ISOLATION AND CHARACTERIZATION OF BACTERIAL ROOT ENDOPHYTES FROM KENYAN BASMATI RICE WITH POTENTIAL TO ENHANCE PLANT GROWTH

FREDRICK NDONYE MBAI

MASTER OF SCIENCE
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Isolation and characterization of bacterial root endophytes from Kenyan basmati rice with potential to enhance plant growth

Fredrick Ndonye Mbai

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DECLARATION

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

Signature ------------------------------- Date------------------------------

      Fredrick Ndonye Mbai

This thesis has been submitted for examination with our approval as University Supervisors:

1. Signature ------------------------------- Date--------------------------------

      Prof. Esther Magiri
      JKUAT, Kenya

2.   Signature ------------------------------- Date----------------------------

      Dr. Vivienne Matiru
      JKUAT, Kenya

3.   Signature ------------------------------- Date--------------------------------

      Dr. Joseph Ng'ang'a
      JKUAT, Kenya

4. Signature ------------------------------- Date--------------------------------

      Dr. Venny C.S. Nyambati
      JKUAT, Kenya

DEDICATION

This work is dedicated to my family especially my parents Mr. and Mrs. Festus Mbai Ndonye as a symbol of my gratitude, respect, and honor for their inspiration and instilling in me values of hard work, self reliance, patience and persistence in my pursuit for excellence. I would never ask for more; because you are the best. Thank you for your support.

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sincerely grateful to God for giving me such a loving family and caring friends in you.

Love and gratitude is what I feel when I look back to the time that was. May good Lord

bless you all.

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LIST OF ABBREVIATIONS

ARA	Acetylene Reduction Assay
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BLAST	Basic local Alignment Search Tool
CFU	Colony forming units
DNA	Deoxyribonucleic acid
dNTPs	Deoxy-nucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
g	Gravity
HPLC	High-performance Liquid Chromatography
IAA	Indoleacetic acid
IBR	Institute of Biotechnology Research
ILRI	International Livestock Research Institute
MIAD	Mwea Irrigation Agricultural Development Centre
MR-VP	Methyl red-Voges-Proskauer test
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PGPR	Plant growth promoting rhizobacteria
RNA	Ribosomal nucleic acid

RsaI   Restriction endonuclease (*Rhodobacter sphaeroides*)

SIM   Sulfur-indole motility

TAE   Tris-Acetic acid Ethylenediaminetetraacetic acid

TE   Tris- Ethylenediaminetetraacetic acid

UV   Ultraviolet

YEM   Yeast manitol agar

16S rDNA Sixteen S ribosomal deoxyribonucleic acid

ABSTRACT

Rice is an important food crop. Its grain serves as a staple food for a large part of the world's human population especially in urban areas. An endophyte is an endosymbiont, often a bacterium or fungus, that lives within a plant for at least part of its life without causing apparent disease. In this study, a survey of bacterial root endophytes was carried out. 73 bacterial pure isolates were obtained from the root samples collected from farmers' and research fields in Mwea and Ahero. They were morphologically characterized and screened for biological activities. The biochemical tests were used to characterize all the isolates. 10 isolates produced indoleacetic acid (IAA) implying potential to enhance plant growth. 67 isolates were positive for phosphate solubilisation. These 73 isolates were further grouped according to morphological and biochemical similarity and 37 were selected for molecular characterization. All the 37 selected isolates were confirmed to have the potential to fix nitrogen based on acetylene reduction assay (ARA). The phylogenetic analysis of 28 potential isolates clustered them into four different genera namely *Pseudomonas*, *Bacillus, Enterobacter, and Micrococcus.* Results of the phylogenetic analysis of the potential isolates were supported by the outcome on morphological and biochemical characteristics. Isolates M31 and M32 obtained from Mwea Basmati 370 rice were shown to promote plant growth. Endophytes are useful in agriculture for enhancement of growth and production which would consequently enhance affordable production of Kenyan basmati rice. This will not only eradicate poverty and increase food security, but also help conserve microbial diversity.

CHAPTER ONE

1.0. INTRODUCTION

1.1. Background information

Endophytic microorganisms are found in virtually every plant on earth. They are ubiquitous and have been found in all the species of plants studied to date (Berg and Hallman, 2006). It is noteworthy that, of the nearly 300,000 plant species that exist on earth, each individual plant is host to one or more endophytes (Strobel *et al.*, 2004). These organisms reside in the living tissues of the host plant in a variety of relationships, ranging from symbiotic to slightly pathogenic. An endophyte is thus an endosymbiont, often a bacterium or fungus, which lives within a plant for at least part of its life without causing apparent disease (Holliday, 1989).

Endophytes may produce a plethora of substances of potential use in fields like modern medicine, agriculture, and industry (Strobel *et al.*, 2004). For instance, novel antibiotics, anti-mycotics, immune-suppressants, and anticancer compounds have been found after the isolation, culture, purification, and characterization of some choice endophytes in the recent past (Strobel *et al.*, 2004).

Endophytes may be transmitted either vertically (directly from parent to offspring) or horizontally (from individual to unrelated individual) (James *et al.*, 2002). They enter the plant tissue primarily through the root zone. However, aerial portions of plants, such

as flowers, stems and cotyledons may also be used for entry. Endophytes either become localized at the point of entry or are able to spread throughout the plant and such isolates can live within cells, in the intercellular spaces, or in the vascular system (James *et al.*, 2002).

Endophytes of rice include diverse types of nitrogen-fixing and non-nitrogen-fixing bacteria, which are found mainly in the roots, culms and seeds of various wild, traditional and cultivated varieties of rice (Barraquio *et al.*, 1997). Endophytic bacteria are considered to originate from the external environment. Various kinds of endophytic bacteria, such as *Methylobacterium*, *Azospirillum*, *Herbaspirillum*, *Burkholderia* and *Rhizobium*, *Bacillus firmus*, *Bacillus fusiformis*, *Bacillus pumilus*, *Caulobacter crescentus*, *Kocuria palustris*, *Micrococcus luteus*, *Methylobacterium fujisawaense*, *Methylobacterium radiotolerans*, and *Pantoea ananatis* have been found in rice tissues (Cocking, 2003).

1.2. Rice production in Kenya

The average area under rice in Kenya has remained low over the years. Rice yield has also been declining from 42 bags/ha in 2003 to 29 bags/ha in 2007 (Emongor *et al.*, 2009). Rice in Kenya is produced in the irrigation schemes in Nyanza (West Kano and Ahero, covering an area of 3520 ha), Western (Bunyala scheme covering an area of 516 ha) and Mwea irrigation scheme covering an area of 9000 ha. In total the irrigation areas in Kenya cover approximately 13000 ha (Emongor *et al.*, 2009).

National rice consumption is estimated at about 300,000 tones against an annual domestic production of between 45,000 to 80,000 tonnes (Emongor *et al*., 2009). This huge gap between consumption and production is met through importation of rice from countries including Pakistan, Thailand, Uganda and Tanzania. In 2008, rice imports into Kenya were valued at Ksh. 7 billion (Emongor *et al*., 2009). Promotion of rice production in Kenya will therefore improve food security, household incomes and reduce the rice import bill. Annual rice consumption is increasing at the rate of 12 % compared to wheat (4%) and maize (1%) (Emongor *et al*., 2009). These changes are attributed to change in eating habits of the population. It is therefore expected that demand for rice in the country will continue to increase in the future. Furthermore, promotion of rice production and consumption in Kenya will help remove over-reliance on maize as a staple food hence improve rural and urban households' incomes and food security.

1.3. Morphological characterization of bacteria

In the characterization process, determination of the morphological state of the microorganism is among the first activities carried out. The Gram staining method is named after the Danish bacteriologist Hans Christian Gram (Cappuccino and Sherman, 2002). It is a differential staining method that places bacterial species into gram-positive and gram-negative based on properties of their cell walls (Cappuccino and Sherman, 2002).

1.4. Biochemical characterization of bacteria

Biochemical characterization of microorganisms is also essential in the characterization process, and facilitates understanding of the metabolic properties of the different isolates.

1.5. Molecular characterization of bacteria

In the context of this undertaking, molecular characterization of bacterial endophytes involved the use of 16S rDNA gene for identification and phylogenetic analysis.

Ribosomal DNA sequence analysis has been extensively used to study phylogenetic relationships between microorganisms as well as for taxon identification (Woese *et al*., 1990). Sequence information from the conserved regions of the 16S rDNA gene is useful for studying phylogenetic relationships as well as for the design of specific or generic oligonucleotide probes and primers used for identification by hybridizations and discriminant PCR-amplifications, respectively (Givannoni, 1991). Variable regions of 16S rDNA provide sequence data to develop specific probes and primers for detection of bacteria by hybridization or with polymerase chain reaction (Stahl and Amann, 1991). The availability and use of PCR based amplification methods and sequencing of the PCR products on automated sequencers has dramatically expanded DNA databases. Sequences of over 16 000 rDNA molecules from different organisms have been catalogued (Ludwig and Schleifer, 1999). This wealth of sequence information is now readily available in public databases for ever finer identification of new bacterial isolates by sequence comparisons.

This study focused on the search for rice bacterial root endophytes. This involved isolation and characterization of bacterial root endophytes and assessment of any phenotypic effects on rice seedlings by the bacterial root endophytes.

1.6. Statement of the problem

Rice farmers in Kenya mostly depend on application of chemical fertilizers for nutrient supply to their plants. The increased cost of fertilizers is a constraint that limits growth and production since not all farmers are able to afford the fertilizers. In addition, chemical fertilizers are not eco-friendly as they also reduce microbial diversity (Mahdi *et al*., 2010). Hence there is need to exploit an alternative source of nutrient supply, such as biofertilizers. One possibility is through the use of bacterial or fungal endophytes which have been shown to have such potential. There is however no information so far on Kenyan basmati rice bacterial root endophytes and their role in promoting growth and production.

1.7. Justification of study

Rice is one of the world's most important grain food crops (Hossain and Fischer, 1995). As a cereal grain, it is the most important staple food for a large part of the world's human population, especially in East and South Asia, the Middle East, Latin America, and the West Indies. It is the grain with the second-highest worldwide production, after maize (corn) (Ladha *et al.,* 1997).

It is the third most important staple food in Kenya after maize and wheat (Emongor *et al*., 2009). Rice has a high consumption rate especially for urban population because it is easy and cost effective to prepare. The population is growing at a rapid rate; therefore,

rice yields will need to be enhanced to match the increased consumption. However its production is limited by nutrient supply in the case where chemical fertilizers have to be used, yet not all farmers can afford them since they are expensive. An alternative to the increased use of chemical fertilizers is to explore and improve the ability of rice to obtain nitrogen from biological nitrogen fixation (Ladha and Reddy, 1995: Wu *et al.,* 1995) using endophytes.

Bio-fertilizers are cost-effective relative to chemical fertilizers. They are also environmentally friendly in that they do not only prevents damaging the natural source but also help to some extent cleanse the plant from precipitated chemical fertilizers (Vessey, 2003). Application of endophytes also helps maintain soil microbial diversity (Vessey, 2003). Therefore, a better understanding of endophytic bacteria may help to elucidate their function and potential role more effectively in developing sustainable and affordable systems of crop production such as the development and use of biofertilizers. This will contribute to increased yield and trade in Kenyan basmati rice to eradicate poverty and increase food security. This will also help conserve microbial diversity.

1.8.   Null hypothesis

There are no bacterial endophytes in Kenyan basmati rice roots, with potential to promote plant growth.

1.9. Objectives

1.9.1. General objective

To isolate and characterize bacterial root endophytes from Kenyan basmati rice and assess the effects of the isolates on rice plant growth.

1.9.2. Specific objectives

1. To establish the presence of bacterial endophytes from Kenyan basmati rice root samples

2. To carry out morphological and biochemical characterization tests on the pure isolates

3. To perform molecular characterization of the potential plant growth promoting pure isolates using 16S rDNA based analysis

4. To assess the plant growth promotion activity of the potential isolates.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Bacterial endophytes

Historically, endophytic bacteria were thought to be weakly virulent plant pathogens but have recently been discovered to have several beneficial effects on host plants, such as plant growth promotion and increased resistance against plant pathogens and parasites. Several strains are capable of inducing, both biotic (Chen *et al.,* 1995; Sharma and Nowak, 1998) and abiotic stress resistance (Bensalim *et al.,* 1998; Creus *et al.,* 1998; Nowak, 1998) in inoculated plants. Endophyte communities have also been shown to ameliorate disease development (Benhamou *et al.,* 1996; Sturz and Matheson, 1996) and in some instances, plant– endophyte relationships have been found to be tissue type and tissue site specific. For example, Sturz *et al.*, (1999) found that anti-phytopathogenic activity of endophytes recovered from potato tuber peels were highest in the outermost layers of the peel and decreased progressively towards the centre of the tuber.

In general, endophytic bacteria originate from the epiphytic bacterial communities of the rhizosphere and phylloplane, as well as from endophyte-infested seeds or planting materials (Hallmann *et al*., 1997). Besides gaining entrance to plants through natural openings or wounds, endophytic bacteria appear to actively penetrate plant tissues using hydrolytic enzymes like cellulase and pectinase. Since these enzymes are also produced by pathogens, more knowledge on their regulation and expression is needed

to distinguish endophytic bacteria from plant pathogens (Hallmann *et al*., 1997). In general, endophytic bacteria occur at lower population densities than pathogens in plant tissues (Hallmann *et al*., 1997).

Evolutionarily, endophytes appear to be intermediate between saprophytic bacteria and plant pathogens, but it can only be speculated as to whether they are saprophytes evolving toward pathogens, or are more highly evolved than plant pathogens and conserve protective shelter and nutrient supplies by not killing their host (Hallmann *et al*., 1997). Overall, the endophytic micro floral community is of dynamic structure and is influenced by biotic and abiotic factors, with the plant itself constituting one of the major influencing factors. Since endophytic bacteria rely on the nutritional supply offered by the plant, any parameter affecting the nutritional status of the plant could consequently affect the endophytic community.

Increased microbial biomass and activity in soil has been linked to plant disease suppression as competition amongst soil microorganisms increases (Chen *et al.,* 1988; Sturz *et al.,* 1997). There is evidence that soil organic matter can also influence endophytic communities. For example, Hallmann *et al.*, (1999) found an abundance of the bacterium *Burkholderia cepacia* in cotton roots grown in chitin-amended soil while very little colonized the roots grown in non amended soil.

Establishing beneficial bacterial populations in the rhizosphere seems to be key for obtaining a healthy micro floral balance within plants; soil appearing to be an important and moderating source of bacterial endophytes (Mundt and Hinkle, 1976: Patriquin and Dobereiner, 1978: Lamb *et al.,* 1996: Shishido *et al.,* 1999). Thus, in agricultural soils,

certain cultural practices have been shown to influence the build-up of beneficial endophytic populations. For example, when 1% chitin was added as an organic soil amendment, both exo-root and endo-root bacterial communities were found to be modified in cotton roots (Hallmann *et al.,* 1999). The choice of rotation crops has also been shown to influence endophytic populations.

 According to Sturz *et al.,* (1998), crops in complementary rotations can share the same or similar endophyte bacterial populations and the possibility exists of utilizing beneficial relationships between plants and bacterial endophytes over successive crops to develop more sustainable crop production systems. For instance, Sturz and Christie, (1998) demonstrated that endophytic bacteria could contribute to cultivar specific interactions between red clover *(Trifolium pratense* L.) and potatoes *(Solanum tuberosum* L.) in crop rotations; in which case, the most abundant genus was *Rhizobium,* but species of *Curtobacterium, Pseudomonas,* and *Xanthomonas* were common to all cultivars. Similarly, manipulating plant–microbial ecosystems by inoculating seeds with beneficial bacterial endophytes, or encouraging the early development of beneficial endophyte communities has been suggested as a method of improving crop productivity (Sturz *et al.,* 2000), as well as helping to acclimatize plants to environmental stresses (Lazarovits and Nowak, 1997).

2.2.   Role of bacterial endophytes in plants

Endophytic bacteria ubiquitously inhabit most plant species, and have been isolated from a variety of plants (Lodewyck *et al.,* 2002). It has been reported that endophytic

bacteria may promote plant growth and suppress plant diseases probably by means similar to plant growth-promoting rhizobacteria (Feng *et al*., 2006).

Endophytes may benefit host plants by preventing pathogenic organisms from colonizing them. Extensive colonization of the plant tissue by endophytes creates a "barrier effect", where the local endophytes outcompete and prevent pathogenic organisms from taking hold (Berg and Hallmann, 2006). Endophytes may also produce chemicals which inhibit the growth of competitors, including pathogenic organisms.

The potential of endophytic bacteria to fix nitrogen and promote plant growth has renewed the interest in such associations. They also produce phytohormones which are important in plant growth promotion.

It is well known that the majority of phosphates in the sediments are present as insoluble organic and inorganic forms. Microorganisms play an important role in transformation of phosphorous. The solubilization of phosphorus compounds may be brought about by acids and enzymes of microbial origin. Among the different microbes, bacteria and yeasts are the potential candidates for dissolving the insoluble organic and inorganic phosphorus compounds (Mullen, 2005).

