MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISATION OF BACTERIAL ROOT ENDOPHYTIC ISOLATES WITH POTENTIAL TO ENHANCE PLANT GROWTH FROM KENYAN (MWEA) 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. 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, bioprospecting for bacterial rice root endophytes was explored. 66 bacterial pure isolates were isolated from the 138 root samples collected from farmers' and research fields. They were morphologically characterized and screened for biological activities. All isolates were gram negative rods. Two phosphates were used for phosphate solubilisation, in which case more positive response was observed in calcium phosphate than in Alluminium phosphate. Nine isolates were identified to produce IAA- indoleacetic acid implying potential to enhance plant growth. All isolates tested negative for denitrification test. The biochemical tests carried out helped reduce the number of isolates from 66 to 30 upon similarity and uniqueness. All 30 selected isolates were confirmed to have potential to fix nitrogen based on acetylene reduction assay (ARA). The selected 30 isolates have demonstrated to have the potential to promote plant growth in at least one biological mechanism. This is the first study on the endophytic nature of Kenyan (Mwea) Basmati rice endophytes. These microorganisms may be useful for agriculture (biofertilizer) to enhance affordable production for Kenyan basmati rice farmers, and also used in other applications. This will contribute to increased yield and trade of Kenyan basmati rice to eradicate poverty and increase food security, and help conserve microbial diversity.

Key words: Bacterial, biofertilisers, endophyte, isolation, isolates, rice

1.0 Introduction

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. It is noteworthy that, of the nearly 300,000 plant species that exist on the 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,that lives within a plant for at least part of its life without causing apparent disease (Holliday, 1989).

Due to what appears to be their contribution to the host plant, the endophytes may produce a plethora of substances of potential use to modern medicine, agriculture, and industry (Strobel *et al.*, 2004). Novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds are only a few examples of what has been found after the isolation, culture, purification, and characterization of some choice endophytes in the recent past.

Endophytes may be transmitted either vertically (directly from parent to offspring) or horizontally (from individual to unrelated individual). 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).
Endophytic bacteria ubiquitously inhabit most plant species, and have been isolated from a variety of plants (Lodewyck *et al.,* 2002). Recently, 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. Endophytic non-rhizobial diazotrophs like *Azoarcus* spp., *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. have been isolated from kallar grass, sugarcane and rice respectively.

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.

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). The aim of this qualitative study was to isolate and characterize (morphologically and biochemically) Kenyan basmati rice bacterial root endophytes.

2.0 Materials and Methods

Rice root samples were collected from Mwea Irrigation Agricultural Development Centre (MIAD) research field and farmers' paddy fields in Mwea. The morphological and biochemical characterization was carried out at the Food Science Microbiology Laboratory, Jomo Kenyatta University of Agriculture and Technology, Juja.

Two basmati rice varieties (basmati370 and basmati217) samples were randomly collected from the experimental site (Mwea). A sample size of 245 was used, which was determined according to Walpole (1986). The samples were placed in labeled plastic bags and transported to the laboratory in an ice box.
The samples were weighed, surface sterilized and ground before plating on three different types of media. Briefly, the samples were thoroughly washed with sterile water to remove adhering soil then subjected to a 2 minutes wash in 70% ethanol, followed by 4- to 10-minutes wash in 5% sodium hypochlorite, and finally a five times rinse in sterile water. The samples were then aseptically dried using sterile paper towels. The sterile samples were then ground, an aliquote picked and serially diluted, and plated on nutrient agar, 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), and nitrogen free media (0.5g/L K2PO4, 0.2g/L $MgSO_4$, 0.2g/L NaCl, 15mg/L $FeCl_3$, 6.6g/L $NaMoO_4(H_2O)$, 15g/L agar) in replicates. These were incubated at 30°C to allow growth. Individual unique colonies were then picked and purified upon streaking. All the bacterial isolates were coded for identification from M1 to M66.

