Isolation and Characterisation of Bacterial Root Endophytes with Potential to Enhance Plant Growth from Kenyan Basmati Rice

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Abstract

Rice is an important grain food crop being a staple food for a large part of the world's human population including Kenya. Rice production in Kenya is limited by various factors, the most significant being application of fertilizers, which are expensive, unaffordable and not eco-friendly. In this study, a survey of bacterial root endophytes was carried out. Seventy three bacterial rice root pure isolates from farms and research fields in Mwea and Ahero were characterized using morphological, biochemical and molecular techniques. Ten isolates were identified to produce indoleacetic acid (IAA) while sixty seven showed a positive outcome for phosphate solubilisation. All these isolates were confirmed to have the potential to fix nitrogen based on acetylene reduction assay (ARA). Phylogenetic analysis of the twenty eight selected isolates clustered into four different genera namely Pseudomonas, Bacillus, Enterobacter and Micrococcus which was in agreement with other previous tests. Isolates M31 and M32 from Mwea Basmati 370 rice were shown to promote plant growth which makes them potential candidates for the development of a biofertilizer. Therefore, if used they 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.

Key words: Auxin production, bacterial root endophyte, biofertilizers, growth promotion, isolates, phosphate solubilisation, IAA, rice

Introduction

Rice is an important grain food crop being a staple food for a large part of the world's human population including Kenya. The national rice consumption in Kenya 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. In 2008, rice imports into Kenya were valued at Ksh. 7 billion (Emongor *et al*,. 2009). Enhancement 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 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. However, rice production is affected by various factors including the application of fertilizers which are expensive, unaffordable and not ecofriendly. An alternative to use of chemical fertilizers is exploration and use of endophytes as a nutrient supply method. Endophytes reside in the living tissues of host plants in a variety of relationships, ranging from symbiotic to slightly pathogenic. They may produce a plethora of substances of potential use in fields like modern medicine, agriculture, and industry (Strobel *et al.*, 2004). They may be transmitted either vertically or horizontally (James *et al.*, 2002). They enter the plant tissue primarily through the root zone though aerial portions of plants, such as flowers, stems and cotyledons may also be used for entry.

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. This study sort to isolate and characterize bacterial root endophytes from Kenyan basmati rice and assess the potential in plant growth promotion activity with the view of development of a biofertilizer.

Methods

Collection of Root Samples

Two Kenyan basmati rice varieties were used, namely 370 and 217. A total of 245 basmati 217 and basmati 370 rice root samples (Walpole, 1986) were randomly collected from Mwea and Ahero irrigation schemes rice paddies and transported on ice to the laboratory for endophyte isolation procedures. In the laboratory, the samples were stored at 4°C.

Preparation of Culture Media

Yeast manitol agar (Sigma Aldrich, Steinheim, Germany), nitrogen free medium, and nutrient agar (Sigma Aldrich, Steinheim, Germany), were weighed and dissolved in distilled water by warming on a hot plate. These were then autoclaved under pressure for fifteen minutes at 121°C. The media was then dispensed in sterile petri dishes.

Surface sterilization of samples

The roots were thoroughly washed with sterile distilled water to remove any adhering soil. The samples were then 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.), and finally a five times rinse in sterile distilled water. The samples were then aseptically dried using sterile paper towels.

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). A fivefold serial dilution was performed after which 100µl was 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. The seventy three pure isolates generated were then coded as follows: Mwea isolates were M1 to M66 while those from Ahero formed K1 to K7. These were then used to perform morphological, biochemical and subsequently molecular characterization.

Morphological and biochemical characterization of the bacterial root isolates

Cell morphology was determined by the classical gram staining method (Bathlomew, 1962). Biochemical tests for urease activity, nitrate reduction, citrate utilization, hydrogen sulfide production, phosphate solubilisation, methyl red-Voges-Proskauer, and acetylene reduction assay were done according to the procedures described by Cappuccino and Sherman (Cappuccino and Sherman, 2002). The Salkowski test (Glickmann and Dessaux, 1995) was also performed to assess the production of auxins by the bacterial isolates. All incubations of the bacterial cultures were done in a clean incubator (IN-81, Yamato, Japan) at 30°C.

