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Isolation, Characterization and Identification of Plant Growth-Promoting Rhizobacteria

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Abstract Continuous application of inorganic fertilizers affects the soil conditions and the environment. Thus, recently, agricultural production relies on a more sustainable and eco-friendly approaches like biofertilizers which is an effective alternative for augmenting nutrient supply in the soil. In this study, a total of 25 nitrogen-fixing bacterial strains were isolated and screened for their growth-promoting activities such as indole-3-acetic acid (IAA) production, phosphate solubilization, and starch hydrolysis. Fifty-five isolates produced IAA, 23 solubilized inorganic phosphate, and only 5 hydrolyzed starch. Five promising isolates were selected based on their ability to produce IAA and solubilize inorganic phosphate. Four of the five selected isolates were identified as: *Lactobacillus kefiri, Rothia amarae, Bacillus pumilus* and *Bacillus coagulans* using BIOLOG GEN III Microbial Identification System. Under laboratory conditions, inoculation with N300-21 and *Lactobacillus kefiri* significantly increased the oven dry weight of 14 day-old rice seedlings by 133% relative to uninoculated control. The ability of the selected rhizobacteria to produce growth-promoting compounds, and the significant increase in the growth of rice seedlings due to inoculation suggests its potential as plant growth-promoting inoculant for rice. However, further evaluation of the identified isolates under screenhouse and field conditions is recommended to uncover their efficacy as effective microbial inoculant.

Keywords: rhizobacteria, plant growth-promoting bacteria, Biolog GEN III Microbial Identification System, microbial inoculant, biofertilizers

Introduction

Rice (*Oryza sativa L.)* is grown in different parts of the world which feeds more than half of the world's population (Gonzales, 2004). It is very high in complex carbohydrates and contains almost no fat and cholesterol. In general, rice is considered good source of vitamins, minerals, and some essential amino acids (PhilRice, 2010). However, rice production is affected by

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various factors including the application of fertilizers which are expensive and not eco-friendly.

Due to the possible consequences of increasing use of chemical fertilizers, there is a growing interest in alternative strategies to ensure competitive yields of crops and at the same time maintaining the long-term ecological balance of the soil ecosystem. In this context, the use of soil microorganisms as microbial inoculant in agriculture is considered as one alternate source to meet the nutrient requirement of crops. Soil microorganisms are very important as almost every chemical transformation taking place in soil involves active contributions from them (Yadav and Kumar, 2007). They stimulate plant growth through mobilizing nutrients in soils, producing numerous plant growth regulators, protecting plants from phytopathogens, improving soil structure and bioremediating the polluted soils (Ahemad and Kibret, 2013). Bacteria colonizing around or in the plant roots are referred to as rhizobacteria which are more versatile in transforming, mobilizing and solubilizing the nutrients compared to those from bulk soils (Hayat *et al*., 2010). Glick (2012) reported that the rhizobacteria are the dominant driving forces in recycling the soil nutrients and consequently, they are crucial for soil fertility.

Plant growth promoting rhizobacteria (PGPR) are heterogeneous group of rhizobacteria in association with roots which can improve the extent or quality of plant growth directly or indirectly. The direct promotion by PGPR entails either providing the plant with growth-promoting substances that is synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPRs lessen or prevent the deleterious effect on one or more phytopathogenic microorganisms (Ahemad and Kibret, 2013). According to Kloepper and Schroth (1981), PGPR-mediated plant growth promotion occurs by the alteration of the whole microbial community in rhizosphere niche through the production of various substances such as IAA, gibberellins, siderophore; and through mechanism of different growth-promoting traits such as phosphate solubilization and antifungal activity.

With this, there is a continued interest on these microorganisms for use as microbial inoculant in agriculture due to their potential applications in plant growth and development. Thus, the present study was designed to screen and identify rhizospheric bacterial isolates with multiple plant growth-promoting properties. This study represents a unique opportunity to look for promising growth-promoting bacteria that can improve plant health and contribute to higher crop yield.