2.3.   Rice bacterial endophytes

Rice (*Oryza sativa*) is arguably the most important cereal crop in the world, feeding more than 50% of the world's population (Hossain and Fischer, 1995: Ladha *et al.,* 1997). However, the population is growing at a rapid rate; therefore, rice yields will

need to be enhanced to match the increased consumption. Achieving these higher yields will require at least double the amount of nitrogen fertilizers currently being used because, after water, nitrogen is the most limiting factor for rice growth (International Rice Research Institute, 1993). An alternative to the increased use of chemical fertilizers is to explore and improve the ability of rice to obtain nitrogen from biological nitrogen fixation (Ladha and Reddy, 1995: Wu *et al.,* 1995).

It has long been known that rice can form natural associations with various nitrogen fixing bacteria, both phototrophs and heterotrophs (Barraquio *et al.,* 1982: Barraquio *et al.,* 1983: You and Zhou, 1989: Roger and Ladha, 1992: Malik *et al.,* 1997). All or some of these may be responsible for supplying the plants with fixed nitrogen (James *et al.,* 2000: Ladha and Reddy, 1995).

Moreover, substantial molecular diversity of nitrogen fixing bacteria has been detected in field-grown rice based on retrieval of *nifH* or *nifD* gene fragments from root DNA (da Rocha *et al.,* 1986: Ueda *et al.,* 1995 a: Ueda *et al.,* 1995b). However, the contribution of the bacteria externally associated with rice is insufficient to sustain a high yield (Ladha *et al.,* 1998). It has been suggested that bacteria colonizing the plant interior might interact more closely with the host, with less competition for carbon sources and a more protected environment for nitrogen fixation (Quispel, 1991: Reinhold-Hurek and Hurek, 1998), such as that occurring in the relatively efficient nitrogen fixing symbioses between rhizobia and legumes (Mylona *et al.,* 1995).

Use of nitrogen fertilizer is of great importance in rice production, as nitrogen is the major factor limiting growth under most conditions (Dong *et al*., 1997). Since agriculture is expected to move

toward environmentally sustainable methods, much attention has been paid to natural methods of biological nitrogen fixation. Several diazotrophic bacteria, including *Klebsiella oxytoca* (Fujie *et al.*, 1987), *Enterobacter cloacae* (Fujie *et al*., 1987), *Alcaligenes* (Tou and Zhou, 1989), and *Azospirillum* (Baldani and Dobereiner, 1980), have been isolated from the rhizosphere of wetland rice. *Azoarcus* sp. from Kallar grass, abundantly colonize and express *nif* genes and

nitrogenase protein inside the original host as well as in rice roots (Reinhold-Hurek and Hurek, 1998). *Herbaspirillum seropedicae* strain Z67 colonizes mainly subepidermal regions of rice roots (Barraquio *et al*., 1997).

2.4. Biofertilizers

Biofertilizer is a medium which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003). Biofertilizers add nutrients through the natural processes of nitrogen fixation , solubilizing phosphorus, and stimulating plant growth through the synthesis of growth promoting substances.

They can be expected to reduce the use of chemical fertilizers and pesticides. The microorganisms in biofertilizers restore the soil's natural nutrient cycle and build soil organic matter (Vessey, 2003). Since they play several roles, a scientific term for such

beneficial bacteria is plant-growth promoting rhizobacteria (PGPR) (Vessey, 2003). They are extremely advantageous in enriching the soil fertility and fulfilling the plant nutrient requirements by supplying the organic nutrients (Mahdi *et al*., 2010). Hence, biofertilizers do not contain any chemicals which are harmful to the living soil.

Plant-growth promoting rhizobacteria inoculants seem to promote growth through at least one mechanism; suppression of plant disease (termed Bioprotectants), improved nutrient acquisition (termed Biofertilizers), or phytohormone production (termed Biostimulants) (Kloepper and Schroth, 1978). Species of *Pseudomonas* and *Bacillus* can produce as yet not well characterized phytohormones or growth regulators that cause crops to have greater amounts of fine roots which have the effect of increasing the absorptive surface of plant roots for uptake of water and nutrients (Glick *et al*., 1999).

A variety of beneficial bacteria have been found to colonize the roots and aerial parts of rice, wheat, maize, sugarcane, and other graminaceous plants (Diem *et al.,* 1978: Patriquin and Dobereiner, 1978: Magalhaes *et al*., 1979: Bilal and Malik, 1987: Hurek *et al.,* 1994: James *et al*., 1994: Hassan *et al*., 1998). Interest in the beneficial rhizobacteria associated with cereals has increased due to their potential use as biofertilizers (Dobereiner and Day, 1976: Bashan and Levanony, 1988). Application of bacterial inoculants as biofertilizers has resulted in improved growth and increased yield of cereal crops. Beneficial effects of these plant growth promoting rhizobacteria have been attributed to biological nitrogen fixation and production of phytohormones

that promote root development and proliferation resulting in more efficient uptake of water and nutrients.

Nitrogen-fixing bacteria belonging to the genera *Azospirillum, Acetobacter, Azoarcus, Enterobacter,* and *Herbaspirillum* appear to be frequent colonizers of important cereal crops and grasses (Dobereiner and Day, 1976: Baldani *et al*., 1986: Bilal and Malik, 1987: Reinhold-Hurek and Hurek, 1998). Blue green algae belonging to genera *Nostoc, Anabaena, Tolypothrix* and *Aulosira* fix atmospheric nitrogen and are used as inoculants for paddy crop grown both under upland and low land conditions (Vessey, 2003). *Azolla* is a free-floating water fern that floats in water and fixes atmospheric nitrogen and also enriches soils with organic matter in association with nitrogen fixing blue green algae *Anabaena azollae*. Rice growing areas in South East Asia and other third World countries have had increased interest in the use of the symbiotic nitrogen fixing water fern *Azolla* either as an alternate nitrogen sources or as a supplement to commercial nitrogen fertilizers (Vessey, 2003).

Other types of bacteria, so-called phosphate solubilizing bacteria like *Pantoea agglomerans* strain, and *Pseudomonas putida* strain are able to solubilize the insoluble phosphate from organic and inorganic phosphate sources (Malboobi *et al*., 2009). Due, to immobilization of phosphate by mineral ions such as iron, Alluminium and Calcium or organic acids, the rate of available phosphate (Pi) in soil is well below plant needs. In addition, chemical inorganic phosphate fertilizers are also immobilized in the soil, immediately, so that less than 20 percent of added fertilizer is absorbed by plants (Pandey *et al*., 2006). Therefore, reduction in inorganic phosphate (Pi) resources on one

hand, and environmental pollutions resulting from both production and applications of chemical Pi fertilizer, on the other hand, have already demanded the use of new generation of phosphate fertilizers globally known as phosphate-solubilizing bacteria or phosphate bio-fertilizers (Malboobi *et al.*, 2009). Several soil bacteria and fungi, notably species of *Pseudomonas, Bacillus, Penicillium, Aspergillus etc.* secrete organic acids and lower the pH in their vicinity to bring about dissolution of bound phosphates in soil (Pandey *et al.*, 2006).

Biofertilizers increase crop yield by 20-30%, replaces chemical nitrogen and phosphorus by 25%, and stimulates plant growth. It can also provide protection against some soil-borne diseases. Bio-fertilizers are cost-effective relative to chemical fertilizers. They have lower manufacturing costs, especially regarding nitrogen and phosphorus use. They are also environmentally friendly (Mahdi *et al.*, 2010).

Inoculation of plants with beneficial bacteria can be traced back for centuries. From experience, farmers knew that when they mixed soil taken from a previous legume crop with soil in which nonlegumes were to be grown, yields often improved. By the end of the 19th century, the practice of mixing "naturally inoculated" soil with seeds became a recommended method of legume inoculation in the USA (Smith, 1992). A decade later, the first patent ("Nitragin") was registered for plant inoculation with *Rhizobium* sp. (Nobbe and Hiltner, 1896). Eventually, the practice of legume inoculation with rhizobia became common. For almost 100 years, *Rhizobium* inoculants have been produced around the world, primarily by small companies. Some legumes, like soybean in Brazil, are not fertilized with nitrogen, but are only inoculated. Apart from soybean

inoculation, which has made a major agricultural impact in the USA, Brazil, and Argentina, significant contributions to the production of other legumes were made in Australia, North America, Eastern Europe, Egypt, Israel, South Africa, New Zealand, and, to a lesser extent, Southeast Asia (Smith, 1992).

The immediate response to soil inoculation with associative, non symbiotic plant growth promoting bacteria varies considerably depending on the bacteria, plant species, soil type, inoculant density and environmental conditions. In general, shortly after the bacteria are introduced into the soil, the bacterial population declines progressively (Bashan and Levanony, 1988). This phenomenon together with bacterial biomass production and the physiological state of the bacteria in the inoculants may prevent the buildup of a sufficiently large plant growth promoting bacteria population in the rhizosphere to obtain the intended plant response.

2.5. Molecular characterization of endophytes

The most powerful approaches to taxonomy are through the use of nucleic acids because these are either direct gene products or the genes themselves and comparisons of nucleic acids yield considerable information about true relatedness. Molecular systematics, which includes both classification and identification, has its origin in the early nucleic acid hybridization studies, but has achieved a new status following the introduction of nucleic acid sequencing techniques (O'Donnell *et al.*, 1993). Significance of phylogenetic studies based on 16S rDNA sequences is increasing in the systematics of bacteria and actinomycetes (Yokota, 1997).

16S ribosomal DNA (16S rDNA) is a component of the 30S subunit of prokaryotic ribosomes. The genes coding for it are referred to as 16S rDNA and are used in reconstructing phylogenies. The 16S rDNA gene is used for phylogenetic studies as it is highly conserved between different species of bacteria and archaea (Coenye and Vandamme, 2003). Woese (1990) pioneered this use of 16S rDNA. Universal PCR primers are used to amplify the 16S rDNA gene providing the phylogenetic information. The most common universal primer pair was devised by Weisburg *et al.,* (1991), and is currently referred to 27F and 1492R.

In addition to highly conserved primer binding sites, 16S rDNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. As a result, 16S rDNA gene sequencing has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification (Clarridge, 2004). It has also been used to describe new species that have never been successfully cultured (Schmidt and Relman, 1994; Gray and Herwig, 1996).

Analysis of the 16S rDNA begins by isolating DNA (Hapwood *et al.*, 1985) and amplifying the gene coding for 16S rDNA using the polymerase chain reaction (Siva, 2001). The purified DNA fragments are directly sequenced. The sequencing reactions are performed using DNA sequencer in order to determine the order in which the bases are arranged within the length of sample and a computer is then used for studying the

sequence for identification using phylogenetic analysis procedures (Stackebrandt and Goebel, 1994).

The use of 16S rDNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These include its presence in almost all bacteria; the function of the 16S rDNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time; and the 16S rDNA gene (1,500 bp) is large enough for informatics purposes (Patel, 2001).

 In 1980 in the Approved Lists, 1,791 valid names of microbes were recognized at the rank of species using 16S rDNA. Today, this number has ballooned to 8,168 species, a 456% increase (Clarridge, 2004). The explosion in the number of recognized taxa is directly attributable to the ease in performance of 16S rDNA gene sequencing studies as opposed to the more cumbersome manipulations involving DNA-DNA hybridization investigations.

One of the most attractive potential uses of 16S rDNA gene sequence informatics is to provide genus and species identification for isolates that do not fit any recognized biochemical profiles (Stackebrandt and Goebel, 1994). Difficulties encountered in obtaining a genus and species identification include the recognition of novel taxa, too few sequences deposited in nucleotide databases, species sharing similar and/or identical 16S rDNA sequences, or nomenclature problems arising from multiple genomovars (gene groups) assigned to single species or complexes.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study Site

The root samples were collected from Ahero and Mwea Irrigation Agricultural Development Centre (MIAD) research fields and farmers' fields in the same sites. Isolation and morphological as well as biochemical characterization were carried out at the Food Science microbiology laboratory, while the molecular characterization was carried out in the Biochemistry Department and the Institute of Biotechnology Research (IBR) at Jomo Kenyatta University of Agriculture and Technology, Juja.

3.2. Experimental Design

Samples of Basmati 370 and 217 rice varieties were randomly collected from Mwea and Ahero experimental sites. The root samples were ground for plating on three different types of media and isolates obtained used for morphological, biochemical and molecular characterization process.

3.3. Determination of sample size

A sample size of 245 per site was used. This was determined according to Walpole (1986) formula:

$$n = \frac{Z^2 \, pq}{d^2}$$

$$= \frac{(1.96^2) \ 0.8(1\text{-}0.8)}{(0.05^2)}$$

$$= 245$$

Where: n= the desired sample size

Z= the standard normal deviate at the required confidence level (95% confidence

level was used in this study)

p= the estimated proportion of an attribute that is present in a population.

(In this study, P was obtained upon literature review of an article of a

similar study by Goryluk *et al.,* 2009, in which case P was 0.8).

q= 1-p

d= the level of statistical significance set.

3.4. Collection of Root Samples

Root samples from basmati 217 and basmati 370 rice varieties were randomly collected from rice paddies in Mwea and Ahero irrigation schemes and transported at 4°C to the laboratory for endophyte isolation. The samples were collected from 21 farms in the experimental sites. These were collected across a diagonal line on the farms avoiding the edges because of influence of extraneous factors. The rice plants were gently uprooted from the soil, after which the vegetative part was chopped off. Soil attached to the roots was then gently washed off before placing the sample in a plastic bag. In the laboratory, the samples were stored at 4°C.

3.5.   Preparation of Culture Media

Three different media were used. These were yeast manitol agar, nitrogen free medium, and nutrient agar. The ingredients were weighed and dissolved in distilled water by warming on a hot plate. Each of the media comprised of the following ingredients:

I.   Yeast Manitol agar (10g//L manitol, 0.5g/L K_2PO_4, 0.8g/L MgSO4, 0.2g/L NaCl, 0.01g/L $FeCl_3$, 1g/L yeast extract, 15g/L agar) (Sigma Aldrich, Steinheim, Germany)

II.   Nitrogen free media (0.5g/L K_2PO4, 0.2g/L $MgSO_4$, 0.2g/L NaCl, 15mg/L $FeCl_3$, 6.6g/L $NaMoO_4(H_2O)$, 15g/L agar) (Sigma Aldrich, Steinheim, Germany)

III.   Nutrient Agar (28g/L) (Sigma Aldrich, Steinheim, Germany)

These were then autoclaved under pressure for fifteen minutes at 121°C. Finally, the media was dispensed in sterile petri dishes.

3.6.   Surface sterilization of root samples

The roots were thoroughly washed with sterile distilled water to remove any adhering soil. The samples were subjected to surface sterilization procedure as follows: a 3 minutes wash in 75% ethanol (Scharlab S.L., Spain), followed by a 5 minutes wash in 5% sodium hypochlorite (Reckitt Benckistter East Africa Ltd., Nairobi), and finally a five times rinse in sterile distilled water. The samples were then aseptically dried using sterile paper towels.

3.7. Evaluation of the effectiveness of surface sterilization

Two experiments were carried out to check the effectiveness of the sterilization procedures. First, the surface-sterilized tissues were imprinted onto nutrient agar, incubated at 30°C, and then checked for microbial growth. Second, the surface-sterilized samples were washed in sterile distilled water three times, soaked in 5 ml sterile distilled water, and stirred for 1 min. A 0.2-ml aliquot of the suspension were then inoculated onto nutrient agar plates, incubated at 30°C for 24 hours, and observed for microbial growth. If no microbial growth occurred on the surface of the medium, the surface sterilization was considered successful.

3.8. Isolation and culturing of bacterial endophytic isolates

The samples were aseptically ground in a motor and pestle in potassium dihydrogen phosphate buffer (pH 6.8). An aliquot (1ml) was then picked and placed in the same buffer (9ml) for serial dilution. This procedure was repeated to form a fivefold serial dilution. The serially diluted aliquot (100µl) was then inoculated on yeast manitol agar (YEM), nitrogen free medium, and nutrient agar. The cultures were then placed in an incubator at 30°C for 24 hours to allow for endophyte growth. Individual colonies were picked and streaked on fresh media for purification to generate pure cultures (Appendix 1). The pure cultures were then used to perform morphological, biochemical and subsequently molecular characterization.

3.9. Morphological characterization of endophyte isolates

This was done to determine the cell shape of the bacterial cells, in which case, the classical gram staining method was used (Bathlomew, 1962). Smears of the bacterial isolates were prepared and heat fixed, after which they were flooded with crystal violet (Sigma Aldrich, Steinheim, Germany) and left to stand for a minute. The smears were gently washed with tap water, and then flooded with grams iodine (Sigma Aldrich, Steinheim, Germany). They were rinsed with tap water after a minute. Decolorization with 95% ethanol (Scharlab S.L., Spain) was then done, followed with counterstaining with safranin for forty five seconds. Smears were then gently washed with tap water, and blot dried for observation under oil immersion on a light microscope.