Acetylene reduction assay (ARA)

A loopful of bacteria were grown for three days in nitrogen free medium and then placed in semi solid agar media containing 2.3g of agar per liter. 5ml of the media was placed in 10ml vials and Acetylene added to attain a concentration of 12% v/v. Ethylene production was determined after 12 hours on a Shimadzu Gas Chromatograph (GC-9A, Japan) (Eckert *et al.*, 2001).

Urease Test

The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using Christensen's urea broth (Oxoid ltd., Basingstoke, Hampshire, England) containing the pH indicator phenol red (Cappuccino and Sherman, 2002). A loopful of the bacteria were aseptically inoculated into sterile Christensen's urea broth using a sterile wire loop and incubated for 24 hours.

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 for 72 hours.

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 incubated for two weeks, after which observations were done.

Phytohormones (auxins) production test

Production of indoleacetic acid was detected by a calorimetric method using the Salkowski reagent (Glickmann and Dessaux, 1995). The pure bacterial isolates were aseptically inoculated into sterile nutrient broth (Oxoid ltd., Basingstoke, Hampshire, England), and incubated for 72 hours. The cultures were then centrifuged at 12,000 g, for 5 minutes, at 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 after which observations on color change were done (Eckert *et al.,* 2001).Other biochemical tests carried out included the citrate utilization, Catalase, hydrogen sulfide production and Methyl red Voges-Proskauer tests (Cappuccino and Sherman, 2002).

Molecular characterization of endophytic bacterial isolates

Extraction of genomic DNA from bacterial root endophytic isolates

Genomic DNA was extracted from selected thirty seven isolates using chloroform extraction procedure (Sambrook *et al.,* 1989). Prior to extraction, bacterial cells were harvested from broth by centrifuging at 12,000g on a microcentrifuge (Hettich, Micro 200, Germany) for 5 minutes at 25°C. For this case, 1ml of culture was placed in a 1.5ml eppendorf tube and the supernatant poured out after the centrifugation. The bacterial cells were washed by re-suspending in equal volumes of TE buffer (pH 8), centrifuged for 5 minutes at 12,000 g, 25°C, and the supernatant discarded. The cells were then re-suspended in 200 µl of solution containing 50mM Tris (pH 8.5), 50mM EDTA pH (8.0) and 25% sucrose solution, enriched with 5µl of 20mg/ml lysozyme (Sigma Aldrich, Steinheim, Germany) and 5µl of 20mg/ml RNase A (Sigma Aldrich, Steinheim, Germany) then mixed gently.
The mixture was then incubated at 37°C for 1 hour. About 600µl of solution containing 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 12, 000 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. The mixture was then spun at 12, 000 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 12, 000 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 12, 000 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 0.8% (w/v) agarose (Sigma Aldrich, Steinheim, Germany) gel in 1xTAE buffer for 30 minutes and visualized under UV by staining with ethidium bromide (Sambrook *et al.,* 1989). The DNA was then quantified using an Eppendorf AG model 22331 spectrophotometer (Hamburg, Germany) with the absorbance at 260nm and 280nm to determine the purity of the DNA. The DNA with the ratio of 1.8 - 2.0 was used in the subsequent polymerase chain reaction.

Polymerase Chain Reaction

The 16S rDNA gene sequences were PCR-amplified using bacterial primer pair 27F forward 5'-GAGTTTGMTCCTGGCTCA-3' and 1492R reverse, 5'-TACGGYTACCTTACGACT-3' (Bioneer, USA) derived from *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), 2µg/ml 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 all above and DNA template from *Pseudomonas putida* identified using 16S rDNA based analysis. Reaction mixtures were subjected to the following optimized conditions: Initial denaturation of the template at 94^{o}C for 5 minutes, denaturation at 94°C for 45 seconds, primer annealing at 43^{o}C for 2 minutes, chain extension at 72^{o}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).

