flavimonas oryzihabitans* (43)**	5.40 a	0.26 a	85.30 a	4.54 a	112.08 a	6.08 a	149.75 a	8.24 a	159.20 a	9.57 a
Control	4.20 abc	0.19 a	76.00 a	4.08 a	116.30 a	6.13 a	147.45 a	7.79 a	144.43 a	8.43 a

n=5

Values are means of 5 replicates and means with the same letter are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

6.3.2 Effect of isolates on plant mineral nutrient concentration

In the 1st experiment, inoculation with 5 selected isolates had no significant effect on plant mineral nutrient concentrations (**Table 6.14**). However, plants inoculated with *Rahnella aquatilis* (ME19V2c)had the highest levels of P, K and Ca in their shoots. There was no significant interaction between fertilizer application and bacterial inoculation.In experiment 2, inoculation with the 5 selected bacterial strains did not have a significant effect on P, Mg and Ca concentrations; however, it significantly reduced the Zn and Fe concentrations (**Table 6.15**). Inoculation with *Ewingella americana* (K32V2c) and *Flavimonas oryzihabitans* (K50V2s) did also reduce the Mn concentrations significantly. In experiment 3, inoculation with 24 bacterial strains had neutral to positive significant effects on plant mineral nutrient concentrations as shown in appendix II.

Table 6.14: Effect of inoculation with 5 selected bacterial strains on shoot mineral nutrient content of tissue cultured banana plantsin experiment 1.

Bacterial isolate	**Mg (ppb)**	**P (ppb)**	**Fe (ppb)**	**K (ppb)**	**Ca (ppb)**	**Mn (ppb)**
***Bacillus subtilis subsp. inaquosorum* (M9V1r)**	30.14 a	45.40 a	1.22 a	491.85 a	42.91 a	3.63 a
***Enterobacter ludwigii* (J1V1r)**	28.63 a	45.44 a	1.00 a	492.17 a	37.42 a	4.04 a
***Ewingella americana* (K32V2c)**	31.38 a	48.10 a	1.14 a	530.18 a	39.35 a	3.88 a
***Rahnella aquatilis* (ME19V2c)**	32.72 a	50.86 a	1.21 a	530.97 a	44.36 a	3.74 a
***Flavimonas oryzihabitans* (K50V2s)**	28.60 a	42.96 a	0.93 a	473.41 a	38.05 a	3.68 a
Control	33.81 a	49.33 a	1.26 a	490.72 a	38.90 a	3.44 a

n=8

Values are means of 2 replicates and means with the same letter are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

Table 6.15: Effect of inoculation with 5 selected bacterial strains on shoot mineral nutrient content of tissue cultured banana plantsin experiment 2.

Bacterial isolates	**P (ppm)**	**Mg (ppm)**	**Ca (ppm)**	**Mn (ppm)**	**Zn (ppm)**	**Fe (ppm)**
***Bacillus subtilis subsp. inaquosorum* (M9V1r)**	6.02 a	1.57 a	5.24 abc	0.60 ab	0.13 c	2.56 a
***Enterobacter ludwigii* (J1V1r)**	5.63 a	1.37 a	4.84 c	0.54 abc	0.13 c	2.18 b
***Ewingella americana* (K32V2c)**	5.48 a	1.33 a	4.93 bc	0.29 d	0.10 d	1.62 c
***Rahnella aquatilis* (ME19V2c)**	5.62 a	1.52 a	5.48 a	0.53 bc	0.16 b	2.02 b
***Flavimonas oryzihabitans* (K50V2s)**	5.72 a	1.41 a	5.34 ab	0.38 cd	0.14 c	1.57 c
Control	5.90 a	1.40 a	5.14 abc	0.62 ab	0.19 a	2.48 a

n=10

Values are means of 2 replicates and means with the same letter are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

6.3.3 Effect of isolates on the chlorophyll content

6.3.3.1. SPAD calibration

SPAD measurements were taken for selected leavesrepresenting a range of colors extending from most green, moderately green, moderately yellow and yellow. The values obtained were correlated with absolute values for chlorophyll content for the same leaves expressed on a fresh weight basis following solvent extraction. The calibration procedure is described in Section 5.2.4. The results were analysed to establish the best fit between the SPAD values and chlorophyll content; linear, polynomial and logarithmic relationships were tested and logarithmic relationship proved best. Correlation analysis showed a highly significant relationship between SPAD values and chlorophyll content ($p<0.0001$) with r^2 value of 0.9745 (**Figure 6.5**).

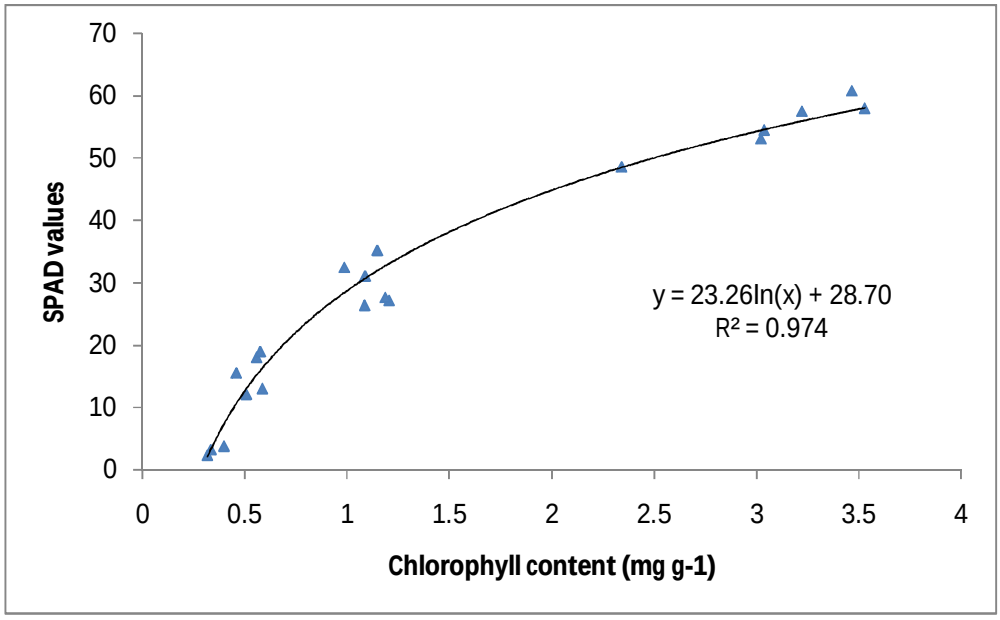


Figure 6.5: Relationship between SPAD values and chlorophyll contents for leaves of tissue cultured banana plants.

6.3.3.2. Chlorophyll analysis

In experiment 1, inoculation of tissue cultured banana plants with selected bacterial isolates at different fertilizer regimes had a neutral to positive effect on the leaf chlorophyll content (**Table 6.16**).At the lowest fertilizer regime (2.5 g), plants inoculated with isolates *Bacillus subtilis subsp. inaquosorum* (M9V1r), *Enterobacter ludwigii* (J1V1r)and *Ewingella americana* (K32V2c)had significantly higher chlorophyll contents than the control plants (uninoculated). At 5 g and 10 g fertilizer regime, plants inoculated with isolate *Enterobacter ludwigii* (J1V1r)and *Ewingella americana* (K32V2c)had significantly higher chlorophyll content than the control, respectively.

At 7.5 g fertilizer regime, plants inoculated with isolate *Ewingella americana* (K32V2c)recorded the highest chlorophyll content though not significantly different from the control.Days after planting had a significant effect (<0.0001) on the leaf chlorophyll content across the different fertilizer regimes. At the lower fertilizer regimes (2.5 g and 5 g), chlorophyll content increased significantly with increased days after plantingupto 42 DAP after which it dropped significantly while at the higher fertilizer regimes (7.5 g and 10 g), chlorophyll content increased significantly upto 56 DAP and dropped thereafter (**Table 6.17**).

Table 6.16: Leaf chlorophyll content (mg g^{-1}fresh weight) of tissue cultured banana plants as affected by inoculation with selected bacterial endophytes at different fertilizer regimes (g)in experiment 1.

Bacterial isolate	**2.5 g**	**5 g**	**7.5 g**	**10 g**
***Bacillus subtilis subsp. inaquosorum* (M9V1r)**	1.81 a	1.90 abc	2.23 a	2.29 bc
***Enterobacter ludwigii* (J1V1r)**	1.76 a	2.23 a	2.32 a	2.78 bc
***Ewingella americana* (K32V2c)**	1.86 a	2.06 ab	2.36 a	3.52 a
***Rahnella aquatilis* (ME19V2c)**	1.72 ab	1.66 c	2.00 a	2.82 b
***Flavimonas oryzihabitans* (K50V2s)**	1.72 ab	2.02 abc	2.29 a	2.10 c
Control	1.47 b	1.83 bc	2.07 a	2.63 bc

n=40

Values are means of 8 replicates and the means with the same letter in a column are not significantly different. Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

Table 6.17: Leaf chlorophyll content (mg g^{-1}fresh weight) of tissue cultured banana plants as affected by days after planting at different fertilizer regimesin experiment 1.

