

SCREENING OF BACTERIAL AND FUNGAL ISOLATES FOR THEIR PLANT GROWTH PROMOTING ACTIVITIES

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Abstract

Rice (*Oryza sativa* L.) is the most important staple food crop in several developing countries, and is ranked third in Kenya after maize and wheat. Most of the rice is grown in Central Kenya; other areas which produce small quantities include Western, Nyanza and Coastal Kenya. Declining soil fertility as a result of continuous cropping without replenishing soil nutrients is a major problem in Kenya. Low soil fertility problem can be minimized by using fertilizers. However the use of chemical fertilizer is currently limited due to rising costs and environmental concerns. This situation can be altered by exploring alternative sources which are cost effective and environmental friendly. Phosphorus and nitrogen are the two most limiting nutrients in rice soils while IAA is an essential natural growth promoter that extensively affects plant growth and development. Many soil micro-organisms are able to solubilize the unavailable phosphorus, increase uptake of nitrogen and also synthesize growth promoting hormones. The phylloplane and the rhizosphere of the rice plants provides conducive habitat for various micro-organisms. It has been documented that inoculation of rice with plant growth promoting bacteria (PGPB) resulted in an increased plant/crop growth and yield. The PGPB acts as bio-fertilizer and bio-enhancer. The aim of this study is to isolate and characterize phylloplane and rhizosphere micro-organisms from Kenyan rice with growth promoting habits. In this study whole plant rice samples were collected from different rice growing regions of Kenya out of which a total of 130 pure bacterial and 120 pure fungal isolates were obtained. These isolates were screened for production of plant growth promoting factors such as phosphate solubilization, nitrogen fixation and IAA production. Out of the 130 bacterial isolates, 99 (76.2 %) were positive for phosphate solubilization, over 80 % for nitrogenase activity and 50 (38.5%) for IAA production. Out of the 121 fungal isolates, 21 (17.5 %) were positive for phosphate solubilization, none for nitrogenase activity and 6 (5 %) for IAA production. This clearly indicates the potential that these micro-organisms have for utilization as bio-fertilizers in rice production.

Key words: Micro-organisms, phosphate solubilization, nitrogen fixation, IAA production

1 Introduction

Rice (*Oryza sativa* L.), is the main staple food for more than one-third of the world's population and it directly provides 20% of human calorie intake (Zeigler and Barclay 2008). It is the most important staple food crop in many developing countries, and is ranked third in Kenya after maize and wheat. The national rice consumption is estimated at about 410,000 tonnes (MoA, 2010), against an annual domestic production of 72,500 tonnes (KACE, 2011). Annual rice consumption is increasing at the rate of 12 % compared to wheat (4%) and maize (1%) (GoK, 2009). Most of the rice is grown in Central, Western and Coast provinces of Kenya (Grain, 2005).

In Kenya, continuous cropping without replenishing soil nutrients is a major problem resulting to declining soil fertility. Chemical fertilizers have been used to solve the low soil fertility problem. However, environmental pollution, caused by excessive soil erosion and use of chemical fertilizers to surface and groundwater, has caused serious environmental problems. There has been a growing interest in organic agriculture by environmentalists thus calling for possible alternatives to chemical-based, conventional agriculture.

Microorganisms are present in soil rhizosphere, rhizoplane and internal of the plant tissues (Hallmann *et al.*, 1997). To improve the growth and yield of agricultural crops, Plant Growth Promoting Rhizobacteria (PGPR) have been used as inoculants and therefore offer an attractive way to replace/supplement chemical fertilizers (Ashrafuzzaman *et al.*, 2009). Utilization of PGPR in order to increase the productivity may be a viable alternative as they also help in reducing the pollution and preserving the environment (Ştefan *et al.*, 2008). Plant Growth Promoting Rhizobacteria or combinations of PGPR and Arbuscular Mycorrhizal Fungi (AMF) can improve the nutrient use efficiency of fertilizers and allow reduced application rates of chemical fertilizers (Adesemoye *et al.*, 2009). The use of PGPR isolates as inoculants biofertilizers is beneficial for rice cultivation as they enhance growth of rice and by inducing other plant growth promoting traits (Ashrafuzzaman *et al.*, 2009). The direct promotion by PGPR entails either providing the plant with plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment (Joseph *et al.*, 2007).

The rice plants represent a habitat for diverse microorganisms, those at the phyllosphere, at the rhizoplane as well as those at the rhizosphere (Kowalchuk *et al.*, 2010). Rice fields comprise a rich diversity of plant growth-promoting bacteria (PGPB) such as N₂-fixers and phosphate-solubilizing bacteria (PSB) (Islam *et al.*, 2010). Rice plants are known to readily form mycorrhizal associations under upland conditions. Reports on the survival and colonization of rice roots by *Glomus etunicatum* (fungi) reveal their high adaptive ability (Barea, 1991). Vesicular arbuscular mycorrhizal fungi inoculation is known to enhance yields of rice (Secilia and Bagyaraj 1994).