Restriction analysis of the PCR products

The genetic diversity of the thirty seven bacterial isolates was determined (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 for thirty minutes and DNA fragments visualized under UV illumination (BTS-20.M, EEC, Taiwan). Similarity among strains was estimated from the banding pattern of shared restriction fragment bands generated.

Purification of PCR products

The PCR products were purified using the QIAquick PCR purification Kit protocol according to manufacturer's (Qiagen, Germany) instructions. The 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).

Sequencing and molecular data analysis

Twenty eight bacterial isolates were selected 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. Sequencing of purified PCR products was done by a commercial service provider (International Livestock Research Institute). 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 (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).

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 on plant growth promotion were considered. These included the denitrification, urease, phosphate solubilisation tests, Acetylene Reduction Assay, and test for Auxin 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 any phenotypic effects on growth of Kenyan basmati rice. The concentrations of the bacterial endophytes used for the drenching process were based on similar work (Zhang *et al.,* 2010; Wang *et al.,* 2010; Kifle and Laing 2011). The plant growth indicators considered included plant height, tillering, and dry weight of shoots and roots (Ramezanpour *et al.,* 2010). Basmati 370 rice seeds as the source of endophytes 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 placed under same conditions in the green house to allow growth. The virgin soil used had been steam sterilized at 100° C. The positive control plants were also grown on virgin sterile soil 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 only without any fertilizer application. 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. Data on rice tillering, height and weight of dry matter of shoots and roots was collected at three and eight weeks after inoculation with the respective isolates.

Results

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 Kisumu. The frequency of endophytic isolates was found to differ among rice plant varieties. Basmati 370 was found to harbor more endophytic bacteria (70% - 51/73) than basmati 217 which harbored only 30% (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.

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 while 26 were gram positive rods and one gram positive cocci (**Table1**). 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.

Biochemical characterization of bacterial root endophytic isolates

The biochemical analysis results are summarized in Table **1**. Based on the various tests carried out, the isolates were categorized into ten groups as shown in Table **1**. The results showed that forty seven isolates tested positive while the twenty six were negative for urease test (**Table 1**). Sixty seven isolates (92%) tested positive for phosphate solubilisation while the other six (8%) tested negative (**Table 1**). The results also showed that ten isolates (14%) were able to produce IAA while the other sixty three (86%) did not (**Table 1**). Eighteen isolates tested positive while fifty five were negative for methyl red test. Further, twenty five isolates were positive, while forty eight tested negative for the Voges-Proskauer test (**Table 1**). It was observed that all the seventy three isolates were positive for Acetylene Reduction Assay, Catalase and the citrate utilization tests. Further, none of the isolates tested positive for nitrate reduction and hydrogen sulfide production tests (**Table 1**).

Molecular characterization of endophytic isolates

The expected band of 1500 bp was amplified for all the thirty seven isolates as representatively shown on Figure **I**. Restriction analysis of the PCR products the restricted PCR products gave varying sizes of bands as shown in Figure **2**. Isolates M3 and M39 gave a similar banding profile of fragments in the ranges 300-400, 400-500, and 600-700 (**Figure 2**).

Similarity was also observed for isolates M53 and M9, which gave an identical banding profile of the ranges 300-400 and 900-1000 (Figure **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 300-400,500-600, and 600-700, while that for M56 were 200-300,300-400, and 900-1000 (**Figure 2**).

Phylogenetic analysis of the sequences for the PCR products

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 3**). 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 3**).
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 3**).
Isolate K3 was phylogenetically identified as *Micrococcus luteus* strain BF1-6/ NSM12 at 94% sequence similarity (**Figure 3**). 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 3**). K7 was closely related to *Bacillus thuringiensis* strain S422B-21 while K6 and K8 were *Bacillus megaterium* strain SZ-3 (**Figure 3**).

Assessment of effect of selected isolates on rice plant growth promotion

The two concentrations of 1×10^5 and 1×10^{10} of the bacterial counts 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 (**Table 2**). 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 (**Table 3**). 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 (**Table 2**). On 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 against the negative control which was 0.24525 (**Table 3**).