Days after planting	2.5 g	5 g	7.5 g	10 g
14	1.106 c	1.236 b	1.120 c	1.229 c
28	1.992 b	2.188 a	2.276 b	2.505 b
42	2.310 a	2.466 a	2.801 a	3.341 a
56	1.974 b	2.416 a	2.907 a	3.423 a
70	1.238 c	1.441 b	1.958 b	2.952 ab

n=48

Values are means of 8 replicates and the means with the same letter in a column are not significantly different. Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

In the 2nd experiment, inoculation of plants with bacterial endophytes did not show significant effect on the leaf chlorophyll content across the different fertilizer regimes (**Table 6.18**). However, at 0 g fertilizer regime, plants inoculated with *Flavimonas oryzihabitans* (K50V2s) showed a significantly higher chlorophyll content than the control while inoculation with*Ewingella americana* (K32V2c) showed a significantly lower chlorophyll content than the control.In regard to the leaf chlorophyll content, days after planting interacted significantly (<0.0001) with fertilizer regimes. At 0 g and 2.5 g fertilizer regimes, the chlorophyll content decreased significantly with increased days after planting, whereas at 5 g and 7.5 g fertilizer regimes, the chlorophyll content increased upto 42 days after planting decreasing thereafter (**Table 6.19**). At the highest fertilizer regime (10 g), the

chlorophyll content increased significantly upto 56 days after planting dropping significantlyafterward.

Table 6.18: Leaf chlorophyll content (mg g^{-1}fresh weight) of tissue cultured banana plants as affected by inoculation with selected bacterial endophytes at different fertilizer regimesin experiment 2.

Bacterial isolate	**0 g**	**2.5 g**	**5 g**	**7.5 g**	**10 g**
***Bacillus subtilis subsp. inaquosorum* (M9V1r)**	0.87 bc	1.33 a	1.43 a	1.80 ab	1.85 a
***Enterobacter ludwigii* (J1V1r)**	0.96 ab	1.42 a	1.70 a	1.88 a	2.00 a
***Ewingella americana* (K32V2c)**	0.71 d	1.25 a	1.33 a	1.57 b	1.72 a
***Rahnella aquatilis* (ME19V2c)**	0.87 bc	1.29 a	1.55 a	1.95 a	1.82 a
***Flavimonas oryzihabitans* (K50V2s)**	1.02 a	1.49 a	1.84 a	1.83 ab	1.83 a
Control	0.87 bc	1.35 a	1.71 a	1.70 ab	1.81 a

n=25

Values are means of 5 replicates and the means with the same letter in a column are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

Table 6.19: Leaf chlorophyll content (mg g^{-1}fresh weight) of tissue cultured banana plants as affected by days after planting at different fertilizer regimesin experiment 2.

Days after planting	0 g	2.5 g	5 g	7.5 g	10 g
14	1.502 a	1.589 a	1.547 a	1.637 b	1.535 b
28	1.014 b	1.494 a	1.626 a	1.778 b	1.715 b
42	0.759 c	1.398 ab	1.692 a	2.079 a	2.153 a
56	0.569 d	1.247 bc	1.528 a	2.011 a	2.205 a
70	0.488 d	1.087 c	1.560 a	1.586 b	1.661 b

n=35

Values are means of 5 replicates and the means with the same letter in a column are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

In the 3rd experiment, inoculation of plants with bacterial endophytes did not show significant effect on the leaf chlorophyll content except with isolate *Serratia ureilytica*(K24V1c), which showed significantly higher chlorophyll content as compared with the control plants (**Table 6.20**).Days after planting did not significantly affect the leaf chlorophyll content (**Figure 6.6**).

Table 6.20: Leaf chlorophyll content (mg g^{-1}fresh weight) of tissue cultured banana plants as affected by inoculation with bacterial endophytesin experiment 3.

Bacterial isolate	**Chlorophyll content (mg g^{-1})**
***Yersinia kristensenii*(E43V2)**	1.337 ab
***Klebsiella granulomatis*(K22V1c)**	1.316 ab
***Pseudomonas psychrophila*(E29V2c)**	1.277 abc
***Pseudomonas protegens*(K23V1c)**	1.409 ab
***Raoultella terrigena*(K29V1c)**	1.133 bc
***Rahnella aquatilis*(E25V2c)**	1.124 bc
***Serratia fonticola*(E17V1c)**	1.273 abc
***Serratia ureilytica* (K24V1c)**	1.656 a
***Pseudomonas protegens*(K39V1s)**	1.366 ab
***Pseudomonas moraviensis*(K10V1r)**	1.404 ab
***Serratia plymuthica*(E2V1r)**	1.416 ab
***Pseudomonas graminis*(E18V1c)**	0.919 c
***Enterobacter amnigenus*(E41V2)**	1.147 bc
***Pseudomonas palleroniana*(K49V2s)**	1.161 bc
***Pseudomonas protegens*(K34V2c)**	1.367 ab
***Serratia proteamaculans*(M20V2c)**	1.143 bc
***Flavimonas oryzihabitans*(K50V2s)**	1.300 abc
***Pseudomonas koreensis*(M28V2s)**	1.438 ab
***Bacillus subtilis subsp. inaquosorum* (M9V1r)**	1.366 ab
***Enterobacter ludwigii* (J1V1r)**	1.389 ab

Bacterial isolate	Chlorophyll content (mg g^{-1})
***Yokenella regensburgei*(M32V1s)**	1.069 bc
***Ewingella americana* (K32V2c)**	1.423 ab
***Serratia glossinae*(J22V1c)**	1.388 ab
***Rahnella aquatilis* (ME19V2c)**	1.076 bc
Control	1.266 bc

n=12

Values are means of 4 replicates and means with the same letter in a column are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.

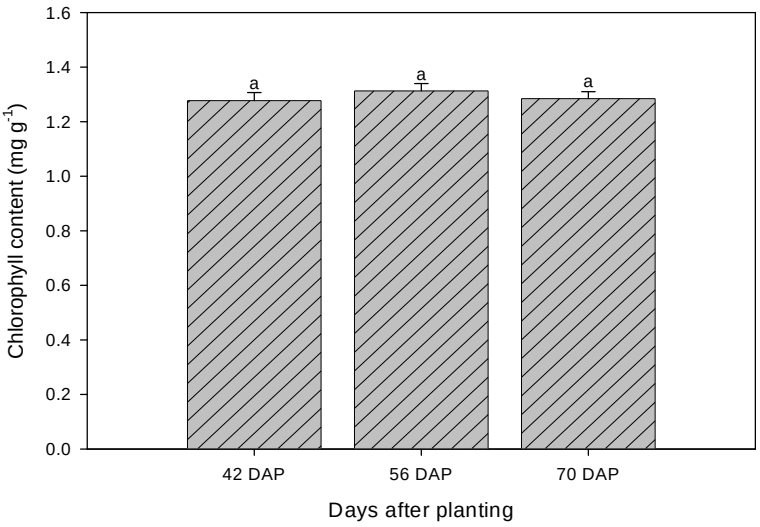


Figure 6.6: Leaf chlorophyll content (mg g^{-1}fresh weight) of tissue cultured banana plants as affected by days after plantingin experiment 3. Values are means of 4 replicates and means with the same letter are not significantly different.Means were separated using Tukey's Studentized Range (HSD) Test at P = 0.05.Vertical bars indicate standard errors. n=104.

6.4. Discussion

In the current study, *Bacillus subtilis subsp. inaquosorum* (M9V1r), *Enterobacter ludwigii* (J1V1r), *Ewingella americana* (K32V2c), *Rahnella aquatilis*(ME19V2c) and *Flavimonas oryzihabitans*(K50V2s) showed neutral to positive effects on banana growth in regard to relative change in plant height, plant diameterand number of green leaves and in regard tofresh and dry weights for bothshoots and roots.Endophytes are increasingly gaining scientific and commercial interest because of their potential to improve plant quality and growth and their close association with internal tissues of host plant (Carroll, 1992; Schulz *et al.*, 1999). Endophytic bacteria have been reported to enhance plant growth in non-leguminous crops and improve their nutrition through nitrogen fixation, phosphate solubilization or siderophore production (Dobereiner and Baldani, 1998; Sturz *et al*., 2000; Sevilla *et al.*, 2001; Hurek *et al*., 2002; Boddey *et al*., 2003; Iniguez *et al*., 2004; Ryan et al., 2008; Uribe *et al*., 2010). *Enterobacter ludwigii, Ewingella americana, Rahnella aquatilis* and *Flavimonas oryzihabitans*have been reported to promote plant growth by various authors. For example, Grimont *et al*., (1983) reported *Ewingella,* whichconsists of only one species;*Ewingella americana*having potential to promote plant growth while*Rahnella aquatilis* has been reported as having potential to improve supply of plants with nutrients like phosphate (Kim *et al*., 1998; Vyas *et al.*, 2010 and Ngamau *et al*., 2012) and to fix free nitrogen (Heulin *et al*., 1994 and Ngamau *et al*., 2012). *Enterobacter ludwigii*has also been reported to promote plant growth through nitrogen fixation, phosphate solubilization and siderophores production (Shoebitz*et al.*, 2009) while *Pseudomonas*species especially *Flavimonas oryzihabitans*have been reported to be high siderophore producers (Gangwar and Kaur, 2009; Tani *et al.*,

2011; Ngamau *et al.*, 2012). Iron chelated by microbial siderophores can be utilized by plants (Chen *et al.*, 1998) and thus improve the iron nutrition of plants.The high efficiency in phosphate solubilization and siderophore production shown by *Rahnella aquatilis*(ME19V2c)and *Flavimonas oryzihabitans*(K50V2s)as free cells, respectively was however not evident *in-planta*.Many bacteria strains seem to exert their beneficial effects at the *in-vitro* level (laboratory culture), while a few of them are successful in greenhouse or field conditions. Understanding the reasons for these inconsistencies may lead to more prospects in biofertilizer research.