The improvement of soil fertility is one of the most common strategies to increase agricultural production. The biological nitrogen fixation and Phosphate solubilization are very important in enhancing soil fertility (Rodriguez *et al.*, 2006). Plant hormones cause physiological responses such as growth and IAA (indole-3-acetic acid) is generally considered the most important native Auxin (Ashrafuzzaman *et al.*, 2009). Cereal plants require large amounts of mineral nutrients including N for their growth, development and grain production. Supply of nitrogen to cereals is critical for attaining yield potential, which is a highly demanding key element since N cannot be stored in the roots. Nitrogen is a mobile element in the plant system and when the supply is suboptimal, plant growth is retarded

(Baset Mia and Shamsuddin, 2010). The ability to reduce and derive appreciable amounts of nitrogen from the atmospheric reservoir and enrich the soil is confined to bacteria (Young, 1992). These include symbiotic nitrogen fixing (N_2 -fixing) forms, and non-symbiotic N_2 -fixing forms. It is well established that nitrogen fixation by bacteria can promote the growth physiology or improve the root morphology of the rice plant (Jha *et al.*, 2009).

Phosphorus (P) is essential macronutrients for plants' growth and development. Most agricultural soils contain large reserves of total P but due to P fixation and precipitation which occur in soil, the concentration of soluble P in soil is usually very low (Fankem *et al.*, 2008) especially in sub Saharan Africa. Phosphate Solubilising Microorganisms (PSM) includes largely bacteria and fungi have provided an alternative solution in sustainable agriculture to meet the P demands of plants (Zaidi *et al.*, 2009). These microorganisms are capable of solubilising the insoluble inorganic P of soil and make it available to the plants. This is an important trait for increasing plant yields. The major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms (Salih, 1989). Soil contains a wide range of organic substrates, which can be a source of P for plant growth. To make this form of P available for plant nutrition, it must be hydrolyzed to inorganic P. Phosphatase enzyme is responsible for mineralization of most organic phosphorous compounds. Some phosphate-solubilizing bacteria behave as mycorrhizal helper bacteria (Frey-Klett, 1997). Phosphate-solubilizing bacteria have been shown to interact with vesicular arbuscular mycorrhizae (VAM) by releasing phosphate ions in the soil, which causes a synergistic interaction that allows for better exploitation of poorly soluble P sources (Ray, 1981).

Auxin is the first phytohormone to be identified among phytohormones which play an important role in root system development and plants yield. It is involved in elongation of plant cells, tropism, apical dominance, root formation and root elongation and promotion of ethylene production. Indole -3-acetic acid (IAA) is the common natural auxin that shows all auxin activities and extensively affects plants physiology. (Etesami, *et al.*, 2009).

Diverse bacterial species possess the ability to produce the auxin phytohormone IAA. Accumulating evidence indicates that PGPR influence plant growth and development by the production of phytohormones such as auxins, gibberellins, and cytokinins (Saharan and Nehra, 2011). The use of PGPR isolates is beneficial for rice cultivation as they enhance the growth of rice by inducing IAA production (Ashrafuzzaman *et al.*, 2009).

To enhance and promote plant growth, at least one or more direct mechanism of action is used by the microorganisms. The exact mechanism may include asymbiotic N_2 fixation and solubilization of mineral phosphates and other nutrients as well as the ability to produce or change the concentration of plant growth regulators like IAA and ethylene, (Sajani and Muthukkaruppan, 2011). Knowledge on which microorganisms can perform these activities is crucial for sustainable rice cultivation since this will reduce the dependence on chemical fertilizers,. Therefore, the present study was undertaken to screen the indigenous microorganisms from rice growing regions of Kenya for their physiological characteristics, including P-solubilization, N-fixation and (IAA) production.

2 Materials and Methods

The 130 bacterial and 120 fungal isolates used for evaluation were distributed as in the table.

Table1: Isolates from various sources

Origin/Site	Number of isolates						
	Rhizoplane		Rhizosphere		Phyllosphere		
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	%
Mwea	29	31	5	6	9	7	34.8
Western	6	16	4	4	19	17	26.4
Coast	27	10	21	14	10	15	38.8
Total	62	57	30	24	38	39	100.0

2.1 Screening for Nitrogen Fixation Ability

The nitrogen-free (NFb) semisolid medium composed of 1 g K_2HPO_4 , 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g $CaCO_3$, 0.2 g NaCl, 5 mg $FeSO_4 \cdot 7H_2O$, 10 g glucose, 5 mg $NaMoO_4$, 2.3 g of agar per liter at pH 7.0 was prepared. Five millilitres of the media were placed in 10 ml cultural tubes and the bacterial cultures were inoculated and incubated at 30°C for 48 hours. The headspace of the cultural tube was replaced with 10% C_2H_2 , and the tube was kept at 30°C for 12 hours. C_2H_4 production in the headspace was assayed using a Shimadzu gas chromatograph (GC-9A, Japan). The column temperature was 120°C while the injection/ detection temperature was 220°C. The carrier gas used was fluoride at 50 ml/ minute. A needle and syringe were used to pick 1 ml of the free space in the cultural tubes, which was then injected into the GC machine that gave a chromatograph showing retention time of 1.4 – 1.5 minutes. An un-inoculated tube of the NFb semi-solid medium was used as a control.