2.1 Morphological Characterisation of Endophyte Isolates

This was done to determine the morphology of the bacterial cells upon observable characteristics such as cell shape, colony color and texture. This was determined by the classical gram staining method as described by Bathlomew, 1962.

2.2 Biochemical Characterization of Endophyte Isolates

All the 66 isolates were subjected to following biochemical tests and analysis;

2.3 Screening of Endophytes for Presence and Activity of Enzyme Catalase (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 production was determined by first growing all the 66 isolates in Tryptic Soy Agar for 24 hours at 30° C, after which 3-4 drops of 3% hydrogen peroxide were added. A positive reaction is indicated by the formation of bubbles (Cappuccino and Sherman, 2002).

2.4 Screening of Endophytes for Presence and Activity of Enzyme Urease (Urease Test)

The *urease* test was used to determine the ability of the bacteria to split urea, through the production of the enzyme *urease* (Harold, 2002). The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined by inoculation and incubation of the isolates in urea broth medium (5g/L peptone, 3g/L beef extract, 1g urea) at 30°C for 24 hours. 2-3 drops of phenol red were then added and observations made: formation of a deep pink color is a positive test (Cappuccino and Sherman, 2002).

2.5 Screening of Endophytes for Nitrate Reduction

The ability of the isolates to reduce nitrates to nitrites or beyond was carried out by inoculating the endophytes (at 30°C for 48 hours) in 2ml of nitrate reduction broth (5g/L peptone, 3g/L beef extract, 1g/L K_2NO_3) in universal tubes containing durham tubes, to capture nitrogen gas (Cappuccino and Sherman, 2002).

2.6 Screening of endophytes for Nitrogen Fixation

2.6.1 Acetylene Reduction Assay (ARA)

ARA measures nitrogenase activity, which ideally is a measure of the total amount of nitrogen that a system or organism has fixed. In this assay, bacteria were grown for three days in nitrogen free medium (15g/L K_2HPO_4, 0.2g/L $MgSO_4 . 7H_2O$, 1g/L $CaCO_3$, 0.2g/L NaCl, 0.0025g/L $FeSO_4 . 7H_2O$, 10g/L glucose, 5g/L $NaMoO_4(H_2O)$, 15g/L Agar). They were then placed in semi solid agar media containing 2.3g of agar per liter. 5ml of the media was placed in 10ml vials with rubber corks. Acetylene was added to attain a concentration of 12% v/v. Ethylene production was determined after 12 hours by sucking the free space (1ml) above the media and injecting into Shimadzu Gas Chromatograph as described by Eckert *et al*., (2001).

2.6.2 Screening of Endophytes for Phosphate Solubilisation

Two phosphate solubilisation medias (3g/L NaCl, 3g/L yeast extract, 5g/L peptone, 5ml of 0.5% bromocrysol green and 15g/L agar, containing either $CaPO_4$ or $Al_2(PO_4)_3$) were used for screening phosphate solubilizing endophytes

using the plate assay method where the ability to grow is associated to the capacity in using $CaPO_4$/ $Al_2(PO4)_3$ as a sole phosphate source. The bacteria were inoculated on a spot on the media and incubated at 30°C for 14 days. The formation of a halo after the 14 days of the incubation implies a positive test for phosphate solubilisation and ability to solubilize inorganic phosphate (Cappuccino and Sherman, 2002).

2.6.3 Assessment of Phytohormones (auxins) Production by Isolates

Production of phytohormones (indoleacetic acid (IAA)) was determined by a colorimetric method using the Salkowski reagent as described by Glickmann and Dessaux, 1995. The pure isolates were inoculated in nutrient broth and incubated for three days, then centrifuged to obtain cell free broth. Salkowski's reagent was then added to the cell free broth and incubated for thirty minutes at room temperature in the dark. A pink color indicated a positive test (Glickmann and Dessaux, 1995).

2.7 Other Biochemical Tests

Other biochemical characterization methods carried out include the citrate utilization, Indole and Hydrogen Sulfide production, and Methyl Red-Voges-Proskauer tests (MR-VP) as described by Cappuccino and Sherman (2002).