According to Van Vuurde and Elvira-Recuenco (2000) application of Gram-negative and -positive bacteria is probably most effective in relatively endophyte-free materials, where niches can still be colonized by the introduced endophyte with relatively low competition with naturally present endophytes. Plant growth-promoting rhizobacteria are also reported to be mostly effective under low-nutrient conditions and have little or no measurable effect on plant growth when the plants are grown in nutrient-rich soil under optimal conditions (Penrose and Glick, 2003). It is in view of this that the 3rdgreenhouse experiment was set up with an attempt to optimize on colonization by inoculating at the *in-vitro* stage before plantlets are exposed to other microorganisms. A sub-optimal fertilizer level of 5 g was also used. Inoculation under these conditions,even for the potential isolates identified at the *in-vitro* stage, did not show significant effect on plant growth. Reasons for theinconsistency in the performance of microbes as free cells (*in-vitro*) and *in-planta*could includevariability of the planting material, limited knowledge of the plant-microbe interactions, the uncontrolled effect of plant varieties and soil types on microbial colonization and functional capacity and lack of adequate formulation

technologies (Rodriguez and Fraga, 1999; Gyaneshwar *et al.*, 2002b; Lucy *et al.*, 2004).To minimize on variability of the planting material, relative growth rates were used in this study instead of absolute growth rate.As expected, the relative growth rate of theplants (height, diameter and number of green leaves) decreased significantly 42 days after planting.

Plant inoculation with *Rahnella aquatilis*(ME19V2c)increased P concentration in plant shootsthough not significantly. *Rahnella aquatilis*(ME19V2c and ME18V2c),as reported in chapter 4, showed ability to solubilize phosphates and since the use of phosphate solubilizing bacteria as inoculants has been reported to increase P uptake by the plant (Rodriguez and Fraga, 1999) the results of this study confirm*Rahnella aquatilis* potential to improve supply of plants with phosphates (Kim *et al.*, 1998; Vyas *et al.*, 2010).The P content of the soil has been suggested as one of the important factors that determine the effectiveness of inoculation with P-solubilizing bacteria (Rodriguez and Fraga, 1999). The use of inert coco peat as support medium (with little or no organic phosphorus) and controlled release fertilizer as source of inorganic P, might explainthe non-significant increase of P concentration in plant shoot.

Chlorophyll content was determined non-destructively using a SPAD-502 meter (Minolta, Japan). Close linear correlations between SPAD values and extractable chlorophyll content have been reported for a wide range of species (Yadava, 1986; Marquard and Tipton, 1987; Finnan *et al.*, 1998; Muthuri, 2004). However, the relationshipbetween SPAD measurements and chlorophyll content is species-dependent (Yadava, 1986; Marquard and Tipton, 1987) and calibration for *Musa* spp. cv. Grande Nainewas therefore necessary. In the present study, a highly

significant relationship ($p<0.0001$) between the SPAD values and the chlorophyll content of *Musa* spp. cv. Grande Naine was found and a logarithmic relationship between the twoestablished to be the best fit with r^2 value of 0.9745.Inoculationof plants with *Bacillus subtilis subsp. inaquosorum*(M9V1r), *Enterobacter ludwigii*(J1V1r)and *Ewingella americana*(K32V2c)significantly increased chlorophyll content in plants.Chlorophyll content is used as a measure of plant wellbeing in relation to the nitrogen levels.Having showed ability to fix atmospheric nitrogen, the bacterial strains may have contributed to the nitrogen economy of the plants as reflected in the increased chlorophyll content.

6.5. Conclusions

The potential of endophytic bacteria in sustainable agriculture is immense. However, their biology and ecology are far from being fully understood.This study has shown the functional potential of *Bacillus subtilis subsp. Inaquosorum*(M9V1r), *Enterobacter ludwigii*(J1V1r), *Ewingella Americana*(K32V2c),*Rahnella aquatilis*(ME19V2c) and *Flavimonas oryzihabitans*(K50V2s) to promote plant growth. However, to facilitate the use of these endophytic bacteria in practical agronomic production, reliable and practical methods of inoculum delivery must be developed.The challenge and goal is to manage microbial communities to favor plant colonization by beneficial bacteria. This would be achieved when a better knowledge on endophyte ecology and their molecular interactions is attained.

The cool and cloudy weather between May and July when the 1st experiment was conducted may have favoured plant growth as compared to the sunny and dry weather between August and October when the 2ndexperiment was done.

CHAPTER SEVEN

7.0 LOCALIZATION AND COLONIZATION OF BANANA ENDOPHYTIC BACTERIA

7.1 Introduction

7.1.1 Localization

Criteria to recognize “true” endophytic bacteria have been published and they not only require isolation from surface-disinfected tissues but microscopic evidence of “tagged” bacteria inside plant tissues must also be provided. The latter criterion is not always fulfilled, and the term ‘putative endophytes’ is used for those not validated microscopically. True endophytes may also be recognized by their capacity to re-infect disinfected seedlings (Rosenblueth and Martínez-Romero, 2006).

The green fluorescent protein (GFP) is widely used as a reporter in studies of gene expression and protein localization in living organisms.The 26.9 kDa polypeptide, from the jellyfish *Aquoria victoria*,exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range (Chalfie *et al*., 1994; Tsien, 1998).Accordingly, GFP together withconfocal laser scanning microscopy (CLSM)have become a powerful tool for studying plant-microbe interactions.

7.1.2 Colonization

Plant colonization by endophytic bacteria is dependent on severalfactors including;1) Endophytes invasion capacity, with some endophytes being better colonizers than others (Dong *et al*., 2003). 2) Plant hosts, which differ in their ability to endophyticallybecolonized suggestingan active host role in the colonization process (Rosenblueth and Martínez-Romero, 2006). 3) Plant defense responsesthat have been shown to limit bacterial populations inside plants allowing entry only tothose that are

of beneficial effects (Rosenblueth and Martínez-Romero, 2006; Senthilkumar *et al*., 2011).4) Plant flavonoids, which are reported to stimulate colonization, for example in wheat where colonization by *Azorhizobiumcaulinodans* and *Azospirillum brasilense* was stimulated byflavonoids (Webster *et al.,* 1998).5) Changes in plant physiology, for example in sugarcane wherea reduction in colonization by *Gluconacetobacter diazotrophicus*was observed in plants under a high nitrogen-fertilizationregime as opposed to low N fertilization. This may be explained by the fact that supply of nitrogen, which alters plant's physiology,may cause a decrease in sucrose needed forthe endophytic growth (Fuentes-Ramírez *et al.*, 1999).6) Competition with indigenous soil bacteria(Cooley *et al*., 2003).

Since plants have a determinant role in controlling endophyticcolonization, colonizationassays should not be performed with plants under suboptimal growthconditions, as they may show unbalanced interactions withendophytes with occasional overestimation of bacterial colonizationby some strains(Rosenblueth and Martínez-Romero, 2006).

The objective of this study was to determine the isolates' endophytic habitat and their capacity to colonize and re-infect bananas inorder to confirm that bacteria isolated from surface-disinfected banana tissues are 'true' endophytes.

7.2 Materials and Methods

7.2.1 Determination ofbacterial strains' endophytic habitat (localization)

In order to assess whether the isolated bacterial strains were actually endophytic, a reporter gene was used to tag the bacteria.

7.2.1.1 Conjugation of bacterial strains with *E. coli*– GFP mutant

Bacillus subtilis subsp. subtilis (M9V1r), *Enterobacter asburiae* (J1V1r), *Ewingella americana* (K32V2c), *Rahnella aquatilis* (ME19V2c) and *Flavimonas oryzihabitans* (K50V2s) strains were each inoculated into LB medium (100µl) in a 1.5 ml eppendorf tube at the clean bench (a relatively large amount of inoculum was used). Very little of *E. coli* S17-1 (mTn5gusA-pgfp21) was then added using a tooth-pick to each of the inoculated strain. The mixture was then spread on to nutrient agar plates and allowed to grow for one night. After one night of growth, 1 ml of LB medium was added to each plate and spread using a spreader. This mixture of colonies and LB medium was then picked and put into a 1.5 ml eppendorf tube. Using a loop, some of this mixture was picked and streaked on citrate agar plus kanamycin plate (0.5 g $NH_4H_2PO_4$, 0.5 g K_2HPO_4, 2.5 g NaCl, 1 g $C_6H_5Na_3O_7.2H_2O$, 0.1 g $MgSO_4.7H_2O$per 500 ml, and 1.5% agar plus 25 µl/l kanamycin, at pH 6.9 ± 0.2 at 25°C). Kanamycin was added to the citrate medium after autoclaving and cooling to about 50°C. Growth was allowed for two nights. Only successfultransconjugantswould grow since *E. coli*does not grow on citrate agar and non-transconjugants would not survive with kanamycin. Colonies of the transconjugantswere then picked from the plates and individually streaked on nutrient agar plus kanamycin.