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 3; for M5 and the cocktail was 4; M32 and M31 was 7. The negative control had 4 tillers, while the positive control had 6. In this case, isolates M31 and M32 gave the highest number of tillers.

The data obtained in this assessment was also compared with the positive control. In this case, analysis with t- test at 95% significance level showed that data on plant height and dry weight for isolates M31 and M32 were not significantly different from the positive control (P values 0.775 and 0.474 respectively at 1×10^{10} Colony forming units). However, data for isolates M5, M16, K7, and the Cocktail were significantly different from the positive control (P values 0.027, 0.047, 0.027 and 0.04 respectively at 1×10^{10} CFU). 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. 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 (i.e 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.

Discussion

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). 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. The retention time on the chromatograph for the standard was 1.378 while that for the experimental isolate was representatively 1.387. These ARA positive results obtained are consistent with other studies on rice plant growth promoting bacteria (Keyeo *et al.,* 2011). Additionally, these isolates were able to grow on nitrogen free media. This clearly showed their potential to fix nitrogen in 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 test is an important aspect in growth and development of rice in the case where fertilizers are applied. 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 reacts with water to form ammonium (NH_4^+) which is a plant available source of nitrogen. 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 phenomenon of positive urease activity has also been observed in other studies on rice endophytes (Tan *et al.,* 2001). This positive test 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. The process is performed primarily by heterotrophic bacteria such as *Paracoccus denitrificans* and various *Pseudomonads* (Carlson and Ingraham, 1983). The rice root endophytic *Pseudomonads* identified in this research 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 (Richardson *et al.,* 2009). 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. 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 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 has been attributed to their capacity to reduce pH by the excretion of organic acids during the assimilation of ammonia (Gyaneshwar *et al.,* 1999).

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 assay for phosphate solubilisation for the isolated basmati rice root endophytic bacteria during this characterization process. This was well observed by the formation of a hallo around the bacterial colonies for the sixty seven isolates that assayed positive for this test. Endophytes have also been shown to promote plant growth by producing the 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. 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 the prevailing conditions. 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.

Molecular characterization of bacterial endophytes

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. 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. Most *Enterobacter* reduce nitrate to nitrite, with exceptions such as *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.

Isolates K1 and M5 were identified as *Enterobacter ludwigii* isolate PSB1/strain 2-1 while M16 and M17 were *Enterobacter cloacae* isolate HQ040619-1 and were shown to have a similar plant growth promotion potential as they assayed positive for nitrogen fixation and phosphate solubilisation. 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) rendering them potential plant growth promoters.

Among plant growth promoting species, *Azospirillum* is one of the best studied IAA producers (Dobbelaere *et al.,* 1999). Other IAA producing bacteria include *Aeromonas* (Halda-Alija, 2003), *Azotobacter* (Ahmad *et al.,* 2008), *Bacillus* (Swain *et al.,* 2007), *Burkholderia* (Halda-Alija, 2003), *Enterobacter* (Shoebitz *et al.,* 2009), *Pseudomonas* (Hariprasad and Niranjana, 2009) and *Rhizobium* (Ghosh *et al.,* 2008) genera. The auxin producers identified here 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 plant growth promoting rhizobacteria (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 were related to *Pseudomonas fluorescens* (M63, M58, M53, M32, and K2) and *Pseudomonas putida* (M9, M59, M1, and M56). The morphological and biochemical characteristics for these rice root endophytic isolates indicated that they are highly closely related to this genus. According to literature, *Pseudomonades* are described as aerobic, rod shaped, Gram-negative bacteria with one or more flagella providing motility. The members of this genus demonstrate a great deal of metabolic diversity, and consequently are able to colonise a wide range of niches (Madigan and Martinko, 2005). The soil bacterium *Pseudomonas putida* and the plant growth promoting *Pseudomonas fluorescens* are among the best studied species of this genus. In the quest for knowledge about Kenyan basmati rice root endophytes, this research identified some of the isolates as *Pseudomonas putida* (isolates M1, M9, M56, and M59) and others as *Pseudomonas fluorescens* (M32, M53, M58, M63, and K2). Further, isolate M32 was shown to promote rice plant growth at the prevailing conditions.