2.2 Screening for Phosphate Solubilization

The phosphate solubilization ability of the bacterial and fungal isolates was tested by plate assay using National Botanical Research Institute's phosphate (NBRIP) growth medium (Nautiyal, 1999). The medium contained in a litre ; 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2$, 0.25 g $MgSO_4$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ and 1.5% agar. The pH of the media was adjusted to 7.0. The plates were incubated at 28°C. Formation of visible halo zones around the microbial colonies/structures in plates containing NBRIP media was an indication of the phosphate solubilization ability of the microorganisms. The halo and colony/structure diameters were measured at 7 and 14 days after inoculation. Halo size was calculated by subtracting colony/ structure diameter from the total diameter.

2.3 Screening for Indole Acetic Acid Production

Detection of IAA production was done as described by Brick (Brick *et al.*, 2004). The pure bacterial and fungal cultures were inoculated and incubated for 48 hours and 72 hours respectively on their respective liquid media at 30°C and 28°C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 m $FeCl_3$ solution). Development of pink colour after 0.5-2 hours incubation at room temperature indicated IAA production.

3 Results

3.1 Nitrogen Fixation Ability

N₂-fixing ability was evaluated and the isolates grouped into 5 groups; Non-fixers (0 nmol of C₂H₄/tube/12h), Low (0.1-20nmol of C₂H₄/tube/12h), Intermediate (21-40 nmol of C₂H₄/tube/12h), High (41-60 nmol of C₂H₄/tube/12h) and Very high (>60 nmol of C₂H₄/tube/12h). All the (30) rhizospheric isolates from the 3 sites showed ARA (Figure 1a).

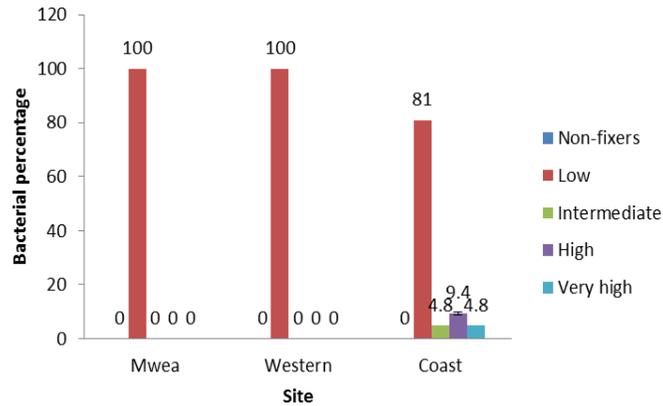


Figure 1a: Acetylene reduction activity by rhizosphere bacterial isolates

Non-fixers = 0, Low = 0.1-20, Intermediate = 21-40, High = 41-60, Very high >60 nmol of C₂H₄/tube/12h

Most of these isolates were low fixers with only Coastal region isolates which had fixing ability in the other groups. Over 70% rhizoplane bacteria from the 3 sites were able to fix nitrogen. All the isolates from Western region (Figure 1b) had the potential for ARA though at low level as opposed to Mwea and Coastal regions.

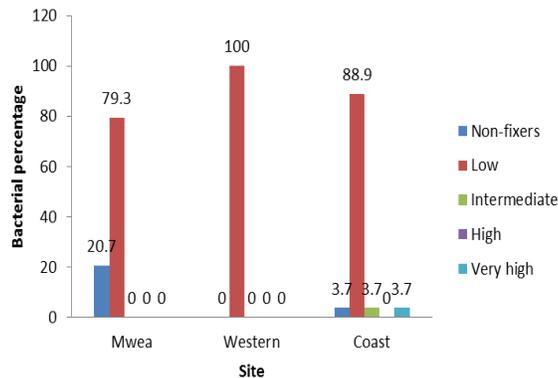


Figure 1b: Acetylene reduction activity by rhizoplane bacterial isolates

Non-fixers = 0, Low = 0.1-20, Intermediate = 21-40, High = 41-60, Very high >60 nmol of C₂H₄/tube/12h

At low levels, over 50% of the phyllosphere bacteria from the 3 sites were able to fix nitrogen (Figure 1c).