7.2.1.2 Free cells fluorescence analysis

Bacterial cells were picked from the nutrient agar plus kanamycin plates and added to 1 ml of 0.9% NaCl in an eppendorf tube. The cells were then mounted to a glass microscope slide for observation and analysis using the Confocal Laser Scanning Microscope (Olympus Fluoview FV1000).Confocal laser scanning microscopy is

used for high-resolution analysis of cell and tissue structures labeled with fluorescent markers like the green fluorescent protein used in this study. Confocal laser scanning microscopy is the prevailing microscope technique for “deeper” analysis at cellular and sub-cellular levels. High resolution is achieved by scanning the sample with a finely focussed laser beam, and exclusion of out-of-focus fluorescence by a confocal aperture. Wild type free cells were used as control.

7.2.1.3 Plant inoculation with GFP mutants

GFP mutantsof *Flavimonas oryzihabitans*(K50V2s)were cultured in 5 ml nutrient broth in 16 cm test tubes in 5 replicates and placed on a RECIPRO shaker model RLS-150N at 105 rpm and left to grow for 20 hours at 30°C. The optical densities of the inocula as well as the colony forming units (CFU) were determined. One milliliter of the bacterial suspension was serially diluted with sterile MilliQ water and plated on nutrient agar plates for cell count. For cell washing, 20 ml cell suspension was centrifuged at 5,000 rpm for 5 minutes and the supernatant was discarded. Sterile MilliQ waterwas than added and the mixture centrifuged at 5,000 rpm for 5 minutes twice. The cells were then re-suspended in 40 ml sterile MilliQ water. For inoculation, 20 ml cell suspensionwas aseptically added to an aseptically maintained rooting jar with 10 individual tissue cultured banana plantlets.The plantlets were 10 weeks old. Each rooting jar was an individual treatment with 10 replicates (each plantlet in the jar constituted a replicate). A control jar of 10 plantlets was inoculated with 20 ml sterile MilliQ water.This was done in duplicates. The inoculation was done aseptically and the bottles re-sealed immediately and left for 24 hours for inoculation to take place. After inoculation, one jar of inoculated plantlets and one of uninoculated plantlets were opened and the plantlets planted in autoclaved

soil in planting trays. They were then maintained in the lab with watering twice daily with tap water for two days.The other set of inoculated and uninoculated jars were left unopened also for twodays. After the two days, sample plantlets were harvested and prepared for fluorescence scanning.

7.2.1.4 Fluorescence scanning of GFP mutant inoculated and uninoculated plants

Plants were thinly sliced (ca. 15 µm) using the plant microtome automatic MT-3 slicer. Slides of the plant slices were prepared and fluorescence analysis done using the Confocal Laser Scanning Microscope (Olympus Fluoview FV1000).

7.2.2 Colonization assayswith K50V2s-GFP mutant

Plants were inoculated with GFP mutant strains as in **7.2.1.3**and planted in sterile soil and maintained in the lab. Two days after inoculation colonization was determined. Two plants per treatment were up-rooted and excess soil shaken off. Each plantlet was then placed in a sterile 50 ml falcon tubein which 25 ml MilliQ water was added and shaken gently.The supernatant was serially diluted and inoculated on nutrient agar medium with and without kanamycin for CFU determination of rhizosphere colonization. Kanamycin was added to the nutrient agar to allow selective growth of only the GFP mutant cells. For internal (endosphere) plant colonization, the plantlets were individually dried using the Kimwiper and weighed for fresh weight.Each plantlet was then surface sterilized with 25 ml of a sterilization solution (50 ml 8-13% sodium hypochrolite, 50 µl Tween 20 topped up to 1000 ml with MilliQ water) in a 50 ml sterile tube, twice with gentle vortex for about 2 minutes each. The individual plantlets were then washed with sterile MilliQ water (vortex and decantation), four times. The plantlets were then aseptically macerated with 5 ml

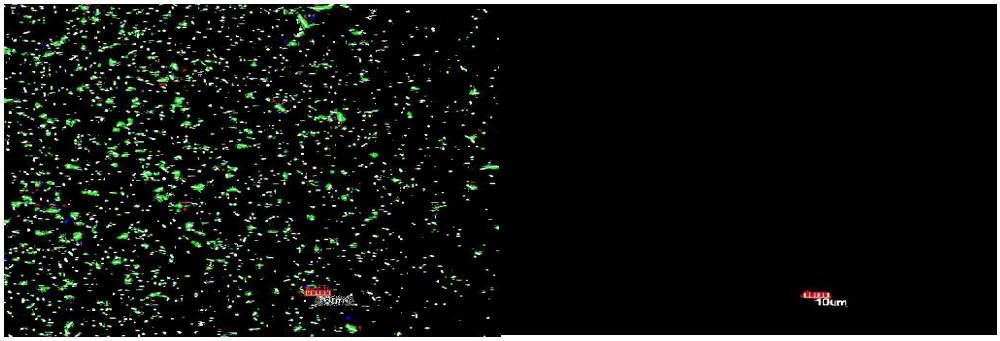
sterile MilliQ water and serially diluted for CFU determination. Nutrient medium with and without kanamycin was used for bacteria growth. The nutrient medium with kanamycin was used to selectively grow only the mutant strains.

7.3 Results

7.3.1 Bacterial strains' endophytic habitat - Localization of GFP mutants

7.3.1.1 Conjugation of bacterial strains with *E. coli* – GFP mutant and fluorescent analysis

As expected no fluorescence was observed withwild type free cells, however of the five tested strains only *Flavimonas oryzihabitans*(K50V2s)GFP mutant free cellsshowed fluorescence (**Plate7.1**).This implied thatonly *Flavimonas oryzihabitans* (K50V2s)strains resulted tosuccessful transconjugants.



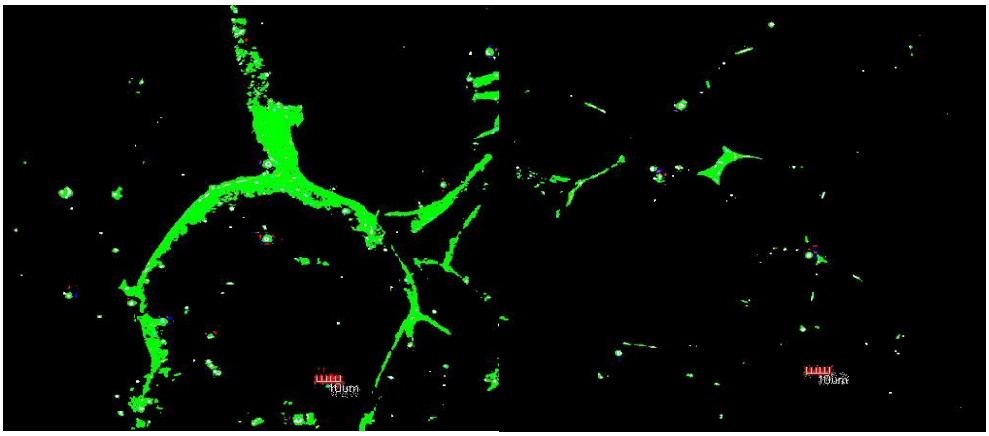
K50V2sGFP mutant free cells K50V2swild type free cells

Plate 7.1: Fluorescence scanning of K50V2s GFP mutant and K50V2s wild type free cells under Confocal Laser Scanning Microscope (Olympus Fluoview FV1000).

7.3.1.2 Plant inoculation with GFP mutant strains and fluorescence scanning

Tissue culture banana plantlets inoculated with *Flavimonas oryzihabitans*(K50V2s)GFP mutant cells and un-inoculated ones (control) were left to stay for 2 days, some in soil and others in the rooting bottles. After the 2 days,

plants were thinly sliced (ca. 15 µm) using the plant microtome automatic MT-3 slicer. Slides of the plant slices were prepared and fluorescence analysis done using the Confocal Laser Scanning Microscope (Olympus Fluoview FV1000). There was more fluorescence in cells of GFP mutant plants as compared to the un-inoculated (control) plants (**Plate7.2**). The fluorescence in the un-inoculated plants may have been caused by other green pigmented elements like the chloroplast.