3.10. Biochemical characterization of endophyte isolates

The isolates were subjected to the following biochemical tests:

3.11.1. Acetylene reduction assay (ARA)

This test measures nitrogenase activity, which ideally is a measure of the total amount of nitrogen that a system or organism has fixed (Eckert *et al.*, 2001). The process for biological nitrogen fixation can be summarized as:

$$N_2 + 8\,H^+ + 6\,e^- \xrightarrow{\text{Nitrogenase}} 2\,NH_3 + H_2$$
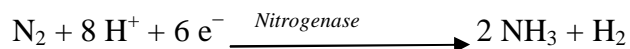
Bacteria were grown for three days in nitrogen free medium. They were then placed in semi solid agar media containing 2.3g of agar per liter. Five ml of the media was placed in 10ml vials. Acetylene was added to attain a concentration of 12% v/v and the ethylene production was determined after 12 hours on a Shimadzu Gas Chromatograph

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(GC-9A, Japan) as described by Eckert *et al.*, (2001). In this case, a needle and syringe was used to pick 1ml of the free space in the reaction vials, which was then injected into the GC machine that gave a chromatograph showing retention time.

3.11.2. Urease test

The urease test was used to determine the ability of an organism to split urea, through the production of the enzyme urease (Harold, 2002).

Summary of the urease test:

$$(NH_2)_2CO + H_2O \xrightarrow{\quad urease \quad} CO_2 + 2NH_3 \text{ (ammonia)}$$
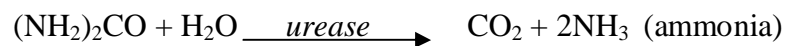
$$\text{Ammonia + phenol red} \longrightarrow \text{deep pink color}$$

Units of any ammonia formed with resulting alkalinity in the presence of the enzyme and the increased pH was detected by a pH indicator. Christensen's urea contains the pH indicator phenol red which under acidic conditions (pH 6.8) is yellow. In alkaline conditions (pH 8.4) the indicator turns the media rose pink. The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using urea broth medium (Oxoid Ltd., Basingstoke, Hampshire, England) containing the pH indicator phenol red (Cappuccino and Sherman, 2002). The bacteria were aseptically inoculated into sterile Christensen's urea broth using a sterile wire loop and incubated in a clean incubator (IN-81, Yamato, Japan) at 30°C for 24 hours, after which observations on color change were done (Cappuccino and Sherman, 2002).

3.11.3. Denitrification test

The ability of the isolates to reduce nitrates to nitrites or beyond was carried out using nitrate reduction broth placed in universal tubes containing Durham tubes. The bacteria were aseptically inoculated into sterile nitrate reduction broth using a sterile wire loop and incubated in a clean incubator (IN-81, Yamato, Japan) at 30°C for 72 hours, after which observations were done (Cappuccino and Sherman, 2002).

Summary of a positive denitrification test:

NO_3^- __*nitrate reductase*__ $\blacktriangleright$ NO_2^- denitrifying bacteria $\blacktriangleright$ $N_{2(g)}$
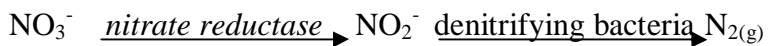
3.10.4. Phosphate solubilisation test

Phosphate solubilisation medium was used for screening phosphate solubilizing microorganisms using plate assay method. The phosphate solubilisation media was prepared and dispensed on sterile petri dishes. A sterile wire loop was then used to place inoculums onto the media. The plates were then placed in a clean incubator (IN-81, Yamato, Japan) at 30°C for two weeks, after which observations were done (Cappuccino and Sherman, 2002). Formation of a hallo around the bacterial colonies indicates a positive test for phosphate solubilisation.

3.11.5. Indoleacetic acid production test

Production of indoleacetic acid was detected by a calorimetric method using the Salkowski reagent as described by Glickmann and Dessaux (1995). The pure bacterial isolates were aseptically inoculated into sterile nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, England), using a sterile wire loop and incubated in a clean incubator (IN-

81, Yamato, Japan) at 30°C for 72 hours. The cultures were then centrifuged (11,965.2 g, 5minutes, 25°C) to obtain cell free broth. Few drops (0.5 ml) of Salkowski's reagent were then added to the cell free broth and incubated for thirty minutes at room temperature (Glickmann and Dessaux, 1995). Formation of a pink color indicates a positive test. Salkowski's reagent is a 35% $HClO_4$ solution containing 10 mM $FeCl_3$, and when mixed with IAA, tris-(indole-3- acetato)iron(III) complex is formed to display pink coloration (Rahman *et al*., 2010).

3.11.6. Citrate utilization test

Simmons' Citrate agar slants were used to determine the capability of the isolates to use citrate as a carbon source for their energy (Harold, 2002). The bacteria were aseptically inoculated into sterile Simmon's Citrate agar (Sigma Aldrich, Steinheim, Germany), using a sterile wire loop and incubated in a clean incubator (IN-81, Yamato, Japan) at 30°C for 24 hours, after which observations on color change were done (Cappuccino and Sherman, 2002).

Bacteria were inoculated in a medium containing sodium citrate and a pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which is utilized as sole source of nitrogen. Utilization of citrate involves the enzyme citrase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO_2. Production of Na_2CO_3 from utilization of sodium citrate results in alkaline pH. This results in change of medium's color from green to blue.

Sodium Citrate $\xrightarrow[\textit{Citrase}]{\textit{Citrate permiase}}$ Pyruvic acid + Oxaloacetic acid + CO_2
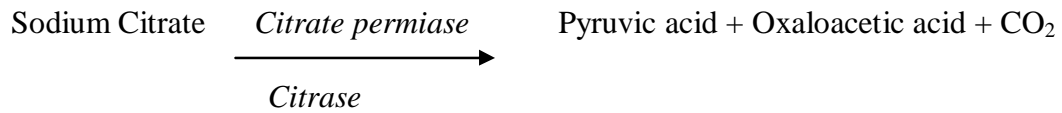
Excess sodium from sodium citrate + CO_2 + H_2O $\longrightarrow$ Na_2CO_3 (Alkaline pH-blue color)

3.11.7. Catalase test

Catalase test detects the catalase enzyme presence in most cytochrome containing aerobic bacteria which form hydrogen peroxide as an oxidative end product of the aerobic breakdown of sugars. Catalase decomposes hydrogen peroxide to water and oxygen.

$2 H_2O_2 \xrightarrow{\textit{Catalase}} 2 H_2O + O_2$
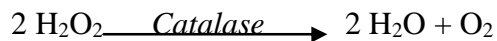
The bacteria were aseptically inoculated into sterile Tryptic Soy agar (Sigma Aldrich, Steinheim, Germany), using a sterile wire loop and incubated in a clean incubator (IN-81, Yamato, Japan) at 30°C for 24 hours, after which Catalase activity was determined by addition of 3% hydrogen peroxide to the cultures. A positive reaction is indicated by the formation of bubbles (Cappuccino and Sherman, 2002).

3.11.8. Hydrogen Sulphide production test

Sulfur-Indole Mortility agar (Oxoid Ltd., Basingstoke, Hampshire, England) media was used to demonstrate the production of cysteine desulfurase by the isolates. Cysteine desulfurase breaks down sulfur containing amino acids producing pyruvate, ammonia and hydrogen sulfide. Iron in the medium reacts with hydrogen sulfide producing the

characteristic black precipitate which is a positive test for hydrogen sulfide production by the isolates (Cappuccino and Sherman, 2002). This can be summarized as:

Cysteine $\xrightarrow{\textit{Cysteine desulfurase}}$ pyruvic acid + ammonia + hydrogen sulfide gas

$H_2S + Fe^{+2} \xrightarrow{\hspace{3cm}} Fe(NH_4)SO_4$

(Black precipitate)
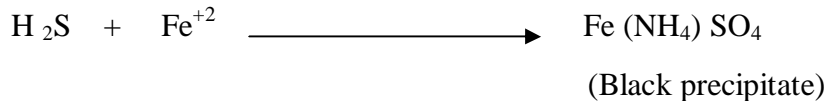
In the test for hydrogen sulfide production, a loopful of the culture was inoculated in Sulfur-Indole Mortility agar (Oxoid Ltd., Basingstoke, Hampshire, England) by stubbing through the media. This set up was then incubated in a clean incubator (IN-81, Yamato, Japan) at 30°C for 24 hours. Hydrogen sulfide production was detected by the blackening of the media (Cappuccino and Sherman, 2002).

3.11.9. Methyl Red-Voges-Proskauer test (MR-VP)

The MR-VP test was done to determine the ability of the isolates to oxidize glucose with the production and stabilization of high concentration of acids as end products. The bacteria were aseptically inoculated into sterile MR-VP broth (Sigma Aldrich, Steinheim, Germany), using a sterile wire loop and incubated in a clean incubator (IN-81, Yamato, Japan) at 30°C for 24 hours. Aliquots (1ml) of each culture were then picked and added with either methyl red indicator (MR test) or Barritts reagent (VP test) then observations on color change were done (Cappuccino and Sherman, 2002).

Summary of MR test:

Positive test: glucose $\xrightarrow{\hspace{2cm}}$ pyruvic acid (1 day)

Pyruvic acid $\longrightarrow$ lactic, acetic, and formic acids

Many acids (pH 4.2) + added methyl red $\longrightarrow$ red color

Negative test: glucose $\longrightarrow$ pyruvic acid (1 day)

Pyruvic acid $\longrightarrow$ neutral end products

Neutral end products (pH6.0) + methyl red $\longrightarrow$ yellow color

Summary of VP test:

Glucose + ½ O_2 $\longrightarrow$ 2 pyruvate $\longrightarrow$ α-acetolactate $\longrightarrow$ acetion $\longrightarrow$ 2,3-butanediol

Acetion + Barritt's reagent $\longrightarrow$ diacetyl + creatine (pink complex)

3.11. Molecular characterization of endophytic bacterial isolates

3.12.1 Extraction of genomic DNA from endophyte isolates

Genomic DNA was extracted from the selected thirty seven isolates using the chloroform extraction procedure (Sambrook *et al*., 1989). Prior to extraction, bacterial cells were harvested from broth by centrifuging on a micro centrifuge (11,965.2 g, 5minutes, at 25°C) (Hettich, Micro 200, Germany). For this case, 1ml of culture was placed in a 1.5ml eppendorf tube and the supernatant poured out after the centrifugation. This was repeated twice to obtain enough cell yields. The bacterial cells were washed by re-suspending in equal volumes of TE buffer (pH 8), centrifuged

(Hettich, Micro 200, Germany) for 5 minutes at 11,965.2 g, at 25°C, and the supernatant discarded. The cells were then re-suspended in 200 µl of solution 1 [50mM Tris (pH 8.5), 50mM EDTA pH (8.0) and 25% sucrose solution], 5µl of lysozyme (20mg/ml) (Sigma Aldrich, Steinheim, Germany) and 5ul of RNase A (20mg/ml) (Sigma Aldrich, Steinheim, Germany) then mixed gently.

The mixture was then incubated at 37°C for 1 hour. Then 600µl of solution 2 [10mM Tris (pH 8.7), 5mM EDTA (pH 8.0) and 1% sodium dodecyl sulphate] and 10µl of 20mg/ml proteinase K (Sigma Aldrich, Steinheim, Germany) was added and mixed gently. The mixture was then incubated at 50°C for 30 minutes. Equal volumes of phenol-chloroform were added and spun for 5 minutes at 11,965.2 g, at 25°C. The upper aqueous layer was transferred carefully into a separate I.5ml eppendorf tube. This step was repeated before adding an equal volume of diethyl ether to wash off the phenol. This mixture was then spun at 11,965.2 g, at 25°C, for 5 minutes and the supernatant carefully discarded. The procedure was repeated twice. The DNA was then precipitated by adding an equal volume of ice cold absolute ethanol and 0.1 volumes of 3M potassium acetate (Sigma Aldrich, Steinheim, Germany) and left overnight at -20°C. The pellet was concentrated by centrifugation at 11,965.2 g, at 25°C, for 30 minutes and the supernatant discarded. Equal volumes of 70% ethanol (Scharlab S.L., Spain) were added and centrifuged at 11,965.2 g for 5 minutes. The supernatant was discarded carefully without disturbing the pellet. This procedure was repeated twice before

leaving the pellet on the bench to air dry completely at room temperature in order to eliminate residual ethanol.

The dry DNA pellet obtained was then re-suspended in 45µl of TE buffer and then kept at -20°C for future use. The DNA was separated on a 1% (w/v) agarose (Sigma Aldrich, Steinheim, Germany) gel in 1xTAE buffer and visualized under UV by staining with ethidium bromide (Sambrook *et al*., 1989).

The DNA was then quantified using on an Eppendorf AG model 22331 spectrophotometer (Hamburg, Germany) with the absorbance at 260nm and 280nm to determine the purity of the DNA. The ratio 1.8 - 2.0 was used in the subsequent polymerase chain reaction since at this ratio the DNA integrity is good and without impurities.

3.12.2. Polymerase Chain Reaction

For the amplification of the 16S rDNA, 1µl of DNA from each of the thirty seven extracts were amplified using Taq polymerase and 10x buffer according to manufacturer's (QIAGEN) instructions.

Nearly full-length 16S rDNA gene sequences were PCR-amplified using bacterial primer pair 27F forward 5'-GAGTTTGMTCCTGGCTCA-3' and 1492R reverse, 5'-TACGGYTACCTTACGACT-3' (Bioneer, USA) according to the position in relation to *Escherichia coli* gene sequence (Embley and Stackebrandt, 1994). Amplification was performed using an Eppendorf AG, model 22331 thermal cycler (Hamburg).

Amplification was carried out in a 50 µl mixture containing 0.2 Units of Taq polymerase, 20pmol of 27F forward primer, 20pmol of 1492R reverse primer, 1.25mM dNTPs mix (QIAGEN), 10x PCR buffer (QIAGEN), 1 µl of template DNA and 29.8 µl of PCR water. The negative control contained all the above except the DNA template. Likewise, the positive control contained 0.2 Units of Taq polymerase, 20pmol of 27F forward primer, 20pmol of 1492R reverse primer, 1.25mM dNTPs mix (QIAGEN), 10x PCR buffer (QIAGEN), 29.8 µl of PCR water and 1 µl of DNA template from a characterized *Pseudomonas putida* identified using 16S rDNA based analysis. Reaction mixtures were subjected to the following temperatures: Initial denaturation of the template at 94°C for 5 minutes, denaturation at 94°C for 45 seconds, primer annealing at 43°C for 2 minutes, chain extension at 72°C for 1.5 minutes and a final extension at 72°C for 5 minutes (Roux, 1995). Denaturation, annealing and extension cycles were repeated for 35 cycles. Amplification products (7.0 µl) were separated on a 1% (w/v) agarose (Sigma Aldrich, Steinheim, Germany) gel in 1X TAE buffer and visualized by ethidium bromide staining (Sambrook *et al*., 1989).

3.12.3. Restriction analysis of the PCR products

The preliminary genetic diversity of the thirty seven bacterial isolates was determined by amplified ribosomal DNA restriction analysis (ARDRA) of 16S rDNA as described by Desaint *et al.,* (2000). An aliquot of the PCR product (8µl) was digested in a final volume of 20 µl for 12 hours at 37°C with 2 units of a restriction endonuclease (RsaI) according to the manufacturer's (Sigma Aldrich, Steinheim, Germany) specifications.

Digested DNA fragments were separated by gel electrophoresis in 1.5 % (w/v) agarose (Sigma Aldrich, Steinheim, Germany) gel for 2 hrs at 80 V. Gels were stained with ethidium bromide and DNA fragments visualized under UV illumination (BTS-20.M, EEC, Taiwan). Similarity among strains was estimated from the proportion of shared restriction fragment bands generated.

3.12.4. Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol according to manufacturer's (QIAGEN, Germany) instructions. Five volumes of binding buffer (PB) was added to 1 volume of the PCR sample and thoroughly mixed. The QIAquick spin column was placed in a 2 ml collection tube, the sample applied, and then centrifuged for 60 seconds at 11,965.2 g, at 25°C. The flow-through was discarded, and the QIAquick column placed back into the same tubes.

To wash the DNA, 0.75 ml washing buffer (PE) was added to the QIAquick column and centrifuged for 1 minute at 11,965.2 g, at 25°C. The flow-through was discarded and the column centrifuged again for an additional 1 minute at 11,965.2 g, at 25°C to remove residual ethanol from buffer PE.

The Qiaquick column was then placed in a 1.5 ml micro centrifuge tube and 30 µl of elution buffer (EB) added to elute DNA. The tubes were then centrifuged for 1-minute at 11,965.2 g, 25°C, after which the spin column was removed and DNA stored at -20°C for further use (Sambrook *et al.,* 1989).

Amplification products (5.0 μl) were separated on a 1% (w/v) agarose (Sigma Aldrich, Steinheim, Germany) gel in 1X TAE buffer and visualized by ethidium bromide staining (Sambrook *et al.*, 1989).

3.12.5. Sequencing and molecular data analysis

A selection criterion was used to obtain twenty eight bacterial isolates for sequencing. The selection process was guided by the morphological and biochemical characteristics and the restriction analysis profiles of the different isolates to identify any notable differences. Objectives of this study on plant growth promotion were also considered. Sequencing of purified PCR products was done by a commercial service provider (ILRI, Nairobi, Kenya). In this case, dye-terminator sequencing technique was used.

The sequences were checked and corrected manually where necessary using the Chromas Pro program based on conserved regions. The 16S rDNA gene sequences were compared to sequences in the public database using Basic Local Alignment Search Tool (BLAST) on the National Center for biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) in order to determine similarity to sequences in the Gene bank database (Altschul *et al.*, 1990; Shayne *et al.,* 2003).