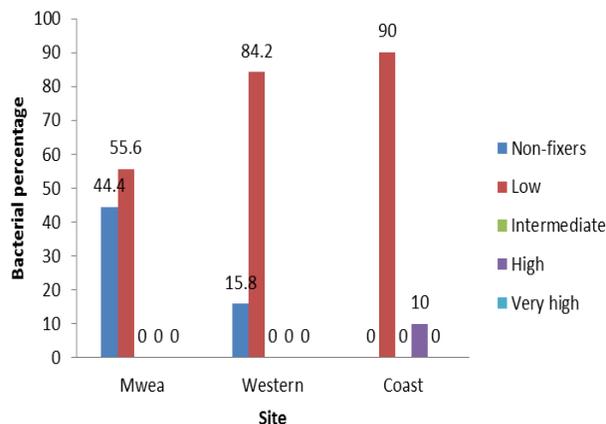


Figure 1c: Acetylene reduction activity by phyllosphere bacterial isolates

Non-fixers = 0, Low = 0.1-20, Intermediate = 21-40, High = 41-60, Very high >60 nmol of C₂H₄/tube/12h

3.2 Phosphate Solubilization Ability

Ninety nine out of 130 bacterial isolates were able to solubilize phosphates while only 21 out of 120 fungal isolates produced halos both in NBRIP growth medium.

Based on the results, the isolates were classified into 4 groups depending on the halo size; Non-solubilizers (0 mm), Low (1-10 mm), Intermediate (11-20 mm) and High (≥ 21 mm) solubilizers. Phosphate solubilization was most frequently encountered in the rhizospheric bacteria where Mwea and Coastal regions had 100% solubilization ability distributed within 2 groups (intermediate and high) (Figure 2a).

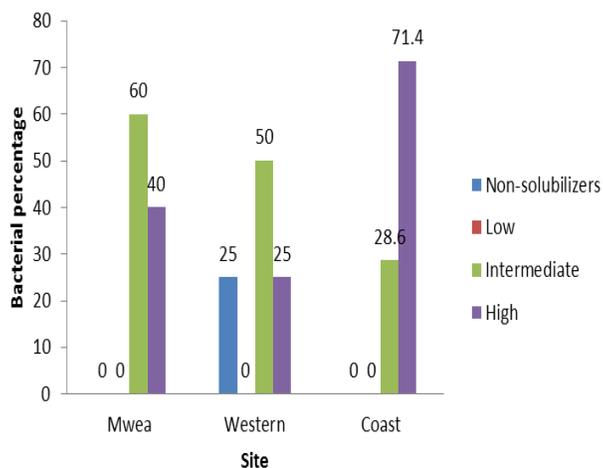


Figure 2a: Rhizospheric P-solubilization by bacterial isolates

Non-solubilizers = 0 mm, Low = 1-10 mm, Intermediate = 11-20 mm, High ≥ 21 mm; Halo size

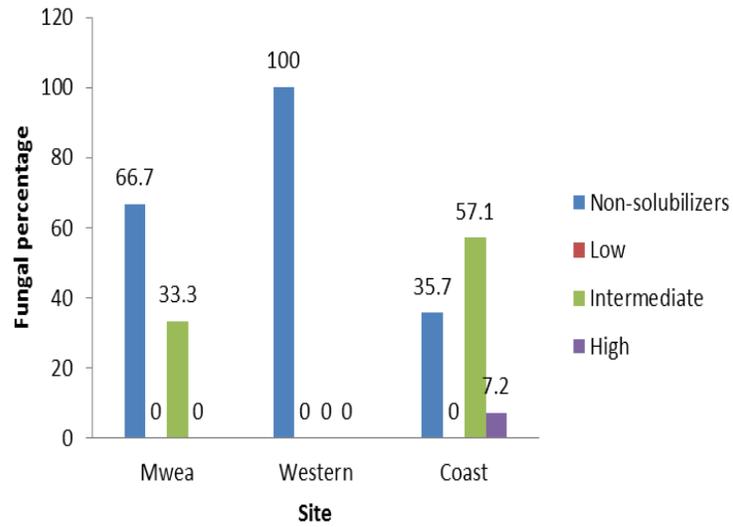


Figure 2b: Rhizospheric P-solubilization by fungal isolates

Non-solubilizers = 0 mm, Low = 1-10 mm, Intermediate = 11-20 mm, High \geq 21 mm; Halo size

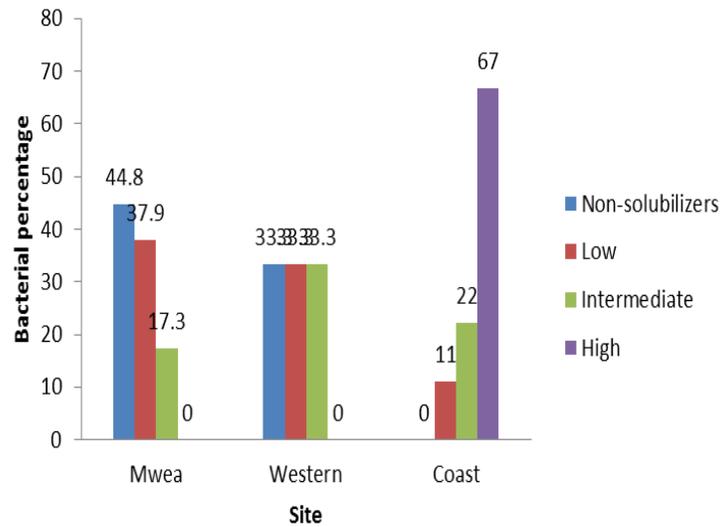


Figure 2c: P-solubilization by rhizoplane bacterial isolates

Non-solubilizers = 0 mm, Low = 1-10 mm, Intermediate = 11-20 mm, High \geq 21 mm; Halo size