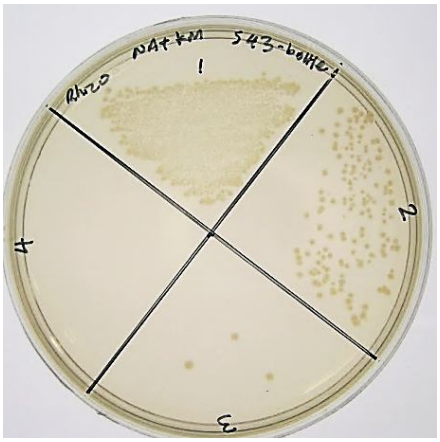


K50V2s GFP mutant plant Control plant

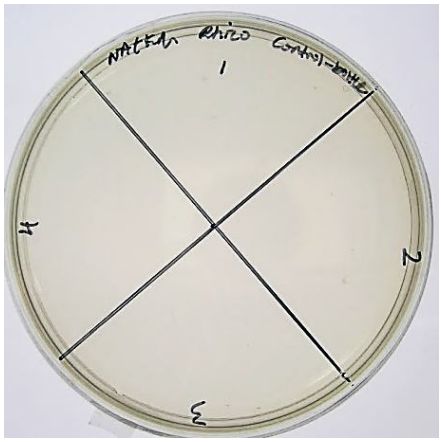
Plate 7.2: Fluorescence scanning of thinly sliced plant tissues of both GFP mutant TC banana plants (inoculated) and control TC banana plants (un-inoculated) under the Confocal Laser Scanning Microscope (Olympus Fluoview FV1000).

7.3.2 Colonization assaywith *Flavimonas oryzihabitans*(K50V2s)-GFP mutants

Only successful transconjugants *Flavimonas oryzihabitans*(K50V2s)strains were used in the colonization assay. There was evidence of colonization both at the rhizosphere and inside the plants with a cell concentration of 1.53 x 10^6cfu/mland 1.4 x 10^5cfu/ml respectively(**Plate 7.3**). The original inoculumwas2.8 x 10^9 cfu/ml. There was no bacterial growth in the control experiments.

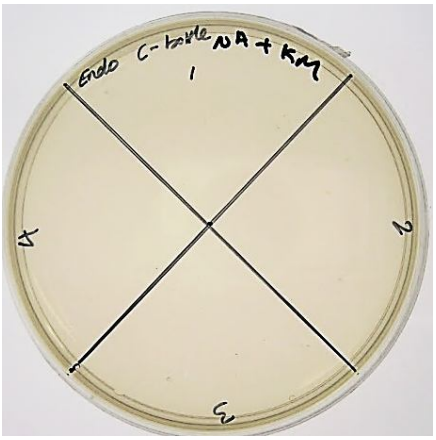


K50V2s -GFP mutant (rhizosphere)
(1.53 x 10^6cfu/ml)



Control (rhizosphere)
No bacterial growth

K50V2s -GFP mutant (endosphere)
(1.4 x 10^5cfu/ml)



Control (endosphere)
No bacterial growth

Plate 7.3: Colonization assay plates. Cell concentration was determined through serial dilution where 10 µl of inoculum dilutes was placed in respective quarter on the plate. The cell concentration was equal to number of colonies per quarter multiplied by $1x10^2$, $1x10^4$, $1x10^6$ and $1x10^8$ for the 1st, 2nd, 3rd and 4th quarter, respectively.

7.4 Discussion

Endophytic habitat of *Flavimonas oryzihabitans* (K50V2s) was confirmed through microscopic evidence of "tagged" bacteria inside plant tissues.The green fluorescent protein reporter gene (mTn5gusA-pgfp21) from*E. coli* S17-1 was used to tag the bacteria while fluorescence scanning was done under theConfocal Laser Scanning Microscope. Onlyconjugation of *Flavimonas oryzihabitans* (K50V2s) with *E. coli* S17-1 (mTn5gusA-pgfp21) resulted to successful transconjugants. Endophytic habitat of *Flavimonas oryzihabitans* (K50V2s) was further confirmed by the fact that the bacterial strains could re-infect tissue cultured banana plantlets. According to Rosenblueth and Martínez-Romero (2006), criteria to recognize true endophytic bacteria require not only isolation from surface-disinfected tissues but microscopic evidence of "tagged" bacteria inside plant tissues and their capacity to re-infect disinfected seedlings as well.

Flavimonas oryzihabitans (K50V2s)showed capacity to colonize both the rhizosphere and the endosphere of tissue cultured banana plantlets with cell concentration of 1.53 x 10^6 cfu/ml and 1.4 x 10^5cfu/ml, respectivelytwo days after inoculation. Plant colonization by endophytic bacteria is dependent among others on the endophytes invasion capacity, with some endophytes being better colonizers than others(Dong *et al*., 2003). According to Kloepper *et al.*(1992) almost all endophytic bacteria are found in the rhizosphere and according toSenthilkumar *et al.* (2011) rhizosphere soil is the primary source for endophytic colonization.Except for bacteria transmittedthrough seeds, colonization of the root surface is critical before entry to the plant(Senthilkumar *et al.* (2011).

Tissue cultured banana plantlets also showed ability to be colonized endophyticallyby *Flavimonas oryzihabitans* (K50V2s)with a cell concentration of1.4 x 10^5 cfu/ml. After root surface colonization, endophytic bacteria enter plants mainly through wounds naturally occurring as a result of plant growth or through root hairs and at epidermal conjunctions. Wounds also allow leakage of plant exudates that serve as a nutrient source for the bacteria (Hallmann *et al*., 1997).Molecular analysis has shown that plant defense responses limit bacterial populations inside plants (Rosenblueth and Martínez-Romero, 2006) with Senthilkumar *et al*. (2011) alluding to the fact that organisms occupying the endosphere have most likely been selected for this niche by the plant because of the beneficial effects they offer their host and their abilities to resist the effects of plant defence products.This is suggestive of the active role that plant hosts play in the colonization process (Rosenblueth and Martínez-Romero, 2006).

7.5 Conclusions

Having confirmed the endophytic habitat and colonization ability of *Flavimonas oryzihabitans*(K50V2s)it can then be concluded that *Flavimonas oryzihabitans* (K50V2s) isolated from surface-disinfected banana tissues were true endophytes. However, endophytic habitat and colonization ability of the other isolated bacterial strains would need to be confirmed as well. It can also be concluded that tissue cultured banana plantlets have the ability to be colonized endophytically.

CHAPTER EIGHT

8.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

8.1 General discussion

Of the ten genera identified in this study, *Rahnella, Yokenella, Raoultella, Yersinia* and *Ewingella* species had previously not been reported in association with bananas suggesting a larger richness of endophytic bacterial species associated with bananas than has so far been reported. This divergence could be as a result of the different agro ecological conditions of study sites and the unique East African highland banana cultivars especially the cooking banana variety (*Musa* AAB) whose endophytic biology has not been widely studied.

*Serratia*species were found to be non-host and non-area specific. The findings are similar to those of Ting *et al*. (2008) whofound *Serratia*species to be non-host specific. *Pseudomonas* species were found to benon-host specific but area specificwhile *Rahnella* species were found to be host and area specific. Kisii Central and Embu Districts, both belonging to the agroecological zone UM1, humid with ando-humic NITISOLS, exhibited the highest bacterial isolates diversity while Imenti North (Meru) District of agroecological zone UM2, sub-humid with humic NITISOLS displayed the lowest diversity with only *Serratia*and *Rahnella* species. Humid conditions and ando-humic NITISOLS soil type seemed to favour bacteriadiversity as compared to sub-humid conditions with humic NITISOLS soil type.According to Kuklinsky-Sobral *et al.*(2004) and Conn and Franco (2004), bacterial species distribution patterns are dependent on plant genotype, tissue sampled, soil and agro-climatic conditions.

Nitrogen fixation is the reduction of atmospheric dinitrogen (N_2) to ammonia (NH_3), a product that is subsequently assimilated into biomass (Staal *et al*., 2001).Screening for nitrogen fixation ability was qualitatively done on solid N-free medium and all the isolates tested showed growth on the medium. This may be explained by the fact that two of the isolation media used were nitrogen free (LGI and NFb). Growth of the bacterial isolates on N-free medium was suggestive of the isolates ability to fix atmospheric nitrogen. In addition, the isolates showed varied levels of nitrogenase activity. It is now clear that many diazotrophic microbes inhabit the tissues of banana plants and there is the potential of exploiting them once conditions have been optimized for their use.

Screening of phosphate solubilizers was done on NBRIP medium agar plates, which is based on formation of visible halo zones on the agar plates as a result of organism's production of organic acids into the surrounding medium (Nautiyal, 1999). Twenty seven isolates were observed to solubilize phosphates, with *Rahnella aquatilis* (ME19V2c and ME18V2c) showing the highest potential having formed the largest visible dissolution halos on the NBRIP growth medium. *Rahnella aquatilis* (ME19V2c and ME18V2c) were therefore considered the most efficient phosphate solubilizers. The findings are consistent with those of Kim *et al*. (1998) who reported *R. aquatilis* having genes that are necessary for mineral phosphate solubilization. Vyas *et al*. (2010) also identified a phosphate-solubilizing bacterial strain from *Hippophae rhamnoides* rhizosphere as *Rhanella* spp. Phosphorus is second only to N among mineral nutrients that limit plant growth (Vessey, 2003). This is despite the fact that soils have large reserves of total P but amounts available to plants are usually a tiny proportion of this total as a result of high phosphorus

fixation. P fixation is worse in the tropics and subtropics because of high soil acidity (Norman *et al*., 1995). Releasing the insoluble and fixed forms of phosphorus is therefore an important aspect of increasing soil phosphorus availability. Phosphate-solubilizing micro-organisms (PSM)dissociate the phosphorus from soil complexes through several mechanisms, such as the production of organic acids, which dissolve or chelate inorganic phosphate, or the production of phosphatases and phytases, which dissociate phosphorus from organic sources (Rodriguez and Fraga, 1999). Phosphate-solubilizing micro-organisms therefore enhance availability of soil phosphate, promoting its uptake by plants (Gyaneshwar *et al*., 2002b). It is therefore possible that under optimal conditions *Rahnella aquatilis* (ME19V2c and ME18V2c) can significantly improve banana growth in soils with unavailable P.