Previous studies have shown that *Pseudomonas putida* tests positive for citrate utilization, and negative for methyl red, and Voges- Proskauer tests. These reviewed biochemical aspects were evidently observed in the respective Kenyan basmati rice root endophytic isolates (M9, M59, M1, and M56) identified as *Pseudomonas putida.* 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.

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* are Gram-negative rod shaped bacteria that inhabit soil, plants and water surfaces (Anzia *et al.,* 2000). The optimum growth temperature is between 25-30° C (Haas and Keel, 2003). 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. Studies related to the capability of *Pseudomonas fluorescens* and *Pseudomonas* sp. to fix nitrogen have also been reported (Chan *et al.,* 1994). These phenomenons of nitrogen fixation and production of auxins were observed during this characterization process by the isolates M32, M58, and K2.

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 was 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. *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^{o}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 (Vendan *et al.,* 2010). Isolate K3 had plant growth promoting characteristics as it assayed 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% rDNA 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.*

Other research shows that *Bacillus* is a genus of Gram-positive rod-shaped bacteria (Turnbull, 1996). 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). *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 investigation 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.,* 2003). 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.

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 promote plant growth in Kenyan basmati rice. The most studied PGPR belong to gram-negative genera, and the greatest number of strains is members of the *fluorescent pseudomonads* (Kloepper, 1993). Many reports also suggest that gram-positive bacteria, such as *Bacillus*, are PGPR (Kloepper, 1993). In this study, majority (Madigan and Martinko, 2005) 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.

There are various studies that have been conducted on 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). Rice plant growth promotion was observed for isolate M32 which was identified as *Pseudomonas fluorescens*. *Pseudomonas fluorescens* has been earlier isolated from plant leaves and roots, and has been shown to contribute to plant growth (Palleroni, 1984). *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 (Keyeo *et al.,* 2011). Isolate M31 was identified as an *Enterobacter*, and was demonstrated to promote rice plant growth. 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).

Conclusions and recommendations

Conclusions

1. Kenyan basmati rice roots harbor plant growth promoting bacterial endophytes that are genetically diverse. Moreover, Basmati 370 harbors more endophytes than basmati 217
2. Plant growth promotion characteristics including nitrogen fixation, phosphorous solubilisation and production of auxins were observed for the rice root bacterial endophytes
3. The phylogenetic analysis of the potential isolates clustered them into four different genera namely *Pseudomonas, Bacillus, Enterobacter, and Micrococcus.* This is in agreement with their morphological and biochemical characteristics
4. Isolates M31 and M32 isolated from Mwea basmati 370 rice demonstrated plant growth promotion at the green house level.

Recommendations

The findings of this study recommend that:

1. Isolate M31 and M32 may be further analyzed for enhancement of plant growth of Kenyan basmati rice at green house level since they have potential
2. Field trials should be done to assess on promotion of plant growth by bacterial endophytic isolates M31 and M32
3. The optimal concentration at which plant growth promotion is observed should also be determined
4. Further molecular characterization should be carried out using more restriction enzymes to establish on any genetic diversity. Molecular characterization can also be performed for the isolates that were not subjected to the same process.

Tables

Table 1: Summary of morphological and biochemical characterization of selected bacterial endophytes; M series- Mwea isolates; K series- Kisumu isolates; + (positive) ; - (negative)

ISOLATE CODE	GRAM TEST	UREASE TEST	PHOSPHATE SOLUBILISATION	MR	VP	AUXIN
M1, M9,M56	-	+	+	-	-	-
M3, M6, M22, 23, M24, M34, M39, M41, M68, K4, K5.	+	+	+	-	-	-
M5, M16, M17, M18, M31, M51	-	-	+	-	+	+
M7, M53, M58, M63	-	+	+	+	-	-
M11, M19	-	+	-	+	-	-
M28, M55, M60, M67, K1, K6	-	-	+	-	+	-
M32, K2	-	+	+	+	-	+
M59	-	-	+	-	-	-
K3	+	-	+	-	-	-
K7	+	+	+	-	+	-