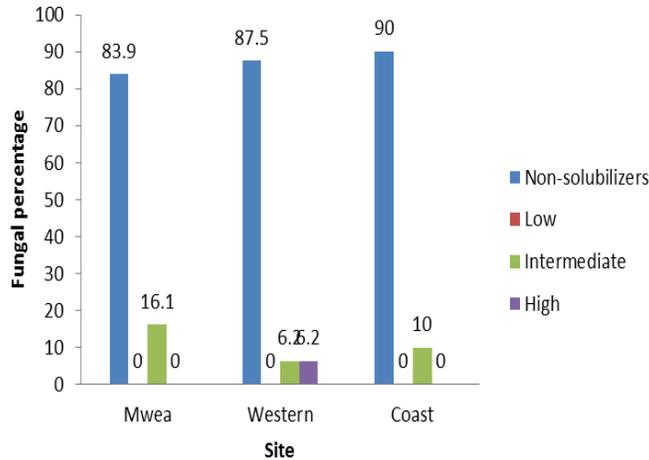


Figure 2d: P-solubilization by rhizoplane fungal isolates

Non-solubilizers = 0 mm, Low = 1-10 mm, Intermediate = 11-20 mm, High \geq 21 mm; Halo size

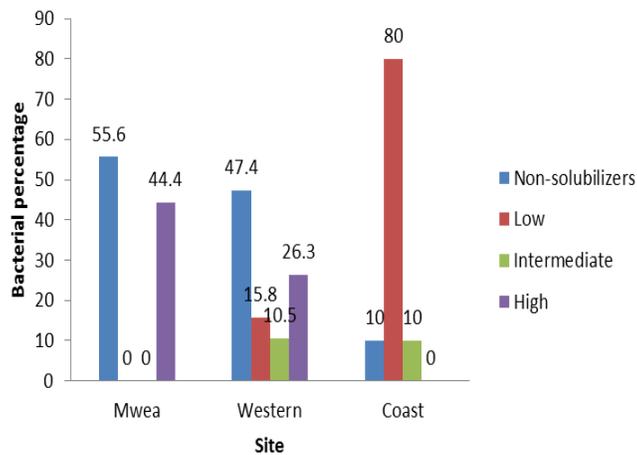


Figure 2e: P-solubilization by phyllosphere bacterial isolates

Non-solubilizers = 0 mm, Low = 1-10 mm, Intermediate = 11-20 mm, High \geq 21 mm; Halo size. Most of the rhizospheric fungi were non-solubilizers (Figure 2b) with Western region having 100% non-solubilization ability. All the 27 rhizoplane bacterial isolates from the Coastal region were able to solubilize phosphates as opposed to those of Mwea and Western region (Figure 2c).

More than 80% of soil fungi from all the 3 regions were non-phosphate solubilizers while Mwea had the highest percentage (16.1%) of soil fungi which could solubilize phosphates (Figure 2d).

Western region had its phyllosphere bacterial isolates distributed within all the 4 groups (Non-solubilizers, Low, Intermediate and High solubilizers), with the majority (47.4%) being non-solubilizers (Figure 2e) as opposed to Mwea and Coastal regions.

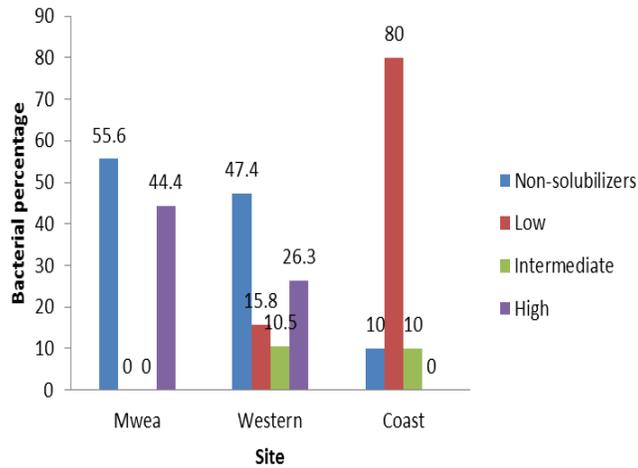


Figure 2e: *P*-solubilization by phyllosphere bacterial isolates

Non-solubilizers = 0 mm, Low = 1-10 mm, Intermediate = 11-20 mm, High \geq 21 mm; Halo size. All the phyllosphere fungal isolates from Mwea and Coastal regions as well as over 80% from Western were non-solubilizers (Figure 2f).