Distinct orange halos were observed with all the 12 *Pseudomonas* isolates with *Flavimonas oryzihabitans* (K50V2s) having the largest orange halos. Orange halos around bacterial colonies on blue Chrome Azurol S (CAS) agar are indicative of siderophore excretion (Schwyn and Neiland, 1987).*Flavimonas oryzihabitans* (K50V2s) is therefore being considered high siderophore producer. These findings are similar tothose of Gangwar and Kaur (2009) who reported *Pseudomonas* spp. isolated from ryegrass as high siderophore producer. Siderophores are small molecule iron scavengers, which have been defined by Sharma and Johri, (2003) as low-molecular-weight compounds with high iron (III) chelating affinity. They are responsible for the dissolution, chelation and transport of iron (III) into microbial cells. Chen *et al*., (1998) have also shown that Fe, chelated by microbial siderophores, can be utilized by plants improving their iron nutrition. Siderophores

can also promote plant growth indirectly by reducing or preventing harm caused by plant-pathogenic microorganisms (Leong, 1986).

In the present study, tissue-cultured banana plantlets (*Musa* spp. cv. Grande Naine) were inoculated with *Bacillus subtilis subsp. inaquosorum*(M9V1r), *Enterobacter ludwigii*(J1V1r), *Ewingella americana*(K32V2c), *Rahnella aquatilis*(ME19V2c)and *Flavimonas oryzihabitans*(K50V2s)and effects of their inoculation on plant growth, physiology and mineral nutrition investigated. Inoculation experiments have been performed to reveal the effects of endophytes (Rosenblueth and Martínez-Romero, 2006). Elimination of resident or indigenous endophytes to achieve bacteria-free plants or seeds has however remained a challenge. Tissue culture has nevertheless been used to eliminate or reduce indigenous endophytes (Holland and Polacco 1994; Leifert *et al*., 1994), making introduction of beneficial endophytic bacteria through sterile produced tissue culture material probably the most effective delivery mechanism (Van Vuurde and Elvira-Recuenco, 2000). *Ewingella americana*(K32V2c)showed a positive significant effect (p=0.05) on the relative change in plant height and number of green leaves while *Enterobacter ludwigii*(J1V1r)significantly (p=0.05) increased shoot dry weight.Inoculation of plants with *Bacillus subtilis subsp. inaquosorum*(M9V1r), *Enterobacter ludwigii*(J1V1r)and *Ewingella americana*(K32V2c)significantly (P=0.05) increased chlorophyll content in plants while*Rahnella aquatilis*(ME19V2c)increased P concentration in plant shoots though not significantly.The high efficiency in phosphate solubilization and siderophore production shown by *Rahnella aquatilis*(ME19V2c)and *Flavimonas oryzihabitans*(K50V2s)as free cells respectively was not evident *in-planta*. Many

bacteria strains seem to exert their beneficial effects at the *in-vitro* level (laboratory culture), while a few of them are successful in greenhouse or field conditions. Understanding the reasons for these inconsistencies may lead to more prospects in biofertilizer research.

Microscopic evidence of "tagged" bacteria inside plant tissues and capacity to re-infect disinfected seedlings provide evidence of endophytic habitat. Conjugation of *Flavimonas oryzihabitans*(K50V2s)with mTn5gusA-pgfp21 from *E. coli S*17-1 resulted to successful transconjugants, which wereinoculated into TC banana plantlets for localization assay. The "tagged" bacteria inside plant tissues were evident under the Confocal Laser Scanning Microscope (Olympus Fluoview FV1000). Plant colonization by endophytic bacteria is dependent among others on the endophytes' invasion capacity, with some endophytes being better colonizers than others (Dong *et al*., 2003), and the plant host ability to be colonized endophytically (Rosenblueth and Martínez-Romero, 2006).*Flavimonas oryzihabitans*(K50V2s), showed capacity to colonize both the rhizosphere and the endosphere of tissue cultured banana plantlets with cell concentration of 1.53 x 10^6 cfu/ml and 1.4 x 10^5 cfu/ml, respectively, two days after inoculation. The endophytic habitat and colonization ability of *Flavimonas oryzihabitans*(K50V2s)was therefore confirmed and tissue cultured banana plantlets showed ability to be colonized endophytically.

8.2 Conclusions

The following conclusions are made:

1. Bananas growing in Kenya have endophytic bacteria, which include *Serratia*, *Pseudomonas*, *Rahnella*, *Enterobacter*, *Yokenella*, *Raoultella*, *Klebsiella*, *Yersinia*, *Ewingella* and *Bacillus* species.
2. Bananas have a larger richness of endophytic bacteria species than has previously been reported. *Rahnella*, *Yokenella*, *Raoultella*, *Yersinia* and *Ewingella* species isolated from bananas in the current study had previously not been reported in association with bananas.
3. Isolate K22V1c is being proposed as a novel species having showed a similarity value of less than 97% (95.27%) with its closest relative *Klebsiella granulomatis*.
4. Endophytic bacteria associated with bananas in Kenya havecapacity tofix free nitrogen having grown on nitrogen-source free medium and showed varied nitrogenase activity.
5. Endophytic bacteria associated with bananas in Kenya have capacity to solubilize phosphates with*Rahnella aquatilis* (ME19V2c)having the highest capacity.
6. *Pseudomonas*speciesassociated with bananas in Kenya have capacity to produce siderophoreswith*Flavimonas oryzihabitans*(K50V2s)havingthe highestcapacity.
7. *Ewingella americana*(K32V2c)showed a positive significant effect (p=0.05) on the relative change in plant height, diameter and number of green leaves while *Enterobacter ludwigii*(J1V1r)significantly (p=0.05) increased shoot dry weight.

8. Inoculation of plants with *Bacillus subtilis subsp. Inaquosorum* (M9V1r), *Enterobacter ludwigii*(J1V1r)and *Ewingella americana*(K32V2c)significantly (P=0.05) increased chlorophyll content in plants while*Rahnella aquatilis* (ME19V2c) increased P concentration in plant shoots though not significantly.
9. The endophytic habitat and colonization ability of*Flavimonas oryzihabitans*(K50V2s)was confirmed.
10. Tissue cultured banana plantlets have ability to be colonized endophytically.

All the alternative hypotheses were accepted.

8.3 Recommendations

To facilitate the use of endophytic bacteria in practical agronomic production, the following is recommended:

1. More research to develop reliable and practical delivery mechanisms of bacterial endophytes, which include efficient inoculation techniques that yield high colonization.
2. A repeat of the*nifH* gene analysis with inclusion of a positive control and optimized PCR conditions for a more precise determination of organisms' N fixation ability.
3. Analysis for more functional potential of the isolates.
4. Localization assays for the remaining isolates to confirm their endophytic habitat.
5. Use of endophytic bacteria in the management of plant nutrients in banana production in Kenya, where farmers are constrained by nutrients availability.

This will contribute to the reduction of total fertilization costs while improving plant growth and productivity.

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APPENDICES

Appendix 1: Sampling site data collection sheet

General

Observer Name: Date:

Farm Identification Number:

Farm location

Village Name: Sub-location:

Location: Division:

District:

Location, Altitude (GPS reading of farm):

Weather

Dry Wet

Household

Farmer Name:

Farmer Age: Farmer Gender:

No. of Children: Marital Status:

Persons working full-time on the farm: No. family members

No. hired

Training on orchard management: Formal:

Informal:

Crop Production

Farm Production: only subsistence / subsistence + market / only market

Types of bananas cultivated: cooking varieties -

Dessert varieties -

Do you consume all bunches, or do you also sell?

What proportion of the bananas do you sell? 0-25% / 25-50% / 50-75% / >75% / N.A

What other banana products do you sell? Leaves / stem

Which other crops do you grow?

Which ones do you sell?

Livestock

Number of local cows: Number of improved cows:

Others:

Do you have access to grazing land? Yes/no

How do you do the grazing? Zero-grazing, semi-zero grazing, free ranging

Livelihoods

Apart from farming, do you have other source of income?

Selling labor/salary/charcoal selling/outside family members support/trading/land rental

Are there months in the year when you experience food shortage?

Observer interpretation of wealth

Quality of house: mud / timber / stone grass thatched / iron sheets / tiles

Provisionary wealth classification: very poor / poor / medium / rich / very rich

Farm description

Farmer's questions

Size of farm (total): Size of farm (with banana):

When was banana orchard established?

What planting material did you use? (Sucker / tissue culture)

Do you irrigate? How often?

Orchard history (previous crops / fallow /)

How have the yields been since the establishment of the plantation (stable / down / up)?