3.0 Results and Discussion

Endophytic bacteria colonizing rice root tissues were found in samples from the two basmati rice varieties. However, the frequency of endophytic isolates was found to differ among plant varieties. Basmati 370 was found to harbor more endophytic bacteria (70%) than basmati 217 which haboured only 30% of the total (66) isolates. It was also observed that the bacteria had different growth rates on the 3 different types of media used. Only 12 hours were required to observe growth for nutrient agar and yeast manitol agar, while for nitrogen free media at least 48 hours were required for growth to be observed.

The morphological analysis revealed that all the 66 endophytic isolates were gram negative rods with varying thickness and length (table 1). Colony color varied among the isolates from one media to the other. In the classical gram stain, gram positive bacteria which have a thick peptidoglycan layer retain the primary stain (crystal violet) during subsequent decolourisation and appear purple when viewed with a microscope (Cappuccino and Sherman(2002). On the other hand, gram negative bacteria which have a thin peptidoglycan layer lose the primary stain during decoulorisation and take up the secondary stain (safranin) to appear red when viewed under the microscope (Cappuccino and Sherman, 2002).

This study also showed that all the 66 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 in this study were Catalase positive, it means they posse the capability to protect themselves from this toxic effects.

Nitrogen is a major limiting factor in rice production. Rice farmers in Kenya mostly depend on application of chemical fertilizers for nutrient supply to their plants. The increased cost of fertilizers is likely to limit 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. Hence there is need to exploit an alternative source of nutrient supply, such as biofertilizers. One possibility is through the use of bacterial endophytes which have been shown to have such potential. This research sought to address this major problem by carrying out the ARA, urease, and nitrate reduction tests

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The acetylene reduction assay was specifically done to establish whether the bacterial endophytes have potential to fix nitrogen. Organisms that are able to fix atmospheric nitrogen (N_2) possess the enzyme

nitrogenase, which reduces nitrogen to ammonia (NH_3) (Cappuccino and Sherman, 2002). Nitrogenase catalyses the reduction of not only nitrogen but also a variety of other substrates, like acetylene (C_2H_2) (Cappuccino and Sherman, 2002). The reduction of acetylene to ethylene (C_2H_4) is widely used as a method of measuring nitrogenase activity in natural samples, isolates, and cell-free extracts (Cappuccino and Sherman, 2002). In the late 1960's Stewart and others developed the acetylene reduction assay (ARA). ARA measures nitrogenase activity, which ideally is a measure of the total amount of nitrogen that a system or organism has fixed. This fact was confirmed in this study by the observation that the bacteria were able to grow on nitrogen free media. Additionally all isolates tested positive for this assay.

The urease test was done to determine the ability of the isolates to split urea, to simple forms of nitrogen which can be readily absorbed by the plants to promote growth. The results showed that 57 isolates tested positive while 9 were negative for the this test. 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 which are readily absorbed by plants.

The nitrate reduction test was also performed to determine the ability of the isolates to reduce nitrates to nitrogen gas. This is an important factor to help maintain the nitrogen cycle in the three phases of the atmosphere, water, and the soil. Surprising, all isolates tested negative for denitrification test. This is a critical feature of these bacteria, as it will give time to the plants to absorb readily available nitrogen before it can be converted to free nitrogen gas by other bacteria, of be leached.