Table 2: Rice plant raw data on phenotypic assessment after a drench with bacterial endophytes with a concentration of 1×10^5

Isolate	Mean Plant height at 3 weeks (cm)	Mean Plant height at 7 weeks (cm)	Mean Dry weight after 7 weeks (g)
M16	18.625	25.05	0.22175
K12	18.525	22.55	0.26875
M31	20.675	42.025	1.567
M5	18.05	26.1	0.31625
M32	21.425	34.275	1.49675
Cocktail	19.9	27.55	0.35925
Positive control	20.5	33	1.3887
Negative control	19.0	24.525	0.363

Table 3: Rice plant raw data on phenotypic assessment after a drench with bacterial endophytes with a concentration of 1×10^{10}

Isolate	Mean Plant height at 3 weeks (cm)	Mean Plant height at 7 weeks (cm)	Mean Dry weight after 7 weeks (g)
M16	21.8	23.85	0.26875
K12	20.525	23.5	0.3215
M31	21.725	32.375	1.301
M5	22.225	22.6	0.282
M32	22.3	32.35	1.20875
Cocktail	22.05	33.4	0.24525
Positive control	20.5	33	1.3887
Negative control	18.65	21.375	0.24525

Figures

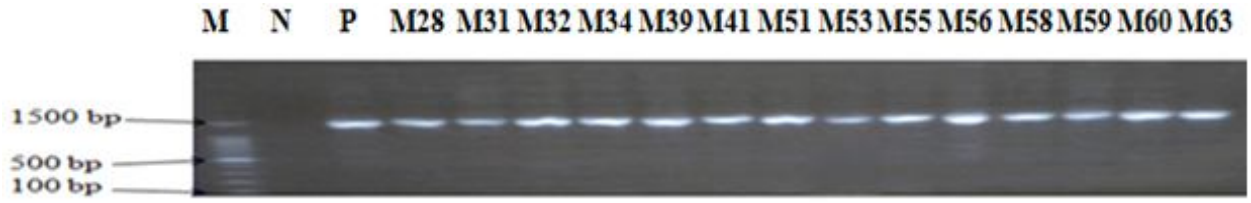

***Figure 1*:** Amplified PCR products of fourteen isolates from Mwea run in 0.8 % (W/V) agarose gel. M- 1500 bp marker, P- positive control, N- negative control, M28, M31, M32, M34, M39, M41, M51, M53, M55, M56, M58, M59, M60, and M63 are the isolates' amplicons

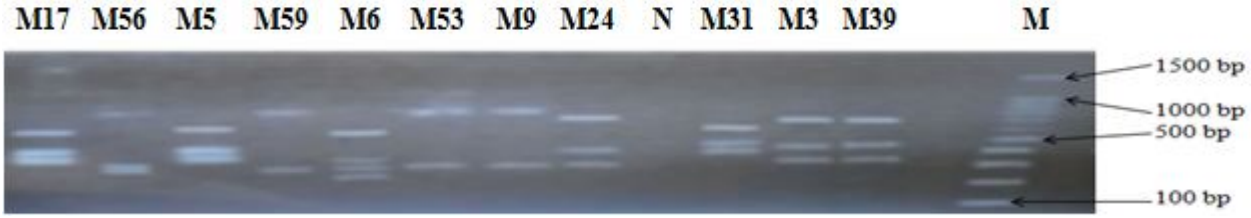

Figure 2: A representative gel photo for restriction products as generated by RsaI digestion run in 1.5% (W/V) agarose gel. M- 1500 bp marker, N- negative control, M17, M56, M5, M59, M6, M53, M9, M24, M31, M3, and M39 are restricted PCR products of the respective isolates