The 16S rDNA gene sequences with high similarities to those determined in the study were retrieved and added to the alignment based on BLAST results. Phylogenetic trees were constructed by Maximum likelihood method. Bootstrap analysis using MEGA 4 for 100 replicates was performed to define confidence estimates for the tree topologies (Saitou and Nei, 1987).

3.12. Assessment of effect of selected isolates on rice plant growth

Five isolates were selected with respect to the objectives of this research on plant growth promotion. In this case, certain relevant tests were considered. These include the denitrification, urease and phosphate solubilisation tests, ARA, and test for indoleacetic acid production. Isolates M31, M32, M16, M5 and K7 were selected in this respect. A cocktail of all these five isolates was also prepared. Inoculums of concentrations 1×10^5 and 1×10^{10} were used to perform the drenching. The selected isolates were all used to perform the drenching and assess for any phenotypic effects on Kenyan basmati rice. The concentrations of the bacterial endophytes used for the drenching process were based on information from literature review on what has been used by other researchers including Zhang *et al*., 2010; Wang *et al*., 2010; Klife and Laing, 2011. The plant growth indicators considered included plant height, tillering, and dry weight of shoots and roots (Ramezanpour *et al*., 2010).

Basmati rice seeds were surface sterilized by first dipping them in 70% ethanol for 10 seconds followed by soaking them in 5% sodium hypochlorite for 5 minutes with gentle swirling. The seeds were then rinsed five times using double distilled water. The seeds were then air dried and planted in virgin sterile soil in pots and placed under same conditions in the green house to allow growth. The virgin soil used was steam sterilized at a hundred degrees Celsius. The positive control plants were also grown on virgin sterile with application of a phosphate fertilizer during planting and a nitrogen fertilizer ten days after planting. The negative control plants were grown on sterile virgin soil without any fertilizer application. All the seedlings' pots (sixty four) were arranged in

rows. Each pot contained one seedling. Viable cells in the inoculums were quantified using the serial dilution – agar plate method (Cappuccino and Sherman, 2002). An inoculation (drench) with the respective potential endophyte isolates was randomly done and observations made against the controls. In this case, each of the promising endophytes was inoculated separately in a four replicate treatment and an additional treatment containing all endophytes was also set up. The rice plants were watered daily. All these inoculations were done on basmati 370, which was the source of the identified endophytes. Data on rice tillering, height and weight of dry matter of shoots and roots was collected at three and seven weeks after inoculation.

3.13. Data analysis

Biochemical characterization procedures were done in replicates of three. Observations were made on these replicates to define the nature of each of the qualitative tests as either positive or negative. This information was used during the generation of phylogenetic trees to identify the rice root bacterial endophytes. The 16S rDNA sequences obtained during molecular characterization were analyzed using Chromas pro program after which phylogenetic trees were generated using MEGA 4. The mean of the seedling tillering, height and weight of dry matter of shoots and roots (for control and experimental) were calculated: and t- test was also used to check on any variation between each of the treatments against the controls (Walpole, 1986).

CHAPTER FOUR

4.0.   RESULTS

4.1.   Isolation and culturing of bacterial root endophytic isolates

Endophytic bacteria colonizing rice root tissues were found in samples from the two experimental sites and basmati rice varieties. Sixty six primary isolates were obtained from Mwea, and seven from Ahero. The frequency of endophytic isolates was found to differ among rice plant varieties. Basmati 370 was found to harbor more endophytic bacteria (seventy percent- 51/73) than basmati 217 which harbored only thirty percent (22/73) of the total (73) isolates. Bacterial endophyte growth was observed on nutrient agar and yeast manitol agar after twenty four hours of incubation, while it took forty eight hours on nitrogen free media. Diversity of the isolates obtained was observed for the samples from the two experimental sites (Mwea and Ahero). Bacterial colonies of different colors such as white, cream white, pink, and yellow were observed. The seventy three pure isolates were then coded as follows: Mwea isolates were M1 to M66 while those from Ahero formed K1 to K7 (Table 4.1).

4.2.   Morphological characterization of endophyte isolates

Morphological characterization of the 73 isolates based on Gram Test revealed that 46 of the endophytic bacterial isolates were gram negative rods (Appendix 2, b; Table 4.1) while 26 were gram positive rods (Appendix 2, a; Table 4.1) and one gram positive cocci (Appendix 2, c; Table 4.1). The Gram positive bacteria retained the primary stain

(crystal violet) during subsequent decolourisation and appeared purple when viewed under a microscope while gram negative bacteria lost the primary stain during decoulorisation and took up the secondary stain (safranin) to appear red when viewed under the microscope (Appendix 2, a, b, c). The rods observed had varying thickness and length. Both Basmati 370 and Basmati 217 were found to host more gram negative bacteria than gram positive ones as observed from the seventy three isolates obtained. Among the 51 bacterial isolates obtained from Basmati 370, sixty nine percent (35/51) were gram negative (Table 4.1). On the other hand, out of the 22 bacterial isolates from Basmati 217, fifty five percent (11/20) were gram negative (Table 4.1). It was further noted that majority (43/66) of the isolates from Mwea were gram negative bacteria (Table 4.1). Conversely for Kisumu bacterial isolates, fifty seven percent (4/7) were gram positive (Table 4.1).

Table 4.1: Summary of results on biochemical characterization of the selected 37 bacterial endophytes; M series- Mwea isolates; K series- Kisumu isolates; + (positive); - (negative)

ISOLATE CODE	GRAM TEST	MORPHOLOGY	CATALASE TEST	UREASE TEST	NITRATE REDUCTION	CITRATE UTILIZATION	HYDROGEN SULFIDE PRODUCTION	PHOSPHATE SOLUBILISATIO	MR	VP	ARA	IAA
M1	-	Rods	+	+	-	+	-	+	-	-	+	-
M3	+	Rods	+	+	-	+	-	+	-	-	+	-
M5	-	Rods	+	-	-	+	-	+	-	+	+	+
M6	+	Rods	+	+	-	+	-	+	-	-	+	-
M7	-	Rods	+	+	-	+	-	+	+	-	+	-
M9	-	Rods	+	+	-	+	-	+	-	-	+	-
M11	-	Rods	+	+	-	+	-	-	+	-	+	-
M16	-	Rods	+	-	-	+	-	+	-	+	+	+
M17	-	Rods	+	-	-	+	-	+	-	+	+	+
M18	-	Rods	+	-	-	+	-	+	-	+	+	+
M19	-	Rods	+	+	-	+	-	-	+	-	+	-
M22	+	Rods	+	+	-	+	-	+	-	-	+	-
M23	+	Rods	+	+	-	+	-	+	-	-	+	-
M24	+	Rods	+	+	-	+	-	+	-	-	+	-
M28	-	Rods	+	-	-	+	-	+	-	+	+	-
M31	-	Rods	+	-	-	+	-	+	-	+	+	+
M32	-	Rods	+	+	-	+	-	+	+	-	+	+
M34	+	Rods	+	+	-	+	-	+	-	-	+	-
M39	+	Rods	+	+	-	+	-	+	-	-	+	-
M41	+	Rods	+	+	-	+	-	+	-	-	+	-
M51	-	Rods	+	-	-	+	-	+	-	+	+	+
M53	-	Rods	+	+	-	+	-	+	+	-	+	-
M55	-	Rods	+	-	-	+	-	+	-	+	+	-
M56	-	Rods	+	+	-	+	-	+	-	-	+	-
M58	-	Rods	+	+	-	+	-	+	+	-	+	-
M59	-	Rods	+	-	-	+	-	+	-	-	+	-
M60	-	Rods	+	-	-	+	-	+	-	+	+	-
M63	-	Rods	+	+	-	+	-	+	+	-	+	-
M67	-	Rods	+	-	-	+	-	+	-	+	+	-
M68	+	Rods	+	+	-	+	-	+	-	-	+	-
K1	-	Rods	+	-	-	+	-	+	-	+	+	-
K2	-	Rods	+	+	-	+	-	+	+	-	+	+
K3	+	Cocci	+	-	-	+	-	+	-	-	+	-
K4	+	Rods	+	+	-	+	-	+	-	-	+	-
K5	+	Rods	+	+	-	+	-	+	-	-	+	-
K6	-	Rods	+	-	-	+	-	+	-	+	+	-
K7	+	Rods	+	+	-	+	-	+	-	+	+	-

4.3. Biochemical characterization of bacterial root endophytic isolates

All the seventy three bacterial endophytic isolates were subjected to various biochemical tests, and the results are presented in Table 4.1. Both positive and negative outcomes were observed for the urease test, citrate utilization test, indole production test, hydrogen sulfide production test, phosphate solubilisation test, Methyl Red-Voges-Proskauer test, IAA production test, Catalase test, Acetylene reduction assay, and denitrification test.

4.3.1. Screening of endophytes for nitrogen fixation

Nitrogenase catalyses the reduction of acetylene (C_2H_2) to ethylene. The reduction of acetylene to ethylene (C_2H_4) is widely used as a method to assess nitrogenase activity in natural samples, isolates, and cell-free extracts. The retention time for acetylene on gas chromatography is 1.5 minutes. It was observed that the retention time for ethylene gas (standard) on the gas chromatograph was 1.378 minutes (Appendix 6) while that for the experimental isolate was 1.392 minutes (Appendix 7). Appendix 7 is representative for the chromatographs obtained for all the isolates. All isolates tested positive for ARA (Table 4.1, Appendix 16). This was established upon comparison of retention times on chromatographs of the standard (Appendix 6) and experimental sample (Appendix 7). Both gram negative (sixty three percent- 46/73) and gram positive (thirty seven percent-27/73) isolates obtained in this study were positive for ARA. Forty seven of the urease positive isolates were also positive for ARA. Ninety two percent (67/73) of the total isolates were positive for both ARA and phosphate solubilisation assay. The ten isolate that were observed to produce IAA were also positive for ARA (Table 4.1).

4.3.2. Assessment for activity of urease on the endophytic isolates

It was observed that forty seven isolates gave a rose pink color (Appendix 4, a), while twenty six gave a yellow color (Appendix 4, b) upon incubation of the respective isolates' inoculums on Christein's urea broth. These results implied that the forty seven isolates tested positive while the twenty six were negative for this test (Table 4.1, Appendix 16). Christensen's urea contains the pH indicator phenol red which under alkaline conditions turns the media rose pink (Appendix 4, a) implying a positive test. In acidic conditions the indicator is yellow (Appendix 4, b) implying a negative test (Cappuccino and Sherman, 2002). Thirty four of the urease positive isolates were from basmati 370, while thirteen were from basmati 217. Forty five percent (21/47) of the urease positive isolates were gram negative endophytic bacteria, while fifty five percent (26/47) were gram positive. Majority of the samples from both experimental sites were urease positive, ninety one percent (43/47) being from Mwea and nine percent (4/47) from Ahero. Eighty nine percent (42/47) of the urease positive isolates were also positive for phosphate solubilisation. On the contrary, eleven percent (5/47) of the isolates were positive for urease test but negative for phosphate solubilisation. All the urease negative isolates were positive for acetylene reduction assay (Table 4.1, Appendix 16).

4.3.3. Denitrification test on the endophytic isolates

All seventy three isolates tested negative for denitrification test (Table 4.1, Appendix 16). This is because, no air bubble was observed in the Durham tube with any of the isolates (Appendix 5) following the inoculation and incubation of the bacterial isolates'

inoculums on nitrate reduction broth. Presence of air bubble in the Durham tube implies a positive nitrate reduction test.

4.3.4. Screening of endophytes for ability to solubilise phosphorous

Sixty seven isolates (ninety two percent) tested positive for phosphate solubilisation while the other six (eight percent) tested negative (Appendix 8 and 16; Table 4.1). The presence of a halo around the bacterial colonies after fourteen days of incubation of plates at 30°C implied a positive test for phosphate solubilisation (Appendix 8). Forty two of the phosphate solubilizing rice root bacterial isolates were gram negative, while twenty five were gram positive. Fifty isolates from basmati 370 were positive for phosphate solubilisation; while those from basmati 217, only fifteen were positive for phosphate solubilisation. Fifty eight percent (42/73) of the total isolates were positive for both phosphate solubilisation and the urease test. The ten isolates observed to produce IAA were also positive for phosphate solubilisation (Table 4.1, Appendix 16).

4.3.5. Assessment of indoleacetic acid production by root endophytic Isolates

The results showed that fourteen percent (10/73) of the isolates were able to produce IAA while the other eighty six percent (63/73) did not (Table 4.1, Appendix 16). Addition of few drops of Salkowski's reagent to the cell free broth gave a pink color for a positive test, (Appendix 9, b) after thirty minutes incubation at room temperature. No color change was observed for negative test (Appendix 9, a). Nine of the isolates (M5, M16, M17, M18, M27, M31, M32, M42 and M51) identified to produce IAA were from Mwea and one (K7) from Ahero. Out of the ten Auxin producing endophytic isolates, eighty percent (8/10) were from basmati 370 while twenty percent (2/10) were from

basmati 217. The ten Auxin producers were gram negative and positive for phosphate solubilisation. Among these ten isolates, twenty percent (2/10) were noted to be positive for urease test while eighty percent (8/10) tested negative (Table 4.1, Appendix 16).

4.3.6. Assessment of Catalase activity in endophytic isolates (Catalase test)

This study showed that all the seventy three isolates were Catalase positive as implied by the formation of bubbles upon addition of hydrogen peroxide to the cultures (Table 4.1, Appendix 16). Catalase decomposes hydrogen peroxide to water and oxygen. A positive reaction was indicated by the formation of bubbles on addition of hydrogen peroxide to the cultures (Appendix 3, a).

4.3.7. Assessment for utilisation of citrate by the endophytes

Seventy three isolates were shown to test positive for citrate utilization test (Table 4.1, Appendix 16). Bromothymol blue indicator incorporated in the media turns from green to Prussian blue indicating positive test for citrate utilization (Appendix 10, b). There is no color change for a negative test. The positive endophytic isolates for citrate utilization were both gram negative and gram positive (Table 4.1, Appendix 16).

4.3.8. Assessment for the production of Hydrogen Sulphide by the bacterial endophytes

None of the isolates were positive for hydrogen sulphide production test (Appendix 12 and 16: Table 4.1). Hydrogen sulfide production is implied by blackening of the media.

4.3.9. Methyl Red-Voges-Proskauer test (MR-VP)

Eighteen isolates tested positive while fifty five were negative for methyl red test (Table 4.1, Appendix 16). Further, twenty five isolates were positive, while forty eight tested negative for the Voges-Proskauer test (Table 4.1, Appendix 16). Positive test was indicated by formation of red color upon addition of the methyl red indicator for methyl red test while absence of red coloration is an indication of a negative test (Appendix 13). Positive test was indicated by formation of pink color upon addition of the Barrit's reagent for Voges-Proskauer test while absence of red coloration was an indication of a negative test (Appendix 14). The methyl red positive isolates (eighteen) were gram negative bacteria, of which, fourteen were from basmati 370, and four from basmati 217. Twenty four of the Voges-Proskauer positive isolates were gram negative while one was gram positive: and eighteen of these were from basmati 370 while the other seven were from basmati 217.

4.4. Molecular characterization of endophytic isolates

All the seventy three isolates were grouped into four groups based on similarity of their morphological and biochemical characteristics. Thirty isolates from Mwea and seven from Ahero were then selected among these groups. Selection was done with bias to the objectives of this study where only bacterial endophytic isolates with potential to enhance plant growth were selected. Attributes that were considered most important included Acetylene reduction assay (nitrogen fixation), production of IAA, phosphate solubilisation, and activity of enzymes Catalase, urease and nitrate reductase. The other biochemical tests were also considered to identify any differences among the isolates.