Qualitative phosphate solubilization activity was assayed for all the 66 isolates. It was noted that 43 isolates tested positive while the other 23 tested negative for phosphate solubilisation (table 1). However, the isolates displayed variable efficiencies for the two phosphates used, with bigger halos being observed for calcium phosphate solubilisation. It should be noted that Phosphorus is one of the main nutrients limiting plant growth and is rapidly immobilized after addition to soil as a soluble fertilizer, becoming unavailable to the plant (Wakelin *et al.,* 2004). 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). Other previous studies had showed that in *Bacillus*, the main compounds involved in the phosphate solubilization are the lactic, itaconic, isovaleric, isobutyric and acetic acids (Vazquez *et al.,* 2000). Therefore, bacterial solubilization activity is highly important as indicators for phosphorus solubilization process that support plant growth. The identification in this study of 43 isolates from Kenyan basmati rice with the ability to solubilize phosphorous is noteworthy achievement since this the first such 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 9 isolates (M5, M11, M16, M17, M31, M51, M53, M58 and M60) were able to produce IAA (table 1). It was further noted that not all phosphate solubilizers were auxin producers. For instance phosphate solubilisation was not observed in isolate M58, M53, M11. These data indicate 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.

For the other biochemical tests (citrate utilization, Methyl red, and Voges-Proskauer) performed: 41 isolates gave a positive outcome while the other twenty five tested negative for citrate utilization test ; 8 tested positive while 58 were negative for VP; and 33 tested positive while 33 were negative for MR test (table 1). This is additional data for further studies in identification of the isolates since the tests have no bias to the objective of the study in plant growth and development promotion.

Table 1: Summary of the biochemical tests performed for the 30 selected isolates

ISOLATE	GRAM TEST	MORPHOLOGY	CATALASE TEST	UREASE TEST	NITRATE REUCTION	CITRATE UTILIZATION	INDOLE PRODUCTION	HYDROGEN SULFIDE PRODUCTION	PHOSPHATE SOLUBILISATION (CALCIUM	PHOSPHATE SOLUBILISATION (ALUMINIUM PHOSPHATE	MR	VP
M39	-	Rods	+	+	-	-	-	-	+	+	+	-
M24	-	Rods	+	+	-	+	-	+	+	+	+	-
M28	-	Rods	+	+	-	-	-	-	+	+	+	-
M41	-	Rods	+	-	-	-	-	-	+	+	+	-
M5	-	Rods	+	+	-	+	-	+	+	-	-	-
M7	-	Rods	+	+	-	+	-	-	+	-	+	-
M16	-	Rods	+	+	-	+	-	-	+	+	-	-
M18	-	Rods	+	+	-	+	-	+	-	+	+	-
M31	-	Rods	+	+	-	+	-	+	+	+	-	-
M23	-	Rods	+	+	-	+	-	-	-	+	-	-
M22	-	Rods	+	+	-	+	-	-	-	+	+	-
M51	-	Rods	+	+	-	+	+	-	+	+	+	-
M55	-	Rods	+	+	-	+	-	-	-	-	-	-
M60	-	Rods	+	+	-	+	-	+	+	+	-	+
M63	-	Rods	+	+	-	+	-	-	+	+	-	-
M17	-	Rods	+	+	-	+	-	+	+	+	-	-
M6	-	rods	+	+	-	-	-	-	+	-	+	-
M68	-	rods	+	+	-	-	-	-	+	+	+	+
M3	-	rods	+	+	-	-	-	-	+	-	+	-
M56	-	rods	+	-	-	-	-	-	+	+	+	-
M32	-	rods	+	-	-	+	-	-	+	+	-	-
M34	-	rods	+	+	-	+	-	+	-	-	-	-
M19	-	rods	+	+	-	-	-	-	-	-	-	-
M1	-	Rods	+	+	-	+	-	+	+	-	+	-
M9	-	Rods	+	+	-	-	-	-	-	-	-	-
M11	-	Rods	+	+	-	+	-	-	-	-	-	-
M53	-	Rods	+	+	-	+	-	-	-	-	-	+
M67	-	Rods	+	+	-	+	-	-	-	-	+	-
M58	-	Rods	+	+	-	+	-	-	-	-	-	-
M59	-	Rods	+	+	-	+	-	-	-	-	-	-

4.0 Conclusion

The endophytes have different growth rates on the different types of medias used;the isolates solubilized calcium phosphate better than to Alluminium phosphate based on size of halo formation and 30 isolates have demonstrated the potential to promote plant growth in at least one biological mechanism. Therefore it can be concluded 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.

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