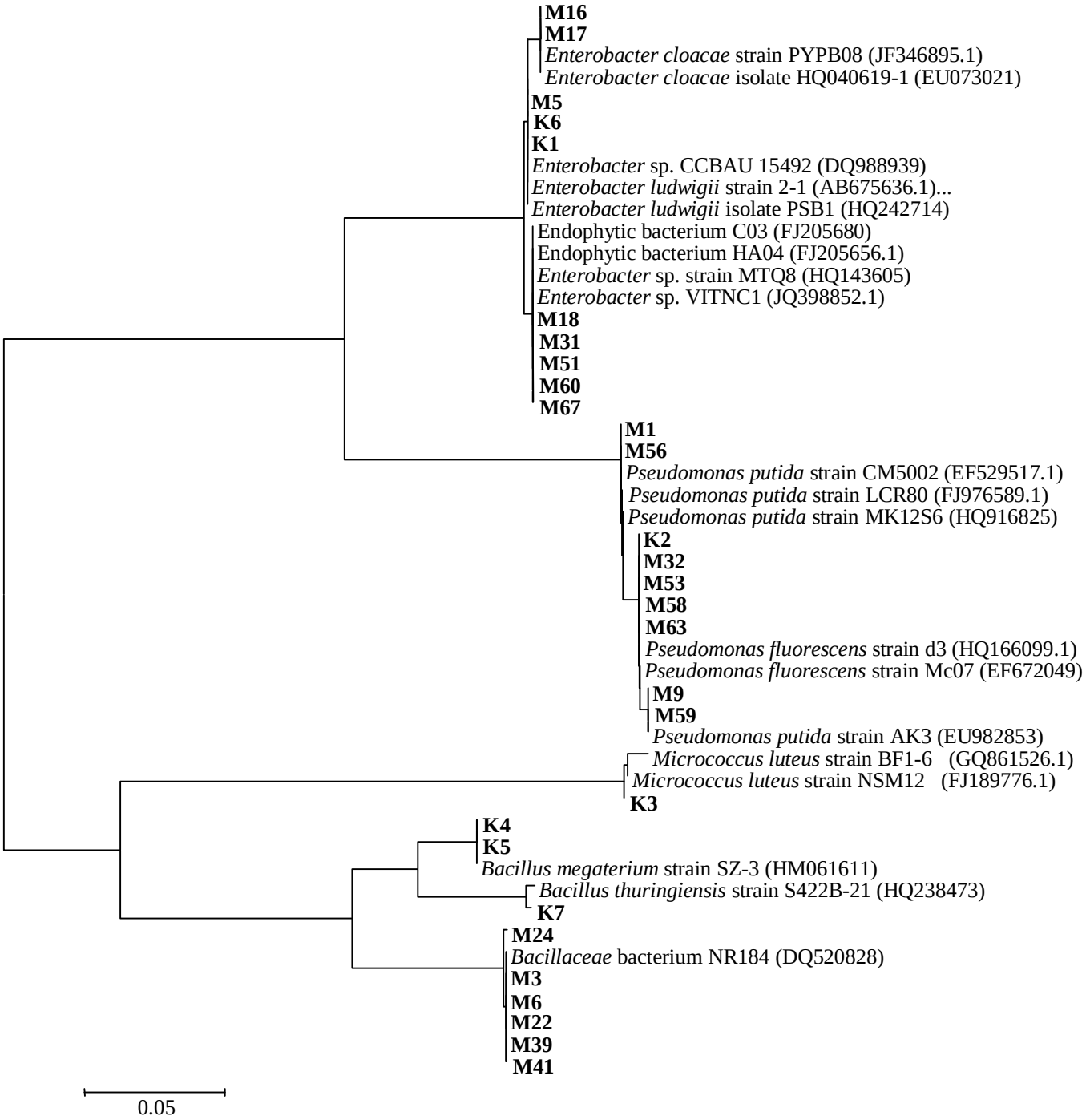

Figure 3: Neighbour joining phylogenetic tree showing the position of *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Micrococcus* basmati rice root bacterial endophytes. The bar indicates the estimated substitution per nucleotide position

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