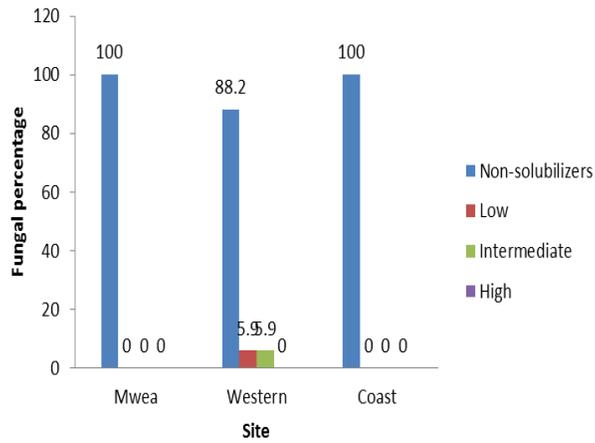


Figure 2f: *P*-solubilization by phyllosphere fungal isolates

Non-solubilizers = 0 mm, Low = 1-10 mm, Intermediate = 11-20 mm, High \geq 21 mm; Halo size

3.3 Indole Acetic acid production Ability

The ability of the microorganisms to produce IAA was detected by the development of pink colour after the addition of salkowski reagent to the cultures as shown in Figure 3.



Figure 3: Isolates showing differences in ability for IAA production

All the Mwea rhizospheric bacterial isolates and 75% of Western isolates were unable to produce IAA whereas over 70% (Figure 4a) of those from the Coastal region were IAA producers.

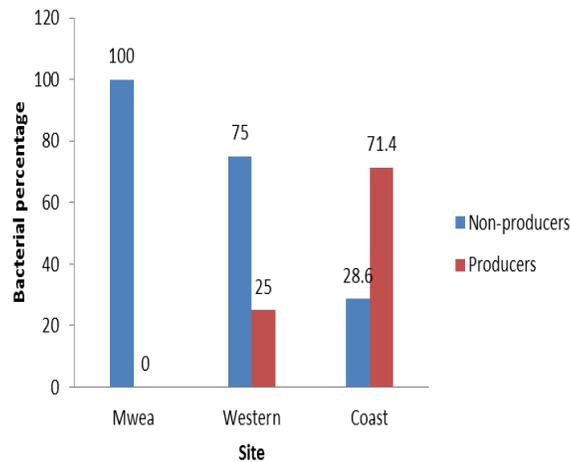


Fig 4a: Rhizospheric IAA production by bacterial isolates

Hundred percent of the fungal isolates (Figure 4b) from the 3 regions were unable to produce IAA.

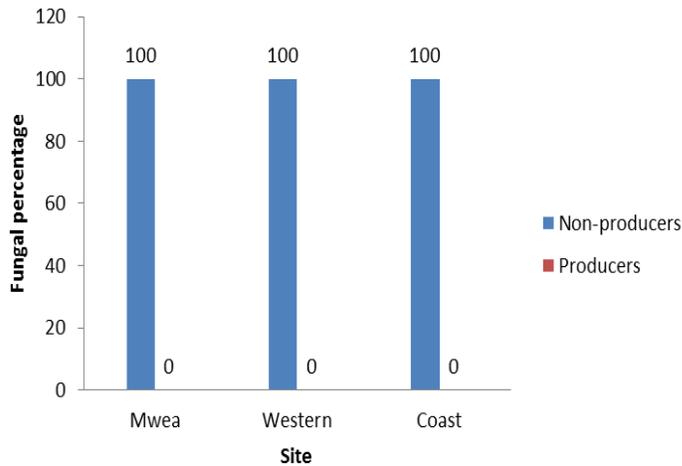


Fig 4b: Rhizospheric IAA production by fungal isolates

The rhizoplane of the 3 regions contained both the IAA bacterial producers and non-producers at varying intensities (Figure 4c). Western region had more (83.3%) IAA producers than non-producers (16.7%) as opposed to the other 2 regions.

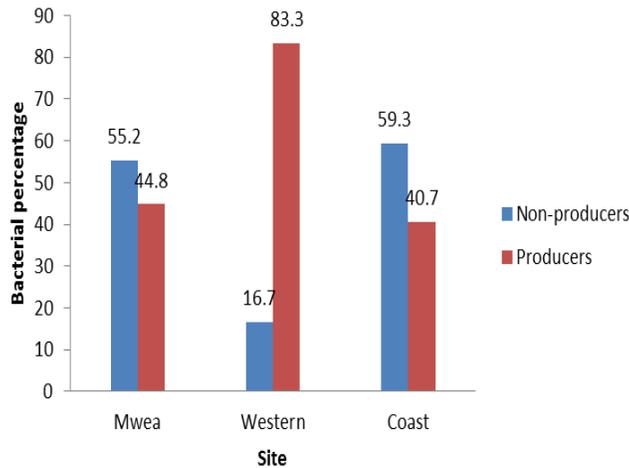


Figure 4c: IAA production by rhizoplane bacterial isolates

Fungal non-producers dominated the rhizoplane of the 3 regions (over 90%) with Coastal region having no IAA producer (Figure 4d).