Genomic DNA for the selected thirty seven isolates was isolated and amplified using 27F and 1492R primers. These primers targeted the 16S rDNA region of the genomic DNA used. The expected band of 1500 bp was amplified for all the thirty seven isolates and is representatively presented in Figure 4.1- 4.3.
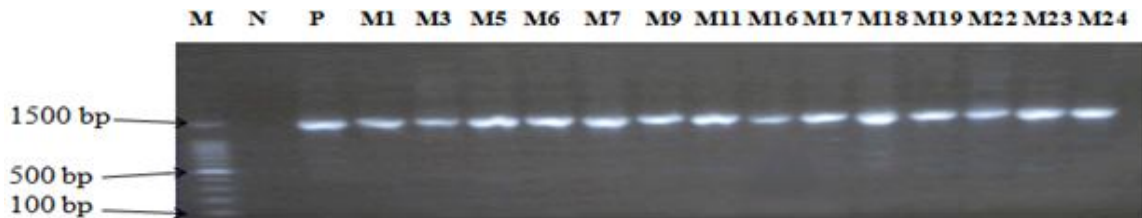
Figure 4.1: Amplified PCR products of 14 isolates from Mwea run in 0.8% (W/V) agarose gel. M- 1500bp marker, P- positive control, N- negative control, M1, M3, M5, M6, M7, M9, M11, M16, M17, M18, M19, M22, M23, and M24 are the isolates' sample PCR products
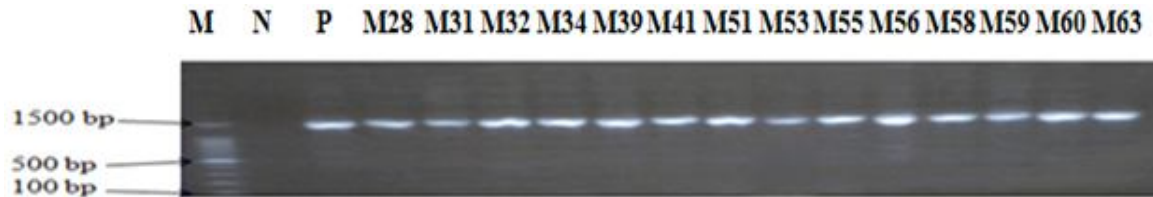
Figure 4.2: Amplified PCR products of fourteen isolates from Mwea run in 0.8 % (W/V) agarose gel. M- 1500 bp marker, P- positive control, N- negative control, M28, M31, M32, M34, M39, M41, M51, M53, M55, M56, M58, M59, M60, and M63 are the isolates' amplicons
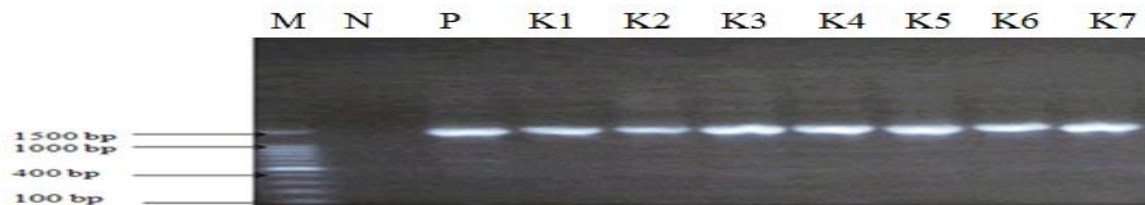
Figure 4.3: Amplified PCR products of seven isolates from Ahero run in 0.8% (W/V) agarose gel. M- 1500 bp Marker, P- Positive control and N- Negative control, KI, K2, K3, K4, K5, K6, and K7- isolates' sample PCR products

4.4.1. Restriction analysis of the PCR products

The PCR products for the thirty seven endophytic bacterial isolates were restricted using RsaI restriction enzyme. This was performed to check on any preliminary genetic diversity among the isolates so that repetition in sequencing the same isolates is minimized as much as possible.

The restricted PCR products gave different sizes of bands. The results for this section of the characterization process of the thirty seven rice root bacterial endophytes are representatively presented in Figure 4.4 and Table 4.2. Isolates M3 and M39 gave a similar banding profile of fragments in the ranges 300-400 bp, 400-500 bp, and 600-700 bp (Figure 4.4, Table 4.2). Similarity on banding was also observed for isolates M53 and M9, which gave an identical banding profile of the ranges 300-400 bp and 900-1000 bp (Figure 4.4, Table 4.2). Some of the isolates were observed to give banding profiles that were distinctively unique from the other isolates. For instance, isolate M17 bands were in the ranges 300-400 bp,500-600 bp, and 600-700 bp, while that for M56 were 200-300 bp,300-400 bp, and 900-1000 bp (Figure 4.4, Table 4.2).
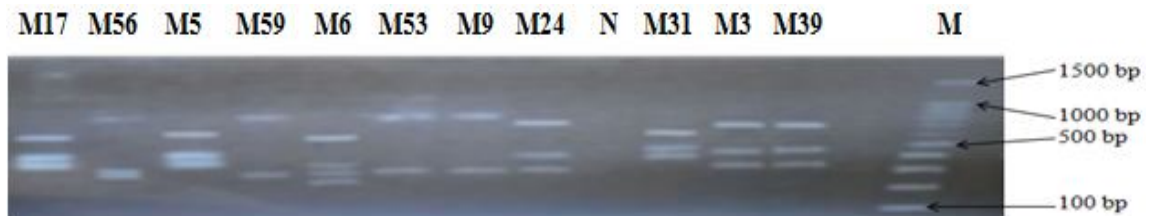
Figure 4.4: Restriction products as generated by RsaI digestion run in 1.5% (W/V) agarose gel. M- 1500 bp marker, N- negative control, M17, M56, M5, M59, M6, M53, M9, M24, M31, M3, and M39 are restricted PCR products of the respective isolates

The summary of the different banding profile for the bacterial isolates is given in Table 4.2:

Table 4.2: Restriction fragments of different isolates as generated by RsaI digestion; Key: (+) – Presence of fragment; (-) – Absence of fragment; M1, M3, M5, M6, M9, M17, M22, M24, M31, M32, M39, M41, M51, M53, M56, M59, M60, M67, K2 and K6 are endophytic bacterial isolates' restricted PCR products

Fragment size (bp)	M1	M3	M5	M6	M9	M17	M22	M24	M31	M32	M39	M41	M51	M53	M56	M59	M60	M67	K2	K6
100-200	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
200-300	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-
300-400	-	+	+	+	+	+	-	+	-	-	+	-	-	+	+	+	-	+	-	+
400-500	-	+	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-
500-600	+	-	+	+	-	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+
600-700	-	+	+	+	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	+
700-800	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
800-900	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-
900-1000	+	-	-	-	+	-	+	+	-	+	-	-	+	+	+	+	-	-	+	-

4.4.2. Sequencing of the PCR products

A selection criterion was used to obtain twenty eight bacterial isolates for sequencing, which was done by a commercial service provider (ILRI, Nairobi, Kenya). Fragment sizes generated by the different isolates upon restriction analysis were compared, and similar isolates grouped together. The morphological and biochemical characteristics of the different isolates were also considered to identify any notable differences among the

isolates. For instance, restriction of the PCR products for isolate M31 using RsaI gave fragment sizes of the ranges 400-500 bp, 500-600 bp, and 600-700 bp (Figure 4.4, Table 4.2). M31 is a gram negative endophytic bacterium which is urease negative but positive for Voges-Proskauer test and production of auxins. Restriction of the PCR products for isolate M3 gave fragment sizes of the ranges 300-400 bp, 400-500 bp, and 600-700 bp (Figure 4.4, Table 4.2). Further, isolate M3 is a gram positive bacterium, which was found to be positive for the urease test but negative for Voges-Proskauer test and production of auxins. It is clear that these two bacteria are different based on their morphological and biochemical characteristics and their restriction analysis profile. Such criterion was used to come up with twenty eight rice root endophytic bacteria for sequencing in this research. Sequence chromatographs for the twenty eight bacterial isolates were obtained from ILRI and used for further characterization process.

4.4.3. Phylogenetic analysis of the sequences for the PCR products

The gene sequences obtained were analyzed using Chromas Pro and MEGA4 programs to generate phylogenetic trees which identified the isolates to belong to various genera.

The analysis of the 16S rDNA sequences suggested that isolates M5, K1, K6, M67, M16, M17, M18, M31, M51, and M60 are phylogenetically related to *Enterobacter* with 95% sequence similarity (Figure 4.5). K1 and M5 were identified as *Enterobacter ludwigii* isolate PSB1/strain 2-1; M16 and M17 *as Enterobacter cloacae* isolate HQ040619-1/PYPB08; M67 *as Enterobacter* species strain MTQ8; M18, M31, M51, and M60 as Endophytic bacterium C03/HA04; and K6 as *Enterobacter* sp. CCBAU

15492 (Figure 4.5). These rice roots bacterial endophytic isolates were found to be gram negative, and positive for Catalase test, citrate utilization, phosphate solubilisation, Voges-Proskauer test and ARA; but negative for urease test, nitrate reduction, hydrogen sulfide production, and methyl red test. Indoleacetic acid production varied among the isolates with some such as M31 and M5 being positive and while others such as K1 and K6 were negative (Table 4.1, Appendix 16).

Phylogenetic positioning of some of the isolates showed that M63, M58, M53, M32, and K2 were related to *Pseudomonas fluorescens* strain Mc07/d3; while M9 and M59 were *Pseudomonas putida* strain AK3; and M1 and M56 were *Pseudomonas putida* strain MK12S6/LCR80/CM5002 at 97% sequence similarity (Figure 4.5). These rice roots bacterial endophytic isolates identified to be closely related to genus *Pseudomonads* were found to be gram negative and tested positive for Catalase test, urease test, citrate utilization, phosphate solubilisation assay, and the ARA, but negative for nitrate reduction, hydrogen sulfide production and Voges-Proskauer test. Indoleacetic acid production varied among the isolates with some such as M32 being positive and while others were negative (Table 4.1, Appendix 16).

Isolate K3 was phylogenetically identified as *Micrococcus luteus* strain BF1-6/ NSM12 at 94% sequence similarity (Figure 4.5). Moreover, this basmati rice root bacterial endophyte was shown to be positive for Catalase test, citrate utilization, phosphate solubilisation and acetylene reduction assay; but negative for urease test, nitrate

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reduction test, production of hydrogen sulfide and Indole acetic acid, and methyl red Voges-Proskauer test (Table 4.1, Appendix 16).

Phylogenetic analysis of isolates M3, M6, M22, M24, M39, M41, K4, K5, and K7 suggested that they were related to members of the genus *Bacillus* with 96% rDNA sequence analysis similarity (Figure 4.5). Isolate K7 was closely related to *Bacillus thuringiensis* strain S422B-21 while K6 and K8 were *Bacillus megaterium* strain SZ-3 (Figure 4.5). All isolates identified to belong to the genus *Bacillus* were observed to be gram positive. Further, their biochemical characteristics showed that they were positive for Catalase test, urease test, and citrate utilization; but negative for nitrate reduction, hydrogen sulfide production, Methyl-red test, and production of Indoleacetic acid (Table 4.1).
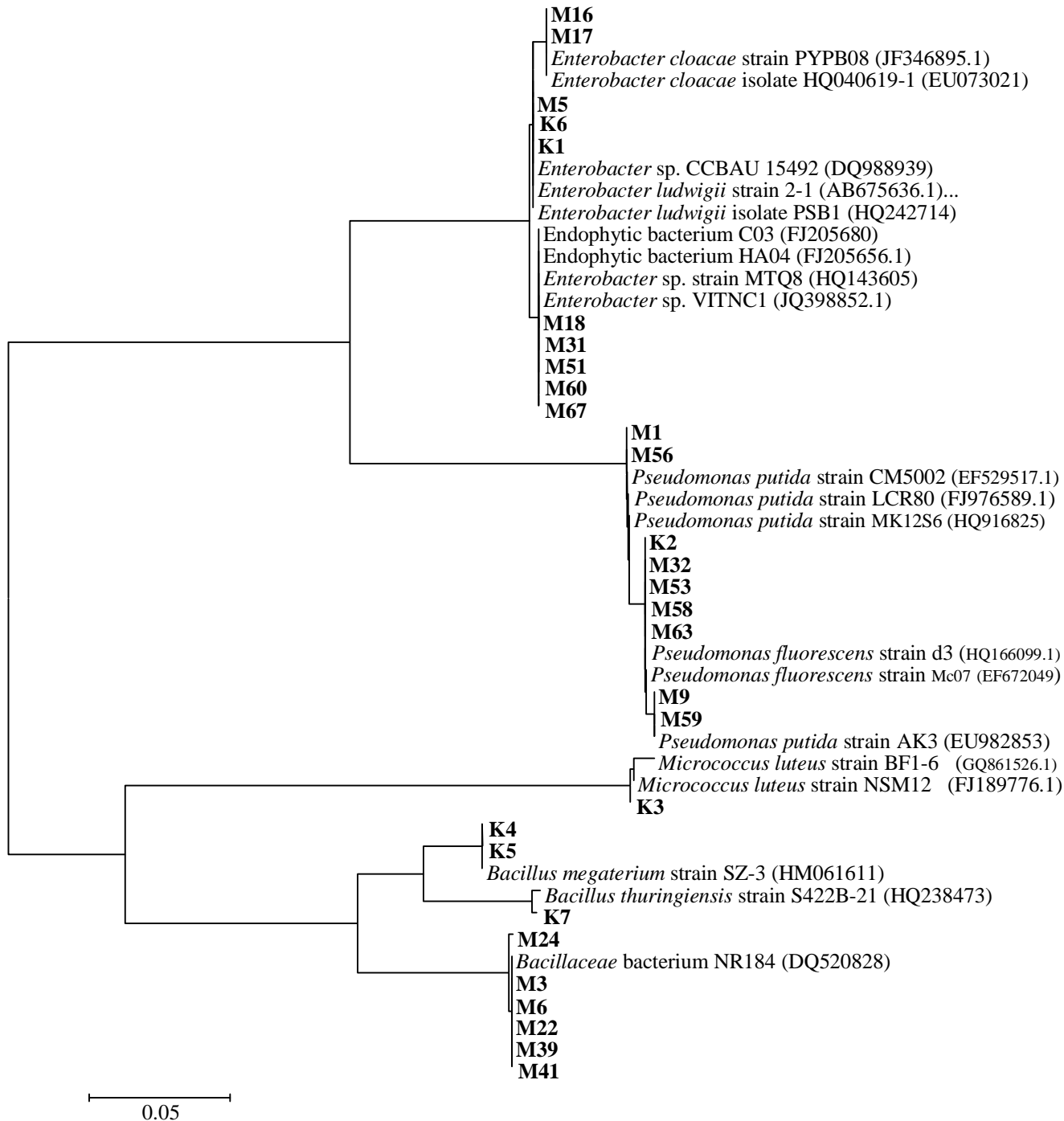
Figure 4.5: Neighbour joining phylogenetic tree showing the position of *Bacillus, Enterobacter, Pseudomonas,* and *Micrococcus* basmati rice root bacterial endophytes. The bar indicates the estimated substitution per nucleotide position

4.5.   Assessment of effect of selected isolates on rice plant growth promotion

Data on plant height (Appendix 15; Appendix 16; Figure 4.6; Figure 4.7) and dry weight of shoot and roots (Appendix 15; Appendix 16; Figure 4.8; Figure 4.9) of the rice seedlings on which drenching was done were collected after three and seven weeks and presented in tables and graphs. The data was collected from four replicates.

Bacterial counts of 1×10^5 and 1×10^{10} CFU used gave varying results. From the data obtained, it was clear that the concentration of 1×10^5 was the better as it gave the highest figures in terms of plant height and dry weight of shoot and roots. For instance, the mean plant height (cm) at concentration 1×10^5 at seven weeks for isolates M16, K7, M31, M5, M32, and the Cocktail were 25.05, 22.15, 42.025, 26.1, 34.275, and 27.55 respectively against the negative control which was 24.525 (Figure 4.6; Appendix 14). On the other hand, the mean plant height (cm) at concentration 1×10^{10} at seven weeks for the same isolates were 23.85, 23.5, 32.375, 22.6, 32.35, and 23.4 respectively against the negative control which was 21.375 (Figure 4.7; Appendix 15). Similar observations were made in the case of mean dry weight for shoots and roots where the mean dry weight (g) at concentration 1×10^5 at seven weeks for isolates M32, M5, M31, K7, M16 and the Cocktail were 1.49675, 0.31625, 1.567, 0.26875, 0.22175, and 0.35925 respectively, against the negative control which was 0.363 (Figure 4.8; Appendix 14). In the contrary, the mean dry weight (g) at concentration 1×10^{10} at seven weeks for isolates M32, M5, M31, K7, M16 and the Cocktail were 1.20875, 0.282, 1.301, 0.3215, 0.26875, and 0.24525 respectively against the negative control which was 0.24525 (Figure 4.9; Appendix 15).

The data obtained in this assessment was compared with the positive control. In this case, analysis with t- test at 95% significance level showed that data on plant height (Figure 4.10 and Figure 4.11) and dry weight for isolates M31 and M32 were not significantly different from the positive control. The P values for analysis of data on plant height for isolates M31 and M32 were 0.775 and 0.474 respectively at 1×10^{10} Colony forming units. However, data on plant height for isolates M5, M16, K7 and the Cocktail were significantly different from the positive control (P values 0.027, 0.047, 0.027 and 0.004 respectively at 1×10^{10} CFU). The P values obtained on analysis of data on dry weight of shoots and roots at 1×10^{10} CFU were upon comparison with the positive control for isolates M5, M16, M31, M32, K12 and the cocktail were 0.011, 0.009, 0.779, 0.490, 0.006 and 0.007 respectively. Similar observations were made at 1×10^{5} Colony forming units, where the P values for isolates M16, M32, K7 and the Cocktail on plant height were 0.018, 0.532, 0.008 and 0.211 respectively: Further, in terms of dry weight of shoots and roots at 1×10^{5} Colony forming units, the P values upon comparison with the positive control for isolates M5, M16, M31, M32, K12 and the cocktail were 0.005, 0.006, 0.479, 0.758, 0.016 and 0.019 respectively. It is clear from these results that the isolates' data which was significantly different with the negative control (M31 and M32) gives a converse outcome with the positive control (that is, not significantly different). This shows that the values obtained for the positive control and isolates M31 and M32 were within range. For instance, the mean plant heights (cm) for isolates M31 and M32 after seven weeks with a bacterial drenching

concentration of 1×10^{10} were 32.375 and 32.35 respectively, while that for the positive control was 33 (Figure 4.10).

In terms of tiller numbers, it was observed that on average, isolates M16 and K7 had three; M5 and the cocktail had four; M32 and M31 had seven; while the positive control had six. In this case, isolates M31 and M32 gave the highest number of tillers.