What are the causes of the changes in yield? Soil fertility / pest& diseases / market / water / planting material / any other

Kindly rate the challenges in a scale of 5 (1-most severe, 5-least severe)

What are plants symptoms of poor soil fertility (carefully describe)?

Scientist observations

Position of the banana orchard within the slope: crest / upper slope/ middle slope/ lower slope / valley bottom

Slope: flat / gentle slope / moderate slope / steep slope / very steep slope

Soil texture: sand / sandy loam / loam / sandy clay / clay loam / clay (cracking soil)

Surface coarse fragments (gravel, rock fragments): none (<2%) / few (2-5%) / common (5-15%) / many (>15%)

Erosion: none / some (rill) / moderate (small gully) / severe (large gully)

Soil management

Tillage (describe tool, frequency, area covered):

Soil and water conservation measures: mulching / grass bunds / stone rows / terracing / ditches & bunds system / water traps / live ground cover

Weeding: none / light (plot is weedy for 3 months) / moderate (plot is weedy for 4-6 weeks) / intensive (always clean)

Mulching material: self-mulch (leaving crop residues) / grass mulch / other

Mineral fertilizer type: Amount:

Times per year: Way of application:

Manure /compost application: Amount:

Times per yr:

Cropping system: mono-culture (banana alone) / mixed cropping (banana + other crop(s)?)

Soil sampling

Soil sample: no……./ site ……. / farm…… / depth…….. (0-20 and 20-40 cm)

Other remarks

Appendix 2: Effect of inoculation with 24 representative isolates on shoot mineral nutrient content of tissue cultured banana plants at 5 g fertilizer regimein **e**xperiment 3.

Bacterial isolates	**P (ppm)**	**Mg (ppm)**	**Ca (ppm)**	**Fe (ppm)**	**Zn (ppm)**	**Mn (ppm)**
***Yersinia kristensenii*(1)**	2.88 cdef	0.98 a	0.93 bcd	0.74 abcde	1.11 a	1.21 ab
***Klebsiella granulomatis*(2)**	2.12 g	0.82 abcd	0.72 ef	0.63 cde	0.22 bcde	0.40 ef
***Pseudomonas psychrophila*(3)**	2.60 defg	0.83 abcd	0.88 cd	0.70 abcde	0.20 bcde	0.43 def
***Pseudomonas protegens*(4)**	2.89 cde	0.85 abcd	1.05 b	0.72 abcde	0.22 bcde	0.33 f
***Raoultella terrigena*(5)**	3.09 abcde	0.74 bcdef	0.87 cd	0.69 abcde	0.26 bc	0.71bcdef
***Rahnella aquatilis*(7)**	2.65defg	0.82 abcd	0.80 cd	0.78 abcde	0.23 bcde	0.70 bcdef
***Serratia fonticola*(8)**	2.57 defg	0.71 cdefg	0.61 fg	0.65 bcde	0.15 de	1.07 abcd
***Serratia ureilytica (*9)**	2.46 defg	0.53 fghi	0.26 j	0.82 abcde	0.21bcde	0.90 abcdef
***Pseudomonas protegens*(10)**	3.03 abcde	0.44 hij	1.52 a	0.82 abcde	0.16 de	1.07 abcd
***Pseudomonas moraviensis*(12)**	3.01 abcde	0.86 abcd	0.93 bcd	0.69 abcde	0.17 cde	0.90 abcdef
***Serratia plymuthica*(13)**	2.43 efg	0.89 abcd	0.93 bcd	0.64 bcde	0.16 de	0.89 abcdef

Bacterial isolates	P (ppm)	Mg (ppm)	Ca (ppm)	Fe (ppm)	Zn (ppm)	Mn (ppm)
***Pseudomonas graminis*(16)**	2.46 defg	0.52 ghi	0.37 ij	0.69 abcde	0.19 bcde	1.14 abc
***Enterobacter amnigenus*(17)**	2.82 defg	0.69 defg	0.43 i	0.61 cdef	0.15 de	1.06 abcde
***Pseudomonas palleroniana*(22)**	2.74 defg	0.53 fghi	0.66 efg	0.50 efg	0.20 bcde	1.21 ab
***Pseudomonas protegens*(23)**	2.82 defg	0.92 abc	1.05 b	0.67 bcde	0.16 de	1.27 ab
***Serratia proteamaculans*(25)**	3.53 abc	0.89 abcd	0.95 bcd	0.63 cde	0.23 bcde	1.12 abc
***Flavimonas oryzihabitans*(27)**	2.91 bcde	0.79 abcde	1.01 bc	0.54 def	0.27b	1.17 ab
***Pseudomonas koreensis*(28)**	2.64 defg	0.73 cdefg	0.94 bcd	0.65 bcde	0.24 bcde	1.00 abcde
***Bacillus subtilis subsp. inaquosorum* (30)**	3.14 abcd	0.96 ab	0.52 ghi	0.79 abcde	0.24 bcd	1.39 a
***Enterobacter ludwigii* (31)**	2.17 fg	0.83 abcd	0.98 bc	0.93 abc	0.20 bcde	1.29 ab
***Yokenella regensburgei*(33)**	3.71 a	0.59 efgh	0.27 j	0.85 abcd	0.14 ef	1.46 a
***Ewingella americana* (39)**	3.70 a	0.34 ij	0.51 ghi	1.01 a	0.18 bcde	1.27 ab
***Serratia glossinae*(41)**	3.60 ab	0.82 abcd	0.42 i	0.96 ab	0.19 bcde	1.30 ab
***Rahnella aquatilis* (42)**	2.98 bcde	0.78 abcde	0.44 hi	0.96 ab	0.18 bcde	1.41 a

Bacterial isolates	P (ppm)	Mg (ppm)	Ca (ppm)	Fe (ppm)	Zn (ppm)	Mn (ppm)
Control	2.45 defg	0.29 j	0.25 j	0.20 g	0.001 g	0.40 ef

n=3

Means with the same letter in a column are not significantly different.

Appendix 3: Soil analysis of sampling sites at Juja. Soil samples were collected in February 2009 around the mats of sampled banana plants. Analysis was done at the Department of Horticulture, JKUAT.

Sample ID / soil depth (cm)	pH (H_2O) (1:2.5)	Ec (H_2O) (1:2.5)	%N	Avail P (ppm)	Extrac. K (Cmol/kg)
J2V1 (0-20)	7.30	0.57	0.37	38.50	0.60
J2V1 (20-40)	6.67	0.14	0.29	14.20	0.14
J3V1 (0-20)	6.80	0.07	0.26	10.00	0.10
J3V1 20-40	6.60	0.05	0.38	Trace	0.05
J3V2 (0-20)	7.65	0.12	0.06	1.10	0.12
J3V2 (20-40)	6.79	0.08	0.23	2.40	0.08

Key: J1 – Juja farm 1; J2 – Juja farm 2; J3 – Juja farm 3;V1 – dessert banana cultivar; V2 – cooking banana cultivar.

Appendix 4: Soil analysis of sampling sites at Maragua. Soil samples were collected in February 2009 around the mats of sampled banana plants. Analysis was done at the Department of Horticulture, JKUAT.

Sample ID / soil depth (cm)	pH (H_2O) (1:2.5)	Ec (H_2O) (1:2.5)	%N	Avail P (ppm)	Extrac. K (Cmol/kg)
M1V1 (0-20)	5.46	0.24	0.33	Trace	1.87
M1V1 (20-40)	5.36	0.16	0.30	Trace	1.13
M1V2 (0-20)	5.60	0.12	0.28	Trace	2.50
M1V2 (20-40)	5.55	0.04	0.38	Trace	0.82
M2V1 (0-20)	5.48	0.11	0.15	Trace	1.10
M2V2 (20-40)	5.11	0.05	0.22	Trace	0.97
M2V2 (0-20)	5.25	0.22	0.21	Trace	1.81
M2V1 (20-40)	4.79	0.05	0.20	Trace	0.82
M3V1 (0-20)	6.63	0.14	0.21	0.65	0.95
M3V2 (20-40)	6.81	0.14	0.28	Trace	0.50
M3V2 (0-20)	6.91	0.11	0.28	6.03	1.23
M3V1 (20-40)	6.50	0.05	0.18	1.09	0.50
M4V1 (0-20)	5.00	0.11	0.22	Trace	1.40
M4V1 (20-40)	5.21	0.04	0.25	0.70	0.82
M4V2 (0-20)	7.50	0.50	0.34	10.20	7.20
M4V2 (20-40)	7.50	0.30	0.20	Trace	5.10

Sample ID / soil depth (cm)	pH (H_2O) (1:2.5)	Ec (H_2O) (1:2.5)	%N	Avail P (ppm)	Extrac. K (Cmol/kg)
M5VI (0-20)	4.98	0.12	0.20	Trace	3.70
M5V1 (20-40)	5.70	0.04	0.18	Trace	0.95
M5V2 (0-20)	6.20	0.04	0.30	Trace	1.74
M5V2 (20-40)	6.09	0.04	0.33	Trace	2.22

Key: M1 – Maragua farm 1; M2 – Maraguafarm 2; M3 – Maragua farm 3; M4 – Maragua farm 4; M5 – Maragua farm 5;V1 – dessert banana cultivar; V2 – cooking banana cultivar.