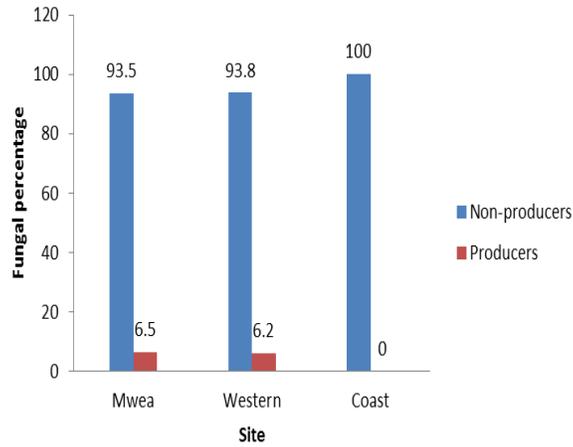


Figure 4d: IAA production by rhizoplane fungal isolates

The composition of phyllosphere bacterial isolates was very minimal with the exception of Coastal region where the IAA producers were 40% (Figure 4e).

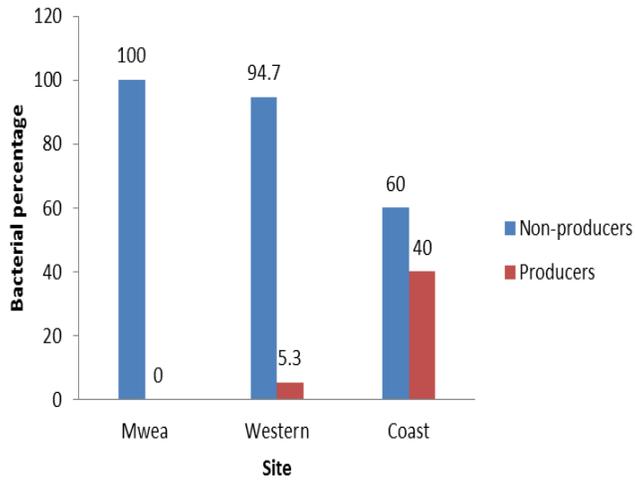


Figure 4e: IAA production by phyllosphere bacterial isolates

Over 80% of the phyllosphere fungal isolates were non-producers with Mwea region having 100% IAA non-producers (Figure 4f).

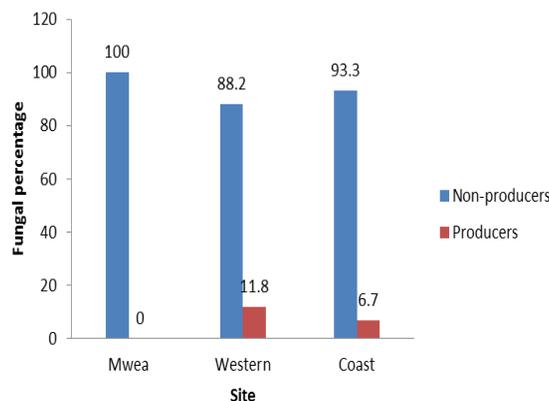


Figure 4f: IAA production by phyllosphere fungal isolates

4 Discussion

Acetylene reduction assay (ARA) is a measure of nitrogenase activity equivalent to the measure of the total amount of nitrogen fixed by an organism. To assess nitrogenase activity in natural isolates, the method which is widely used is the reduction of acetylene (C_2H_2) to ethylene (C_2H_4). The usefulness of ARA methodology in screening plants and microorganisms for presence of nitrogenase activity is beyond doubt (Zafar *et al.*, 1986). The capability of rhizosphere, phyllosphere and rhizoplane bacterial isolates from Mwea, Western and Coastal regions to fix nitrogen was examined based on isolates ability to reduce (C_2H_2) to (C_2H_4). There was a wide range of variation in nitrogenase activity among the 130 isolates tested in accordance to the regions and samples. All (30) rhizospheric isolates from the 3 regions showed ARA ability (Figure 1a) albeit at different levels. All (100%) of the Mwea and Western regions isolates were low fixers as well as 81% of the Coastal isolates. The remaining 29% of the Coastal isolates were distributed in the other 3 groups (Intermediate, High and very high). The rhizosphere is known to be a biologically active zone that contains soil-borne microbes including bacteria and fungi. Despite of the ability of rhizoplane isolates to fix nitrogen in the 3 regions, most (> 70%) of them were low fixers (Figure 1b). These findings are similar to those of Saharan and Nehra, (2011) who reported a large number of microorganisms such as bacteria and fungi coexist in the rhizosphere and that bacteria are the most abundant among them.