These data was further analyzed using t-test, and it was noted that only data for isolate M31 (Endophytic bacterium) and M32 (*Pseudomonas fluorescens*) were significantly different   against the negative control in terms of height (P values 0.035 and 0.042; 0.031 and 0.015 respectively) and dry weight of shoots and roots (0.011 and 0.021; 0.009 and 0.009 respectively) at 95% significance level. Data on plant height at three weeks and tillering was not significantly different against the negative control because the P values for the bacterial isolates were greater than 0.05.

The number of tillers observed for both counts of bacterial endophytes used was different among the different isolates. On average, number of tillers for isolates M16 and K7 was three; for M5 and the cocktail was four; M32 and M31 was seven; while the negative control was four. In this case, isolates M31 and M32 gave the highest number of tillers.

Figure 4.6: Mean plant height (cm) at seven weeks with a bacterial drenching concentration of 1×10^{5}; CTRL- negative control, CK- bacterial endophyte cocktail, M31, M5, K12, M16, M32- bacterial endophyte treatments
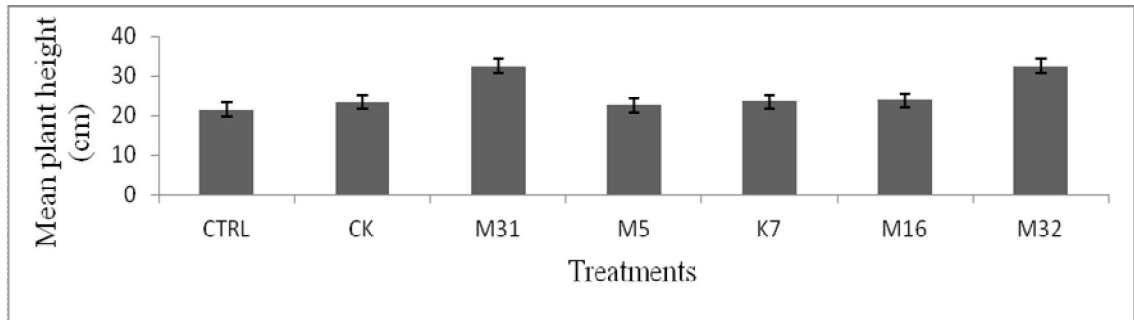
Figure 4.7: Mean plant height (cm) at seven weeks with a bacterial drenching concentration of 1×10^{10}; CTRL- negative control, CK- bacterial endophyte cocktail, M31, M5, K12, M16, M32- bacterial endophyte treatments
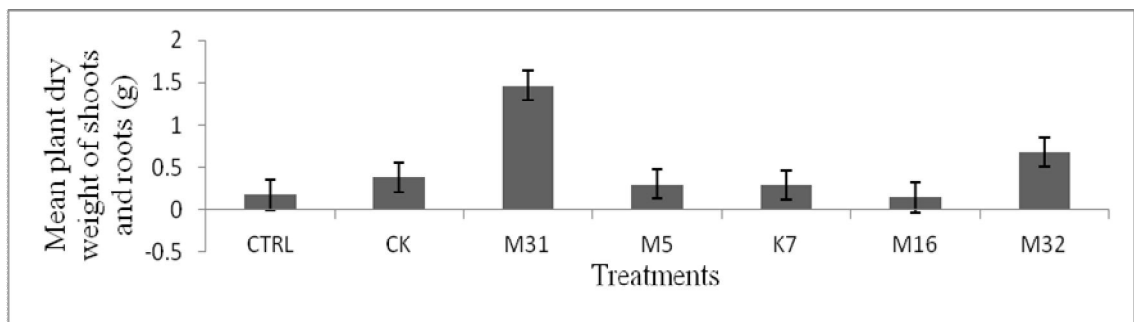
Figure 4.8: Mean plant dry weight of shoots and roots (g) at seven weeks after drenching with a bacterial count of 1×10^{5}; CTRL- negative control, CK- bacterial cocktail, M31, M5, K12, M16, M32- bacterial isolates' treatments
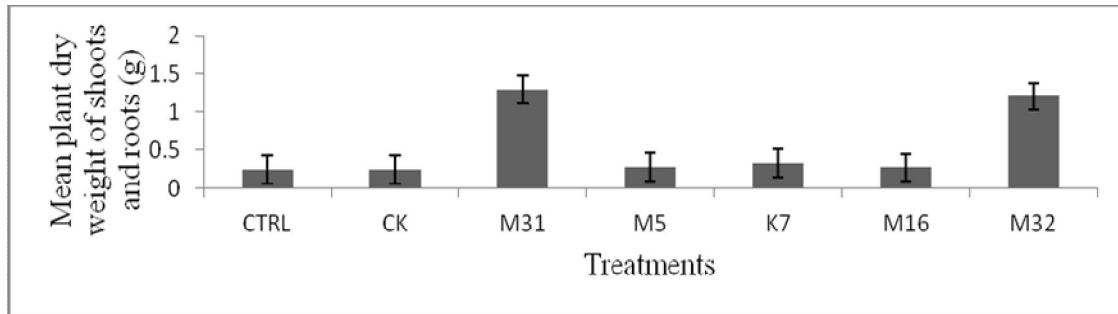
Figure 4.9: Mean plant dry weight of shoots and roots (g) at seven weeks after drenching with a bacterial count of 1×10^{10}; CTRL- negative control, CK- bacterial cocktail, M31, M5, K7, M16, M32- bacterial isolates' treatments
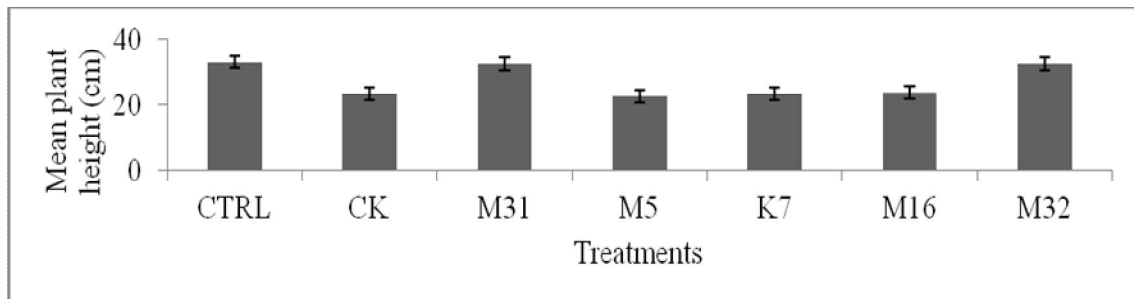
Figure 4.10: Mean plant height at seven weeks with a bacterial drenching concentration of 1×10^{10}; CTRL- positive control, CK- bacterial endophyte cocktail, M31, M5, K7, M16, M32- bacterial endophyte treatments
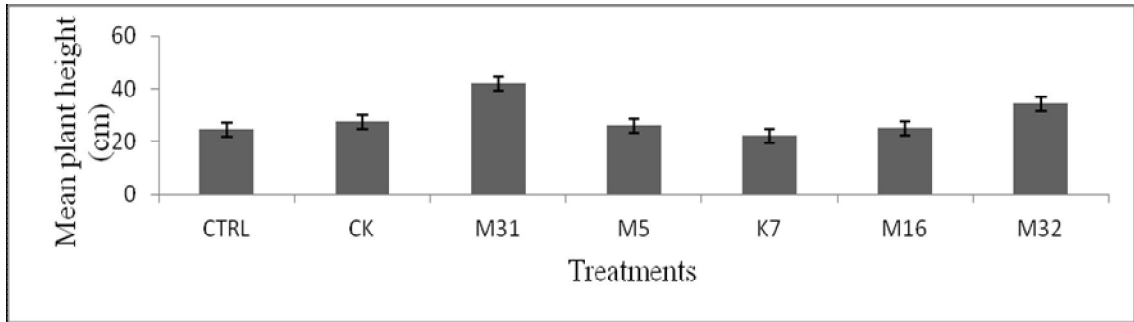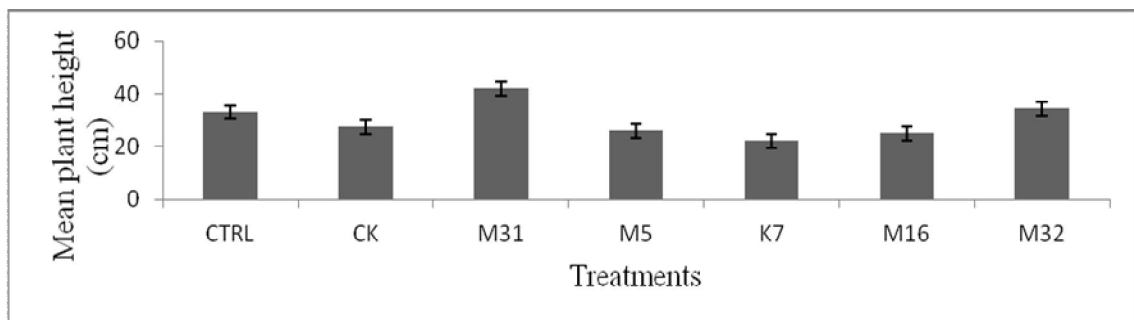

Figure 4.11: Mean plant height (cm) at seven weeks with a bacterial drenching concentration of 1×10^{5}; CTRL- positive control, CK- bacterial endophyte cocktail, M31, M5, K7, M16, M32- bacterial endophyte treatments

CHAPTER FIVE

5.0. DISCUSSION

5.1. Biochemical characterization of bacterial root endophytic isolates

The acetylene reduction assay was specifically done to establish whether the isolated rice root bacterial endophytes have potential to fix nitrogen. Organisms that are able to fix atmospheric nitrogen possess the enzyme nitrogenase, which reduces nitrogen to ammonia (Cappuccino and Sherman, 2002). In the late 1960's Stewart and others developed the acetylene reduction assay (ARA) which measures nitrogenase activity. Nitrogenase catalyses the reduction of not only nitrogen but also a variety of other substrates, like acetylene (Cappuccino and Sherman, 2002). The reduction of acetylene to ethylene is widely used as a method of measuring nitrogenase activity in natural samples, isolates, and cell-free extracts (Cappuccino and Sherman, 2002). The activity of Nitrogenase was examined for all the seventy three rice root bacterial endophytes. In this case, positive results were obtained as observed on the chromatographs. These ARA positive results obtained are consistent with other studies. For instance, the research on rice plant growth promoting bacteria by Keyeo *et al*., 2011 and Koomnok *et al*., 2007. Additionally, these isolates were able to grow on nitrogen free media. This clearly showed their potential to fix nitrogen into the soil. This is a crucial aspect for rice as nitrogen is a limiting factor in growth and production of rice.

The urease test was done to determine the ability of the isolates to break down urea, to simple forms of nitrogen which can be readily absorbed by the plants to promote

growth. The positive implication is an important aspect in growth and development of rice in the case where fertilizers are applied, as the bacteria have shown potential to convert urea to simpler forms of nitrogen which are readily absorbed by plants. For plants to absorb nitrogen from urea it must first be broken down. Urease catalyzes the hydrolysis of urea to unstable carbamic acid. Rapid decomposition of carbamic acid occurs without enzyme catalysis to form ammonia and carbon dioxide (Tisdale *et al*., 1985). The ammonia will likely escape to the atmosphere unless it reacts with water to form ammonium (NH_4^+). This is important because ammonium is a plant available source of nitrogen while ammonia is not. The urease test performed in the process of this characterization of rice root bacterial endophytes showed that forty seven isolates have potential to break down urea to simpler forms that can be readily available to the host plant. This is because the respective forty seven isolates tested positive for the urease test. This phenomenon of positive urease activity has also been observed in other studies on rice endophytes such as Tan *et al*., 2001. This positive implication on urease activity was an added advantage to these forty seven bacterial isolates compared to the twenty six that were negative for the urease test but positive for ARA.

The denitrification test was also performed to determine the ability of the isolates to reduce nitrates to nitrogen gas. All the bacterial rice root bacterial isolates tested negative for denitrification test. This is a critical feature of these rice root endophytes, as it will give time for the plants to absorb readily available nitrogen before it can be converted to free nitrogen gas by other denitrifying bacteria that could be present in the

host plant or in the soil/rhizosphere. Denitrification is a microbial facilitated process of nitrate reduction that may ultimately produce molecular nitrogen (N_2) through a series of intermediate gaseous nitrogen oxide products. This is an important factor to help maintain the nitrogen cycle in the three phases namely the atmosphere, water, and soil. This respiratory process reduces oxidized forms of nitrogen in response to the oxidation of an electron donor such as organic matter. The preferred nitrogen electron acceptors include nitrate (NO_3^-) nitrite (NO_2^-) nitric oxide (NO) and nitrous oxide (N_2O) and dinitrogen (N2). The process is performed primarily by heterotrophic bacteria such as *Paracoccus denitrificans* and various *Pseudomonads* (Carlson and Ingraham, 1983), although autotrophic denitrifiers like *Thiobacillus denitrificans* (Baalsrud and Baalsrud, 1954) have also been identified. The rice root endophytic *Pseudomonads* identified in this undertaking were not able to reduce nitrate to nitrogen gas.

Phosphorus is an essential plant nutrient with low availability in many agricultural soils (Wakelin *et al.,* 2004). Today many agricultural soils have a high total Phosphorous content due to the application of Phosphorous fertilizers over long periods of time. On the other hand, much of this Phosphorous is in mineral forms and is only slowly available to plants (Rodriguez *et al.,* 2006; Richardson *et al.,* 2009). The release of inorganic phosphate from organic phosphates is called mineralization and is caused by microorganisms breaking down organic compounds. Fixed phosphate contains inorganic phosphate compounds that are insoluble and organic compounds that are resistant to mineralization by microorganisms in the soil. Phosphate in this pool may

remain in soils for years without being made available to plants and may have very little impact on the fertility of a soil. The inorganic phosphate compounds are more crystalline in their structure and less soluble. Most of the insoluble Phosphorous forms are present as aluminum and iron phosphates in acid soils (Mullen, 2005), and calcium phosphates in alkaline soils (Goldstein and Krishnaraj, 2007). Endophytes are known to promote plant growth by phosphate solubilization (Wakelin *et al.,* 2004). This is supported by other studies which demonstrated that soil inoculation with phosphate-solubilizing *Bacillus spp.* can solubilize fixed soil Phosphorous and applied phosphates, resulting in a better plant development and higher yields (Canbolat *et al*., 2006). The *Bacillus, Enterobacter, Micrococcus and Pseudomonas* genera identified in this study were also shown to have potential to solubilize phosphorous. Previous studies have shown that in *Bacillus*, the main compounds involved in the phosphate solubilization are the lactic, itaconic, isovaleric, isobutyric and acetic acids (Vazquez *et al.,* 2000). The ability of bacteria to solubilize insoluble Phosphorous minerals has been attributed to their capacity to reduce pH by the excretion of organic acids (for example gluconate, citrate, lactate and succinate) and protons during the assimilation of ammonia (Gyaneshwar *et al.,* 1999; Mullen, 2005). Phosphate solubilizing microorganisms grow in media with tricalcium phosphate or similar insoluble materials as the only phosphate source. In this case, these microorganisms not only assimilate the element but also solubilize quantities in excess of their nutritional demands, thereby making it available for plants (Chen *et al.,* 2006). Potential for this phenomenon was demonstrated in the

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results for phosphate solubilisation obtained in this study. A hallo around the bacterial colonies of sixty seven isolates was observed in this study.

Endophytes have also been shown to promote plant growth by producing the phytohormone (IAA) (Mendes *et al.,* 2007). IAA increases root size and distribution, resulting in greater nutrient absorption from the soil (Li *et al.,* 2008). In this study, the isolates were screened for auxin production. The results showed that ten of the isolates (M5, M16, M17, M18, M27, M31, M32, M42, M51 and K7) were able to produce IAA and therefore have the potential to promote plant growth. Among these auxin producers, M31 and M32 were shown to promote plant growth at green house level. It was further noted that not all phosphate solubilizing endophytic bacterial isolates were auxin producers. This information indicates that plant growth promotion in the environment is not driven by a single species but may be due to a composite effect of features present in several symbiotic bacteria.

It was observed that all the isolates were Catalase positive. This is an important aspect required by the bacteria to reproduce avoiding cellular toxicity. Some bacteria contain flavoproteins that reduce oxygen resulting in production of hydrogen peroxide and superoxide, which are extremely toxic to the cell as they are powerful oxidizing agents and can destroy cellular components very rapidly (Cappuccino and Sherman, 2002). Since the bacterial endophytes isolated were Catalase positive, it means they possess the capability to protect themselves from this toxic effect.

5.2. Molecular characterization of bacterial endophytes

Taxonomic classification of the isolates using their 16S ribosomal DNA sequences showed that the isolates belong to the genera *Bacillus, Enterobacter, Pseudomonas*, and *Micrococcus*. The morphological and biochemical characteristics obtained also support these genus assignments.