Appendix 5: Soil analysis of sampling sites at Embu (Central & Manyata). Soil samples were collected in March 2009 around the mats of sampled banana plants. Analysis was done at the Department of Horticulture, JKUAT.

Sample ID / soil depth (cm)	pH (H_2O) (1:2.5)	Ec (H_2O) (1:2.5)	%N	Avail P (ppm)	Extractable K (Cmol/kg)
E1V1 (0-20)	5.64	0.10	0.22	1.54	3.30
E1V1 (20-40)	5.57	0.10	0.06	Trace	2.00
E1V2 (0-20)	6.60	0.20	Trace	Trace	4.90
E1V2 (20-40)	5.87	0.17	0.04	Trace	4.70
E2V1 (0-20)	4.68	0.10	0.21	Trace	1.00
E2V1 (20-40)	4.57	0.10	0.50	Trace	0.60
E2V2 (0-20)	4.67	0.20	0.03	Trace	1.70
E2V2 (20-40)	4.59	0.13	0.28	Trace	0.90

Sample ID / soil depth (cm)	pH (H_2O) (1:2.5)	Ec (H_2O) (1:2.5)	%N	Avail P (ppm)	Extractable K (Cmol/kg)
E3V1 (0-20)	5.28	0.19	0.28	1.68	2.90
E3V1 (20-40)	4.57	0.10	0.32	0.90	1.00
E3V2 (0-20)	5.59	0.31	0.24	1.26	4.20
E3V2 (20-40)	5.53	0.17	0.30	0.36	3.40
E4V1 (0-20)	5.51	0.10	0.28	Trace	0.20
E4V1 (20-40)	5.48	0.10	0.30	Trace	0.90
E4V2 (0-20)	6.16	0.10	0.50	1.34	9.10
E4V2 (20-40)	6.47	0.13	0.50	Trace	4.30
E5V1 (0-20)	5.63	0.10	0.37	0.50	1.30
E5V1 (20-40)	5.55	0.10	0.44	Trace	1.00
E5V2 (0-20)	4.68	0.10	0.23	Trace	1.60
E5V2 (20-40)	4.46	0.10	0.21	0.03	0.70

Key: E1 – Embu farm 1 (Central); E2 – Embu farm 2 (Manyata); E3 – Embu farm 3 (Manyata); E4 – Embu farm 4 (Manyata); E5 – Embu farm 5 (Manyata);V1 – dessert banana cultivar; V2 – cooking banana cultivar.

Appendix 6: Soil analysis of sampling sites at Meru (Imenti North - Miriga Mieru). Soil samples were collected in March 2009around the mats of sampled banana plants. Analysis was done at the Department of Horticulture, JKUAT.

Sample ID / soil depth (cm)	pH (H_2O) (1:2.5)	Ec (H_2O) (1:2.5)	%N	Avail P (ppm)	Extrac. K (Cmol/kg)
ME1V1 (0-20)	6.67	0.05	0.18	38.1	1.25
ME1V1 (20-40)	6.66	0.03	0.20	12.50	1.24
ME1V2 (0-20)	6.52	0.10	0.10	26.60	1.50
ME1V2 (20-40)	6.90	0.03	0.23	62.20	1.00
ME2V1 (0-20)	6.55	0.09	0.14	20.30	1.45
ME2V1 (20-40)	6.03	0.09	0.17	8.00	0.91
ME2V2 (0-20)	6.80	0.15	0.30	13.70	1.31
ME2V2 (20-40)	6.57	0.10	0.20	7.50	1.05
ME3V1 (0-20)	6.19	0.12	0.28	21.20	1.74
ME3V1 (20-40)	6.29	0.18	0.40	26.20	1.33
ME3V2 (0-20)	6.36	0.16	0.29	73.20	1.50
ME3V2 (20-40)	5.90	0.10	0.22	6.50	0.93
ME4V1 (0-20)	6.56	0.10	0.37	17.00	1.70
ME4V1 (20-40)	5.96	0.10	0.32	11.00	0.84
ME4V2 (0-20)	6.50	0.16	0.25	36.80	1.41
ME4V2 (20-40)	6.22	0.12	0.23	56.90	1.00

Sample ID / soil depth (cm)	pH (H_2O) (1:2.5)	Ec (H_2O) (1:2.5)	%N	Avail P (ppm)	Extrac. K (Cmol/kg)
ME5V1 (0-20)	6.73	0.04	0.19	76.90	1.32
ME5V1 (20-40)	7.37	0.10	0.17	29.00	1.16
ME5V2 (0-20)	6.36	0.10	0.25	13.10	1.20
ME5V2 (20-40)	6.01	0.09	0.37	7.30	1.00

Key: ME1 – Meru farm 1; ME2 – Meru farm 2; ME3 – Meru farm 3; ME4 – Meru farm 4; ME5 – Meru farm 5;V1 – dessert banana cultivar; V2 – cooking banana cultivar.

Appendix 7: Soil analysis of sampling sites at Kiogoro, Kisii Central. Soil samples were collectedin January 2010around the mats of sampled banana plants. Analysis was done at the Department of Horticulture, JKUAT.

Sample ID / soil depth(cm)	pH (H_2O) (1:2.5)	Ec (H_2O) (1:2.5)	%Mg	% Ca	%K	Mn (mg/kg)	Zn (mg/kg)	Fe (mg/kg)	%N	%P	Avail. P	%TOC
K1V1 (0-20)	5.7	0.08	3.80	9.69	3.03	54.0	3.70	18.45	0.22	0.06	0.03	0.95
K1V1 (20-40)	5.8	0.03	3.40	9.69	2.15	34.58	4.50	16.36	0.22	0.09	0.04	1.52
K1V2 (0-20)	5.6	0.06	3.57	8.66	2.87	113.27	2.67	26.28	0.30	0.15	0.09	0.38
K1V2 (20-40)	6.2	0.05	3.89	10.82	2.0	105.92	3.95	26.85	0.53	0.19	0.09	1.33
K2V1 (0-20)	5.6	0.06	3.94	10.15	1.54	97.05	1.14	23.47	0.18	0.09	0.06	0.38
K2V1 (20-40)	5.8.	0.04	3.90	11.19	0.82	67.45	Trace	22.55	0.12	0.12	0.06	0.19
K2V2 (0-20)	5.5	0.06	4.61	11.76	0.46	157.13	3.96	31.02	0.28	0.16	0.06	2.29
K2V2 (20-40)	5.7	0.06	4.31	14.17	0.31	170.82	3.57	36.36	0.29	0.14	0.08	0.57
K3V1 (0-20)	5.3	0.06	2.97	11.41	0.41	103.47	1.70	27.11	0.4	0.13	0.06	2.29
K3V1 (20-40)	5.6	0.12	2.79	11.47	0.51	81.03	1.91	21.8	0.4	0.21	0.06	1.14

Sample ID / soil depth (cm)	pH (H_2O) (1:2.5)	Ec (H_2O) (1:2.5)	% Mg	% Ca	% K	Mn (mg/kg)	Zn (mg/kg)	Fe (mg/kg)	% N	% P	Avail. P	% TOC
K3V2 (0-20)	5.6	0.03	3.24	11.29	0.46	74.14	1.57	21.10	0.27	0.17	0.08	1.90
K3V2 (20-40)	6.1	0.05	3.03	13.87	3.08	90.87	1.53	19.91	0.18	0.10	0.05	0.95
K4V1 (0-20)	6.9	0.13	3.74	9.41	3.08	133.58	5.14	28.66	0.29	0.14	0.01	0.38
K4V1 (20-40)	5.9	0.09	2.55	6.90	2.46	67.13	2.19	25.77	0.28	0.12	0.02	2.09
K4V2 (0-20)	6.4	0.12	3.37	8.83	3.03	4.30	4.96	26.16	0.35	0.20	0.01	4.00
K4V2 (20-40)	5.2	0.12	3.07	9.73	1.79	114.30	3.85	22.32	0.33	0.14	0.04	2.6
K5V1 (0-20)	5.1	0.11	2.44	6.43	0.62	173.55	7.47	38.94	0.15	0.09	0.01	0.95
K5V1 (20-40)	5.5	0.05	2.45	5.76	0.41	149.92	6.02	29.63	0.45	0.10	0.04	0.57
K5V2 (0-20)	6.1	0.24	3.92	5.34	1.85	212.55	8.82	27.34	0.28	0.11	0.04	3.05
K5V2 (20-40)	6.4	0.11	4.14	6.30	2.21	192.91	3.47	31.10	0.28	0.13	0.04	1.52

Key: K1 – Kisii farm 1; K2 – Kisii farm 2; K3 – Kisii farm 3; K4 – Kisii farm 4; K5 – Kisii farm 5;V1 – dessert banana cultivar; V2 – cooking banana cultivar.

Ideal soil conditions

pH:5.5-6.8; Nitrogen levels: > 0.25%; Phosphorus levels: >0.2%; Carbon: ≈2.0%; Calcium: ≈ 8-10%; Magnesium: ≈ 1.5 – 4%; Potassium: ≈ 2%; Fe: > 5mg/Kg; Cu: > 1mg/Kg; Mn: > 35mg/Kg; Zn: >1mg/Kg.