In this study most (> 50%) of the phyllosphere bacterial isolates from the 3 regions also showed some ARA as low fixers (Figure 1c). However, most of the non-fixers microbes in the 3 regions were detected in the phyllosphere (Figure 1c) where Mwea and Western regions had 44.4% and 15.8% respectively opposed to the rhizoplane (Figure 1b) where Mwea and Coastal regions had 20.7% and 3.7% respectively and the rhizosphere (Figure 1a) which had 0% non-fixers. The results indicate that the rhizosphere and the rhizoplane microbes have better ARA than those at the phyllosphere. Knief *et al.*, (2011) detected genes encoding dinitrogen reductase and dinitrogenase in the rhizosphere and the phyllosphere metagenome with a few peptides of dinitrogenase reductase identified in the rhizoplane. From their observation, the nitrogen fixing bacteria are present in the rhizosphere, rhizoplane as well as the phyllosphere.

Soil microorganisms are known to be effective in releasing P from inorganic complexes through solubilization. According to Rodríguez and Fraga (1999) the halos formed around the colonies is as a result of pH drop produced by the release of organic acids, which are responsible for phosphate solubilization. In this study most (99) of the bacterial isolates from the 3 regions and 21 fungal isolates were able to solubilize phosphate. The rhizospheric bacteria from Mwea and Coastal regions had 100% solubilization ability (Figure 2a) whereas 25% from Western region were non-solubilizers. These results show that rhizospheric bacteria have the ability to solubilize precipitated phosphates as reported by Verma *et al.*, (2001). Rhizospheric fungi from

Western region were non-solubilizers (Figure 2b) while those from Coast and Mwea were able to solubilize 64.3% and 33.3% respectively. Ability of the rhizoplane bacterial isolates from the Coastal region to solubilize phosphates was 100% (Figure 2c) compared to Western (66.6%) and Mwea (55.2%). Mwea had the highest percentage (16.1%) of rhizoplane fungi which could solubilize phosphates (Figure 2d) compared to Coastal and Western regions. Over 50% of the phyllosphere bacterial isolates from Western and Coastal regions were able to solubilize phosphates (Figure 2e) whereas over 80% of the phyllosphere fungal isolates from the 3 regions were non-solubilizers (Figure 2f). Even though the fungal isolates were able to solubilize phosphates to some extent, they were not comparable to the bacterial isolates. In general the bacterial isolates from the rhizosphere and rhizoplane were more efficient in solubilizing phosphate than the fungal isolates. These results are consistent with those of Alexander (1977) who reported that there are considerable populations of phosphate-solubilizing bacteria in soil and in plant rhizosphere. According to Johri *et al.*, (2003) in addition to rhizobacteria, several fungi such as species of *Aspergillus* can efficiently solubilize P. They also claimed that almost all phosphate solubilizing microorganisms when multiplied on a simple 'C' source, they produce gluconic, glycolic and 2-ketogluconic acids among others in the medium which are responsible for P solubilization.

Out of the many phytohormones, IAA is generally considered to be the most important native auxin. According to Leinhos (1994) bacterial biosynthesis of IAA is known in many rhizobacteria and it is believed that approximately 80% of rhizospheric bacteria can secrete IAA. However in the present study, over 70% (Figure 4a) of rhizospheric bacterial isolates from the Coastal region were IAA producers whereas 75% of Western isolates and all the Mwea isolates were unable to produce IAA. Hundred percent of the rhizospheric fungal isolates (Figure 4b) from the 3 regions were unable to produce IAA. Compared to the rhizospheric bacterial isolates, the rhizoplane bacteria had greater percentage of IAA producers (Figure 4c). These results differed those of Sarwar and Kremer (1992) who reported that isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil. The phyllosphere bacterial and fungal isolates from the Western and Coastal regions produced IAA in small percentages (Figure 4e and 4f). This is in agreement with earlier report of Lindow and Brandl, (2003) who stated that despite of studies focusing on rhizospheric and endophytic plant hormone-synthesizing bacteria, epiphytes are also known to produce such substances.

5 Conclusion

Results suggest that some of the indigenous bacteria and fungi were able to fix nitrogen, solubilize phosphates and induce the production of IAA. However the ability to perform these plant growth promoting activities differed among the tested bacterial and fungal isolates. Most of the bacterial isolates from the rhizosphere, rhizoplane and the phyllosphere had ARA though at low levels. Bacterial isolates from the rhizosphere and rhizoplane had shown to be efficient in P solubilization whereas the fungal isolates were mostly non-solubilizers. Although the percentage for IAA production by the tested isolates was not high, the performance of the bacterial isolates was better than that of the fungal isolates.

The indication of these results is that, these microorganisms have the potential to be utilized as microbial inoculants (biofertilizers) to replace/substitute chemical fertilizers for sustainable rice cultivation in these regions.

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