The isolates M5, K1, K6, M67, M16, M17, M18, M31, M51, and M60 were found to be phylogenetically related to *Enterobacter* with 95% sequence similarity. The morphological and biochemical characteristics obtained for these rice root bacterial isolates indicated that they are highly closely related to this (*Enterobacter*) genus. Members of the *Enterobacter* are known to be Gram-negative rods that are distributed worldwide and may be found in soil, water, plants and animals. As reviewed, most of *Enterobacter* reduce nitrate to nitrite, although exceptions exist (for example *Photorhabdus*), and have varying Catalase reactions. Many members of this family are a normal part of the gut flora found in the intestines of humans and other animals, while others are found in water or soil, or are parasites on a variety of different animals and plants. Most species grow well at 37°C, although some species grow better at 25 - 30°C and are Catalase-positive except *Shigella dysenteriae* type 1. Isolates M5, K1, K6, M67, M16, M17, M18, M31, M5, and M60 were able to grow at 30°C.

Nitrogen-fixing *Enterobacter cloacae* have been isolated from the roots of dryland and wetland rices (Ladha *et al*., 1983). *Enterobacter ludwigii* and *Enterobacter cloacae* have not only been shown to fix nitrogen (ARA positive), but also have phosphate

solubilizing properties (Mauricio *et al.*, 2009; Manoharan, *et al.*, 2011) rendering them potential plant growth promoters. Isolates K1 and M5 identified as *Enterobacter ludwigii* isolate PSB1/strain 2-1; and M16 and M17 as *Enterobacter cloacae* isolate HQ040619-1 were all shown to have a similar plant growth promotion potential as they assayed positive for nitrogen fixation and phosphate solubilisation.

Among plant growth promoting species, *Azospirillum* is one of the best studied IAA producers (Dobbelaere *et al.,* 1999). Other IAA producing bacteria include *Aeromonas* (Hal da-Alija, 2003), *Azotobacter* (Ahmad *et al.,* 2008), *Bacillus* (Swain *et al.,* 2007), *Burkholderia* (Hal da-Alija, 2003), *Enterobacter* (Shoebitz *et al.,* 2009), *Pseudomonas* (Hariprasad and Niranjana, 2009) and *Rhizobium* (Ghosh *et al.,* 2008) genera. The auxin producers identified during this research were members of the genera *Enterobacter* and *Pseudomonas*: these included isolates M5, M16, M17, M18, M27, M31, M32, M42, M51, and K2. Inoculation with IAA producing PGPR has been used to stimulate seed germination, to accelerate root growth and modify the architecture of the root system, and to increase the root biomass. In addition to stimulating root growth, IAA producing bacteria can also be used to stimulate tuber growth (Swain *et al.,* 2007). This study was able to demonstrate enhancement of plant growth by auxin producing basmati rice root endophytic bacterial isolates M31 and M32, identified as endophytic bacterium CO3 and *Pseudomonas fluorescens* strain Mc07/d3 respectively.

Phylogenetic positioning of other isolates showed that they are related to *Pseudomonas fluorescens* (M63, M58, M53, M32, and K2) and *Pseudomonas putida* (M9, M59, M1,

and M56). The morphological and biochemical characteristics of these isolates indicated that they are closely related to this genus. *Pseudomonades* are described as aerobic, rod shaped, Gram-negative bacteria with one or more flagella providing motility. The members of the genus demonstrate a great deal of metabolic diversity, and consequently are able to colonise a wide range of niches (Madigan and Martinko, 2005). The best studied species include *Pseudomonas aeruginosa* in its role as an opportunistic human pathogen, the plant pathogen *Pseudomonas syringae*, the soil bacterium *Pseudomonas putida*, and the plant growth promoting *Pseudomonas fluorescens*. This study identified isolates M1, M9, M56, and M59 as *Pseudomonas putida* and M32, M53, M58, M63, and K2 as *Pseudomonas fluorescens*. These findings are in agreement with literature (Madigan and Martinko, 2005).

Pseudomonas putida tests positive for citrate utilization, and negative for methyl red, and Voges- Proskauer tests. This is supported by results of isolates M9, M59, M1, and M56 identified as *Pseudomonas putida* in this study. The 16S rDNA sequence analysis suggested that isolates M9, M59, M1, and M56, are phylogenetically related to *Pseudomonas putida* strain AK3/MK12S6/LCR80/CM5002 with 97% sequence similarity. *Pseudomonas putida* is a rod-shaped, flagellated, gram-negative bacterium that is found in most soil and water habitats where there is oxygen. It grows optimally at 25-30° C and can be easily isolated. Some of its strains such as *Pseudomonas putida* strain PS9 have been shown to produce IAA and cause phosphorous solubilisation. The isolates clustered as *Pseudomonas putida* in this finding (M9, M59, M1, and M56) were

not IAA producers, but were shown to solubilize phosphorous. *Pseudomonas putida* has several strains including the KT2440, a strain that colonizes the plant roots in which there is a mutual relationship between the plant and bacteria. The plant roots allow the bacteria to thrive from the root nutrients. In turn, the *Pseudomonas putida* induces plant growth and protects the plants from pathogens. *Pseudomonas putida* and *Pseudomonas fluorescens* have been isolated from rice and banana roots in Sri Lanka (Vlassak *et al*., 1995).

The 16S rDNA sequence analysis suggested that isolates M63, M58, M53, M32, and K2, are phylogenetically related to *Pseudomonas fluorescens* strain Mc07/d3 with 97% sequence similarity. *Pseudomonas fluorescens* is a Gram-negative rod shaped bacteria that inhabit soil, plants and water surfaces (Anzai *et al*., 2000). The optimum growth temperature is between 25-30° C (Haas and Keel, 2003). The Pf-5 strain resides in the plant's rhizosphere and produces a variety of secondary metabolites including antibiotics against soil borne plant pathogens (Jay, 2000). A number of strains of *Pseudomonas fluorescens* suppress plant diseases by protecting the seeds and roots from fungal infection (O' Sullivan and O'Gara, 1992). This effect is the result of production of a number of secondary metabolites including antibiotics, siderophores and hydrogen cyanide. In earlier studies, *Pseudomonas fluorescenes* has been shown to have the capacity to produce indole acetic acid (Dey *et al*., 2004) and improve plant growth. The ability of *Pseudomonas fluorescens* and other *Pseudomonas* sp. to fix nitrogen has also been reported (Gowda and Watanabe, 1985; Chan *et al*., 1994). This

characteristic and production of IAA was observed in isolates M32, M58, and K2 that were identified as *Pseudomonas fluorescens* strain Mc07/d3 in this study.

One of the isolates (K3) was phylogenetically related to *Micrococcus (Micrococcus luteus)* with 94% sequence similarity. The morphological and biochemical characteristics for this rice root endophytic bacterial isolate indicated that it is highly closely related to this genus. *Micrococcus* is a genus of bacteria in the Micrococcaceae family. *Micrococcus* occurs in a wide range of environments, including water, dust, and soil. *Micrococci* have Gram-positive spherical coccoidal cells ranging from about 0.5 to 3 micrometers in diameter and typically appear in tetrads. *Micrococcus luteus* is pigmented as it produces yellow colonies. This was observed for isolate K3. Defining characteristics of *Micrococcus* are the ability to aerobically produce acid from glucose, glycerol, and aesculin hydrolysis, major pigment production, motility, and conversion of nitrate to nitrite (Smith *et al*., 1999). *Micrococcus luteus* can be found in many places in the environment, like water, dust, and soil. It can grow well in environments with little water or high salt concentrations. They grow optimally at 37°C and can be easily grown on inorganic nitrogen agar or Simmon's citrate agar (Smith *et al*., 1999). *Micrococcus luteus* has been shown to be positive for plant growth promoting traits, including phosphate solubilisation and positive urease test, indicating their role in plant growth promotion (Vendan, *et al*., 2010). Isolate K3 had plant growth promoting characteristics as it was positive for ARA, urease test, and phosphate solubilisation.

Phylogenetic analysis of isolates M3, M6, M22, M24, M39, M41, K4, K5, and K7 suggested that they were related to members of the genus *Bacillus* with 96% 16SrDNA sequence similarity. Morphological and biochemical characteristics of these rice roots bacterial isolates suggested their close relatedness with members of genus *Bacillus*. Isolate K7 was closely related to *Bacillus thuringiensis*, while K4 and K5 were more related to *Bacillus megaterium.*

Literature shows that *Bacillus* is a genus of Gram-positive rod-shaped bacteria. Characteristically, primary *Bacillus* cultures are Gram-positive, but may become Gram-negative at a secondary stage. *Bacillus* species can be obligate aerobes or facultative anaerobes, and test positive for the enzyme Catalase (Turnbull, 1996). Further, *Bacillus* test positive for methyl red test, and are negative for hydrogen sulfide production, and the Voges Proskauer tests. The results obtained during the biochemical characterization process for the basmati rice root bacterial endophytic isolates obtained were consistent with the above reviewed characteristics. Being ubiquitous in nature, *Bacillus* includes both free-living and pathogenic species being found in dust, soil, water, air and vegetable matter (Kamal *et al*., 2008). All nitrogen-fixing *Bacillus* strains (such as *Bacillus polymyxa, Bacillus macerans, Bacillus azotofixans*) are now assigned to *Paenibasillus* but there are other many spore forming that might fix nitrogen (Emtiazi *et al*., 2008). All nine rice root endophytes (isolates M3, M6, M22, M24, M39, K4, K5, and K7) identified as *Bacillus* during this investigation were shown to have potential to fix nitrogen as they tested positive for ARA. *Bacillus* species used as biofertilizers probably have direct effects on plant growth through the synthesis of plant growth

hormones (Amer and Utkhede, 2000), Nitrogen fixation (Cakmakci *et al*., 2001) and solubilisation of phosphate (Sahin *et al*., 2004). Phosphate-solubilizing *Bacillus* spp. stimulates plant growth through enhanced Phosphate nutrition (Whitelaw *et al*., 1997) increasing the uptake of Nitrogen, Phosphorous, potassium, and iron (Biswas *et al*., 2000). It was demonstrated in this undertaking that the nine isolates belonging to genus *Bacillus* were able to solubilize phosphorous, which with the composite effect of nitrogen fixation pose potential to promote plant growth.

Bacillus thuringiensis is a Gram-positive, soil-dwelling bacterium, commonly used as a biological pesticide. Some strains of *Bacillus thuringiensis* have been shown to solubilize inorganic phosphate (Seshadri *et al*., 2007). Isolate K7 identified as *Bacillus thuringiensis* strain S422B-21 was observed to have potential to enhance plant growth as it gave positive outcome for the phosphate solubilisation assay. *Bacillus thuringiensis* occurs naturally in the gut of caterpillars of various types of moths and butterflies, as well as on the dark surfaces of plants (Madigan and Martinko, 2005).

Bacillus megaterium is a rod-shaped, Gram-positive, endospore forming, species of bacteria used as a soil inoculant in agriculture and horticulture. It weathers rock phosphate and tricalcium phosphate by decreasing the particle size reducing it to nearly amorphous forms. It is one of the largest eubacteria found in soil and is able to survive in some extreme conditions such as desert environments due to the spores it forms. Isolates K4 and K5 were identified as *Bacillus megaterium* strain SZ-3 and were able to

solubilize phosphorous. These two isolates also tested positive for the urease test and ARA which implies their plant growth promoting characteristics.

Preliminary genetic diversity was observed among the basmati rice root endophytic isolates upon restriction analysis of their PCR products. For instance, isolate M17 bands were in the ranges 300-400,500-600, and 600-700, while that for M56 were 200-300,300-400, and 900-1000. This clearly shows that these two isolates are diverse from each other.

5.3. Assessment of effect of selected isolates on rice plant growth

A preliminary study to assess for growth promotion of the endophytes on rice seedlings was also done. The results showed that isolate M31 (Endophytic bacterium) and M32 (*Pseudomonas fluorescens)* had significant effects compared with the controls in terms of height (P values 0.035, 0.042 respectively) and dry weight (P values 0.011 and 0.021 respectively) of shoots and roots at 95% significance level. This implies that the two isolates do promote plant growth in Kenyan basmati rice at the green house level.

The most studied plant growth promoting rhizobacteria (PGPR) belong to gram-negative genera, and the greatest number of strains are members of the *fluorescent pseudomonads* (Kloepper, 1993). Many reports also suggest that gram-positive bacteria, such as *Bacillus*, are PGPR (Beauchamp, 1993; Kloepper, 1993). In this study, majority (47) of the isolates were gram negative rods, though some (25) were gram positive rods and one gram positive cocci. Isolates M31 and M32 isolated and identified in this study were gram negative rods and were demonstrated to have potential to enhance plant

growth. Root colonization is an important first step in the interaction of beneficial bacteria with plants (Kloepper and Beauchamp, 1992). They should also be able to colonize and survive in the rhizosphere of these plants. It is important to give the endophytes time after the bacterial drench to allow them to gain entry into the plant (roots) before any mutual relationship can be established (Kloepper and Beauchamp, 1992).

There are various studies that have been conducted on Plant Growth-Promoting Rhizobacteria (PGPR), since it can be used as a biofertilizer to promote sustainable agricultural practices. As PGPR colonize the plant roots, they are able to promote plant growth based on the ability to solubilize inorganic phosphorous, fix nitrogen and to excrete plant growth regulator such as IAA (Martinez-Viveros *et al*., 2010; Park *et al*., 2005; Ryu *et al*., 2005).

Pseudomonas fluorescens has been earlier isolated from plant leaves and roots, and has been shown to contribute to plant growth (Palleroni, 1984). Rice plant growth promotion was observed for isolate M32 which was identified as *Pseudomonas fluorescens*.

Enterobacter genus such as *Enterobacter cloacae* are promising symbiotic bioinoculants for rice and have been shown to have effective root colonizing ability and growth promoting potential (Shankar *et al*., 2011). Rice plant growth promotion by *Enterobacter* has also been demonstrated by Keyeo *et al*., 2011. Isolate M31 was

identified as an *Enterobacter*, and was demonstrated to promote rice plant growth at the green house level.

A plant is better able to achieve its optimized physical growth when it receives enough nutrients such as fixed nitrogen and this can be influenced by the presence of diazotrophic bacteria in association with the host plants. These biological processes can help reduce overreliance on chemical fertilizer (Ai'shah *et al.*, 2010; Nguyen *et al.*, 2003).

5.4. Conclusions and Recommendations

5.4.1. Conclusions

i. The isolation of bacterial root endophytes from root samples implied that Kenyan basmati rice roots harbor plant growth promoting bacterial endophytes and that these bacteria are genetically diverse. Basmati 370 harbors more endophytes than basmati 217

ii. Plant growth promotion characteristics including nitrogen fixation, phosphorous solubilisation and production of auxins were observed in the rice root bacterial endophytes

iii. The phylogenetic analysis of the isolates clustered them into four different genera namely *Pseudomonas*, *Bacillus, Enterobacter, and Micrococcus.* This is in agreement with their morphological and biochemical characteristics

iv. Isolates M31 and M32 isolated from Mwea basmati 370 rice demonstrated plant growth promotion at the green house level.

5.4.2. Recommendations

The findings of this study recommend that:

i. Isolate M31 and M32 may be used to enhance plant growth of Kenyan basmati rice at green house level since their potential has been demonstrated in this study

ii. Further work should be done on isolates M31 and M32 focusing on field trials, determination of optimal concentration at which plant growth promotion is observed and finally with view of developing a biofertilizer

iii. Molecular characterization can be performed for the isolates that were not subjected to the same process.

iv. Further biochemical characterization on the isolates to establish any importance other than agriculture.

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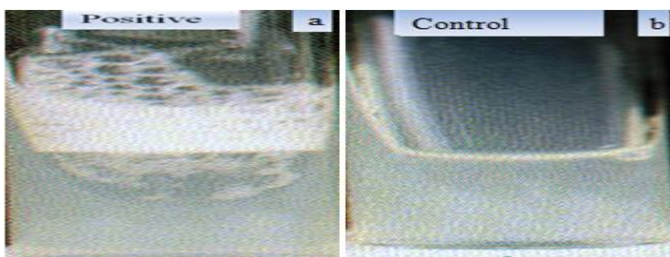
APPENDICES
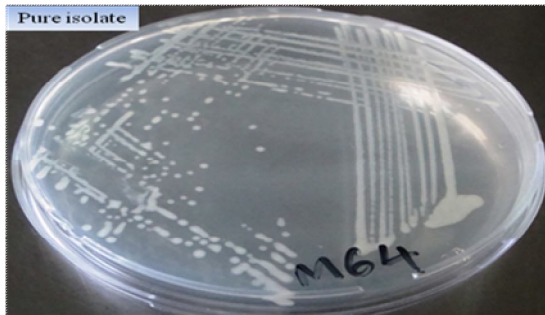
Appendix 1: Pure isolate; Isolate M64 which is a representation of the pure isolates obtained upon streaking of the primary isolates on nutrient agar
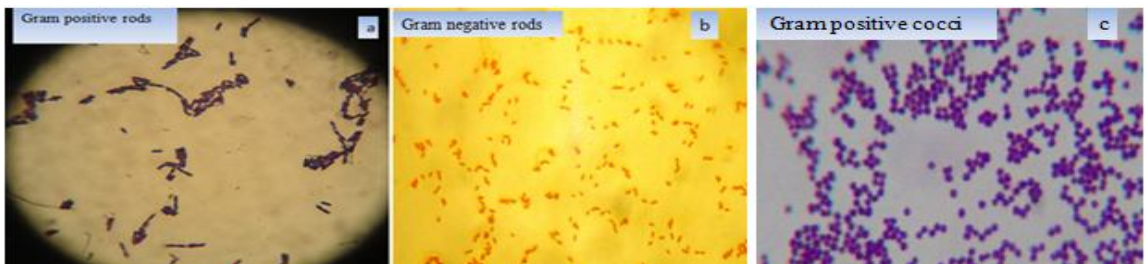

Appendix 2: Gram test; A representation of the gram test, in which case gram positive bacteria appear purple (a and c) while gram negative ones appear red (b)


Appendix 3: Catalase test; bubbles in test tube a imply a positive test; test tube b forms the negative control containing media (Tryptic soy agar) and test reagent (hydrogen peroxide)
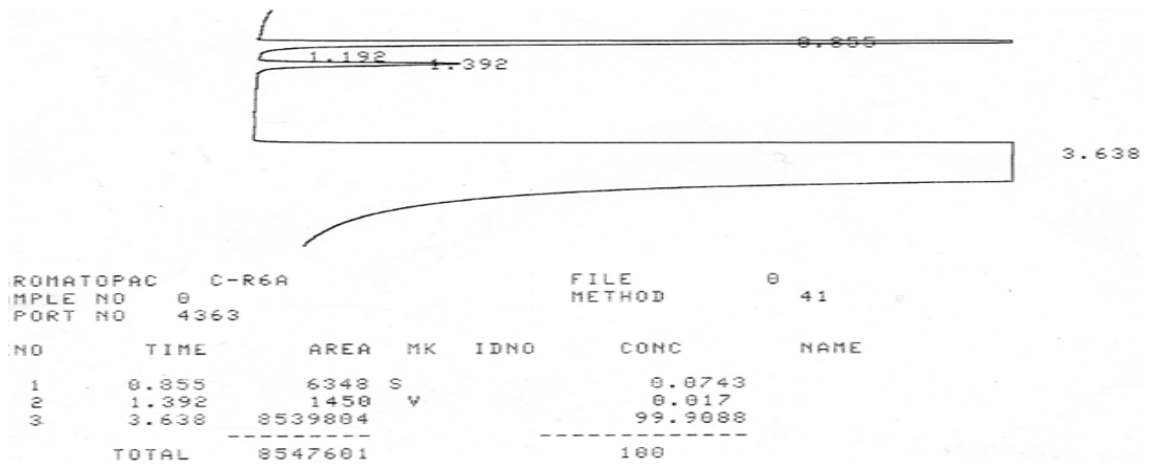
Appendix 4: Urease test: a is rose pink in color indicating a positive test; b is yellow in color indicating a negative test; c is a negative control containing sterile Christein's urea broth only
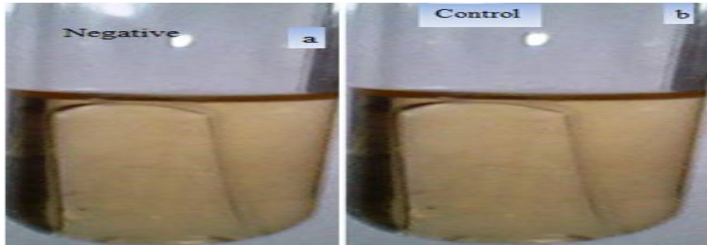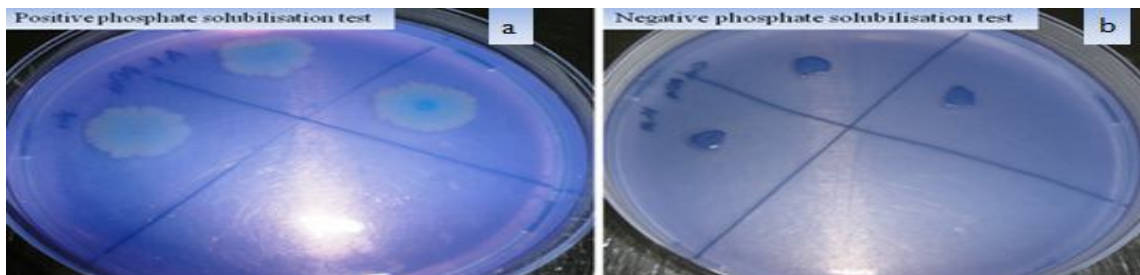

Appendix 5: Denitrification test; a is a negative test indicated by the absence of an air burble in the Durham tube; b is the negative control containing sterile nitrate reduction broth only
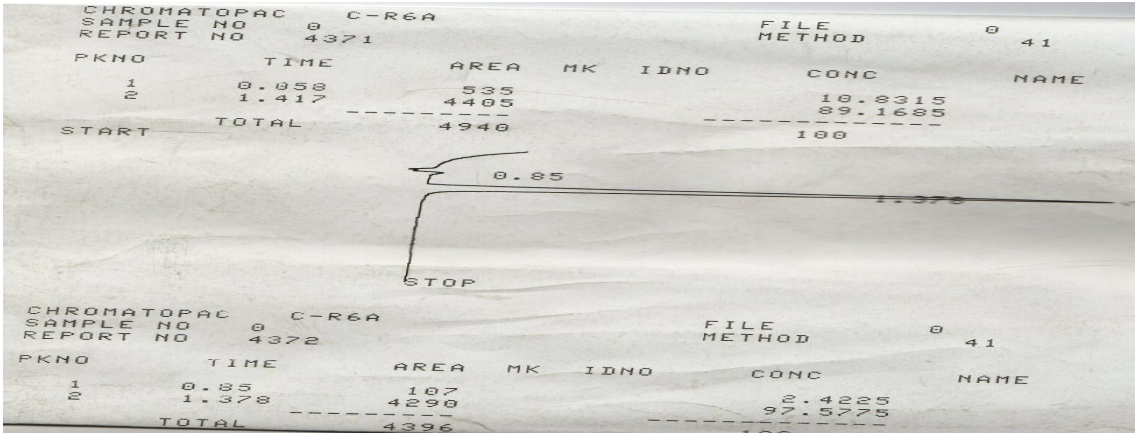
Appendix 6: Standard chromatograph for ARA showing the retention time (1.378) of ethylene

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                              ┌────────────────────────────────── 0.855
         ┌── 1.192
         └───── 1.392
         │
         │
         └────────────────────────────────── 3.638
```

```
ROMATOPAC    C-R6A                        FILE            0
MPLE NO    0                              METHOD             41
PORT NO    4363

NO       TIME          AREA    MK   IDNO        CONC          NAME

1        0.855         6348  S                  0.0743
2        1.392         1450  V                  0.017
3        3.638      8539804                     99.9088
                  ----------                 -------------
         TOTAL      8547601                    100
```

Appendix 7: Chromatograph of a positive test for ARA, showing the retention time (1.387) of ethylene


Appendix 8: Phosphate solubilisation test; a is a positive test, indicated by the hallo around the bacterial colonies; b is a negative test due to absence of a hallo around the bacterial colony

Appendix 9: Assessment for production of IAA; a is a negative test due to the yellow color; b is a positive test as implied by the pink color; c is a negative control containing sterile nutrient broth and test reagent (Salkowski's reagent)

Appendix 10: Citrate utilization test; b is a positive test as implied by the blue color; a is a negative control containing sterile test media (Simon's Citrate agar)
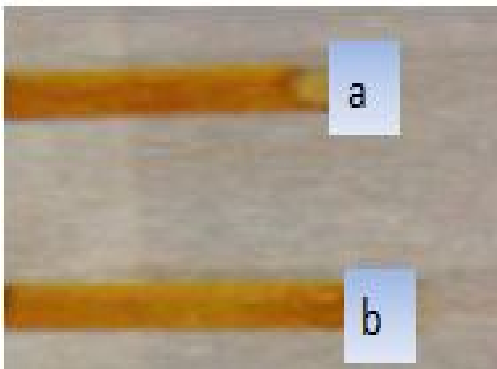
Appendix 11: Hydrogen sulfide production test; b is a negative test due to absence of the black color; a is a negative control containing sterile SIM media

Appendix 12: Methyl red test; a is a negative test as implied by absence of red coloration; b is a positive test as implied by presence of the red coloration; c is the negative control containing sterile MR-VP broth and the test reagent (methyl red)
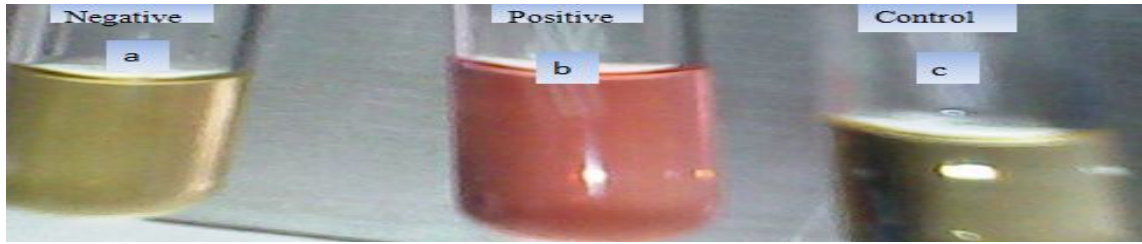
Appendix 13: Voges-Proskauer test; b is a positive test as implied by presence of the red coloration; a is a negative test as indicated by absence of the red coloration; c is the negative control containing sterile MR-VP broth and the test reagent (Barrit's reagent)

Appendix 14: Rice plant data on phenotypic assessment after a drench with bacterial endophytes with a concentration of 1×10^5 CFU. Means within the same column followed by the same alphabet are not significantly different at Turkey's test (P<0.05).

Isolate	DW	HT(3week)	HT (7week)
M31	1.5670±0.14353a	20.6750±0.21747ab	42.025±2.23844a
M32	1.4968±0.28803a	21.4250±0.34970a	34.275±0.66505ab
Crt +ve	1.3888±0.18022a	19.0000±0.73598ab	33.000±1.38984abc
Crt -ve	0.3630±0.10000b	19.0000±0.53072ab	24.525±3.46106bc
Ck	0.3593±0.06581b	19.9000±0.94074ab	27.550±4.06663bc
M5	0.3163±0.04252b	18.0500±0.86265b	26.100±3.01690bc
k7	0.2688±0.05283b	18.5250±0.30104ab	22.150±0.75111c
M16	0.2218±0.03320b	18.6250±0.64340ab	25.050±1.33073bc
$LSD_{0.05}$	**0.6542**	**2.9281**	**11.393**

Appendix 15: Rice plant data on phenotypic assessment after a drench with bacterial endophytes with a concentration of 1×10^{10} CFU. Means within the same column followed by the same alphabet are not significantly different at Turkey's test (P<0.05).

Isolate	DW	HT(3week)	HT (7week)
Crt +ve	1.3888 ± 0.18022 a	19.000 ± 0.73598a	33.000 ± 1.38984a
M31	1.3010 ± 0.15514 a	21.725 ± 0.68845a	32.375 ± 2.67625a
M32	1.2088 ± 0.17958 a	22.300 ± 0.68191a	32.350 ± 1.63783a
K7	0.3215 ± 0.03480 b	20.525 ± 0.35678a	23.500 ± 1.32351b
M5	0.2820 ± 0.02191 b	22.225 ± 1.27957a	22.600 ± 1.18392b
M16	0.2688 ± 0.01692 b	21.800 ± 0.90646a	23.850 ± 1.45402b
CK	0.2453 ± 0.02177 b	22.050 ± 1.07121a	23.400 ± 0.91104b
Crt -ve	0.2453 ± 0.02177 b	18.650 ± 1.16798a	21.375 ± 0.77285b
LSD$_{0.05}$	**0.5015**	**4.2473**	**7.117**

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Appendix 16: Results on morphology and biochemical characterization of the 73 bacterial endophytes; M series- Mwea isolates; K series- Kisumu isolates; + (positive) - (negative)

ISOLATE	GRAM TEST	MORPHOLOGY	CATALASE TEST	UREASE TEST	DENITRIFICATION TEST	CITRATE UTILIZATION	HYDROGEN SULFIDE PRODUCTION	PHOSPHATE SOLUBILISATION	MIR	VP	ARA	IAA
M1	-	Rods	+	+	-	+	-	+	-	-	+	-
M2	-	Rods	+	+	-	+	-	+	+	-	+	-
M3	+	Rods	+	+	-	+	-	+	-	-	+	-
M4	+	Rods	+	+	-	+	-	-	-	-	+	-
M5	-	Rods	+	-	-	+	-	+	-	+	+	+
M6	+	Rods	+	+	-	+	-	+	-	-	+	-
M7	-	Rods	+	+	-	+	-	+	+	-	+	-
M8	+	Rods	+	+	-	+	-	-	-	-	+	-
M9	-	Rods	+	+	-	+	-	+	-	-	+	-
M10	-	Rods	+	-	-	+	-	-	-	+	+	-
M11	-	Rods	+	+	-	+	-	-	+	-	+	-
M14	-	Rods	+	+	-	+	-	+	+	-	+	-
M15	-	Rods	+	+	-	+	-	+	+	-	+	-
M16	-	Rods	+	-	-	+	-	+	-	+	+	+
M17	-	Rods	+	-	-	+	-	+	-	+	+	+
M18	-	Rods	+	-	-	+	-	+	-	+	+	+
M19	-	Rods	+	+	-	+	-	-	+	-	+	-
M20	+	Rods	+	+	-	+	-	+	-	-	+	-
M21	+	Rods	+	+	-	+	-	+	-	-	+	-
M22	+	Rods	+	+	-	+	-	+	-	-	+	-
M23	+	Rods	+	+	-	+	-	+	-	-	+	-
M24	+	Rods	+	+	-	+	-	+	-	-	+	-
M25	+	Rods	+	+	-	+	-	+	-	-	+	-
M26	-	Rods	+	-	-	+	-	+	-	+	+	-
M27	-	Rods	+	-	-	+	-	+	-	+	+	+
M28	-	Rods	+	-	-	+	-	+	-	+	+	-
M29	-	Rods	+	+	-	+	-	+	+	-	+	-
M30	-	Rods	+	+	-	+	-	-	+	-	+	-
M31	-	Rods	+	-	-	+	-	+	-	+	+	+
M32	-	Rods	+	+	-	+	-	+	+	-	+	+
M33	+	Rods	+	+	-	+	-	+	-	-	+	-
M34	+	Rods	+	+	-	+	-	+	-	-	+	-
M35	+	Rods	+	+	-	+	-	+	-	-	+	-
M36	+	Rods	+	+	-	+	-	+	-	-	+	-

ISOLATE	GRAM TEST	MORPHOLOGY	CATALASE TEST	UREASE TEST	DENITRIFICATION TEST	CITRATE UTILIZATION	HYDROGEN SULFIDE	PHOSPHATE SOLUBILISATION	MR	VP	ARA	IAA
M37	+	Rods	+	+	-	+	-	+	-	-	+	-
M38	-	Rods	+	+	-	+	-	+	+	-	+	-
M39	+	Rods	+	+	-	+	-	+	-	-	+	-
M40	-	Rods	+	+	-	+	-	+	+	-	+	-
M41	+	Rods	+	+	-	+	-	+	-	-	+	-
M42	-	Rods	+	-	-	+	-	+	-	+	+	+
M43	+	Rods	+	+	-	+	-	+	-	-	+	-
M44	+	Rods	+	+	-	+	-	+	-	-	+	-
M45	-	Rods	+	-	-	+	-	+	-	+	+	-
M46	-	Rods	+	+	-	+	-	+	+	-	+	-
M47	-	Rods	+	-	-	+	-	+	-	+	+	-
M48	-	Rods	+	-	-	+	-	+	-	+	+	-
M49	-	Rods	+	-	-	+	-	+	-	+	+	-
M50	-	Rods	+	-	-	+	-	+	-	+	+	-
M51	-	Rods	+	-	-	+	-	+	-	+	+	+
M52	-	Rods	+	-	-	+	-	+	-	+	+	-
M53	-	Rods	+	+	-	+	-	+	+	-	+	-
M54	-	Rods	+	+	-	+	-	+	+	-	+	-
M55	-	Rods	+	-	-	+	-	+	-	+	+	-
M56	-	Rods	+	+	-	+	-	+	-	-	+	-
M57	+	Rods	+	+	-	+	-	+	-	-	+	-
M58	-	Rods	+	+	-	+	-	+	+	-	+	-
M59	-	Rods	+	-	-	+	-	+	-	-	+	-
M60	-	Rods	+	-	-	+	-	+	-	+	+	-
M61	-	Rods	+	+	-	+	-	+	+	-	+	-
M62	-	Rods	+	-	-	+	-	+	-	+	+	-
M63	-	Rods	+	+	-	+	-	+	+	-	+	-
M64	+	Rods	+	+	-	+	-	+	-	-	+	-
M65	-	Rods	+	-	-	+	-	+	-	+	+	-
M66	+	Rods	+	+	-	+	-	+	-	-	+	-
M67	-	Rods	+	-	-	+	-	+	-	+	+	-
M68	+	Rods	+	+	-	+	-	+	-	-	+	-
K1	-	Rods	+	-	-	+	-	+	-	+	+	-
K2	-	Rods	+	+	-	+	-	+	+	-	+	+
K3	+	Cocci	+	-	-	+	-	+	-	-	+	-
K4	+	Rods	+	+	-	+	-	+	-	-	+	-
K5	+	Rods	+	+	-	+	-	+	-	-	+	-
K6	-	Rods	+	-	-	+	-	+	-	+	+	-
K7	+	Rods	+	+	-	+	-	+	-	+	+	-