Materials and methods

Isolation of Bacteria from the Rice Rhizosphere

Bacteria were isolated from rice root samples collected from the screenhouse of the Philippine Rice Research Institute, Maligaya, Science City of Muňoz, Nueva Ecija. Samples were collected at the early growth stage, active tillering and panicle initiation stages of rice. Rhizosphere samples were collected by removing the roots and shaking the adhering soils into polyethylene bag.

Serial Dilution and Spread Plate Method

By means of aseptic technique, rhizospheric soil was serially diluted to make a series of ten-fold dilutions. Then, 0.1 mL of 10^{-3} and 10^{-4} dilutions was spread on duplicate Burk's agar plates. The plates were incubated at room temperature and morphologically different colonies appearing on the medium were isolated and sub-cultured for further analysis. The different bacterial isolates were cultured in a nitrogen-free broth and agar medium.

In vitro Screening of Rhizobacteria for their Growth-Promoting Activities

Indole-3-acetic acid (IAA) production

To measure the IAA production of bacteria, a loopful of the bacterial isolates were inoculated in a nitrogen-free broth. After 7 days of incubation, the cultures were centrifuged for 10 min at 13,000 rpm in 4^{o} C. The IAA in the supernatant was, then, detected colorimetrically. One mL of the supernatant was reacted with 2.0 mL Salkowski's reagent. Pink to red color transformation indicated positive reaction. Then, the absorbance was read at 590 nm in a spectrophotometer and the quantity of IAA produced was estimated against the IAA standard.

Phosphorus solubilization

Phosphate solubilization activity of the isolates was investigated using the modified Pikovskaya's medium. The bacterial isolates were spot-inoculated onto the surface of the agar. Clearance or halo zone around the bacterial growth or colony indicates phosphate solubilization. The presence of clearing zone around the bacterial growth as the indicator of P solubilization was noted after seven days of incubation.

Starch hydrolysis

The bacterial isolates were streaked onto the surface of the agar. After 24 hours of incubation, the surface of the plate was, then, submerged with iodine to detect the presence or absence of starch in the vicinity around the bacterial growth. Microbial starch hydrolysis was revealed as a clearing zone surrounding the bacterial growth (Collins *et al,.* 1995).

Selection and identification of bacteria

The five isolates were selected based on their IAA production and phosphorus solubilization properties. Only those with intense IAA production and positive (+) phosphorus solubilization activity were chosen. They were identified using biological and biochemical tests. Identification was done at the Philippines Center for Postharvest Development and Mechanization (PhilMech) Science City of Muňoz, Nueva Ecija.

BIOLOG GEN III System Analysis

The selected isolates were further characterized using BIOLOG GEN III System. An unknown bio-pattern was compared to the database of reactions for each taxon, and a numerical probability calculation was performed. Various qualitative levels of identification were assigned based on the numerical probability calculation. Test data from an unknown organism was compared to the respective database to determine a quantitative value for each of the database taxa. Each of the composite values was compared to the others to determine if the data were sufficiently unique or closed to one or more data taxa. If a unique identification pattern is not recognized a list of possible organism was given, or the strain is determined to be outside the scope of the database.

Evaluation for the Effectiveness of Selected Bacteria in Enhancing Rice Growth

Magenta jar set-up was used to test the effectiveness of five selected bacteria in enhancing the growth of rice seedlings. The materials used in the set-up were sterilized. Pre-germinated and surface-sterilized rice seeds were planted and inoculated with 1 mL seven day old bacterial broth culture per seedling. Plant height was measured at 7 and 14 days after sowing (DAS). Root length and oven dry weight of the seedlings were also measured at 14 DAS. Data gathered were subjected to statistical analysis using SAS.

Results

Isolation of Bacteria from the Rice Rhizosphere

Fifty-five nitrogen-fixing bacterial isolates were obtained from the rhizosphere of rice samples (Table 1). Burk's medium was used in the isolation and purification of nitrogen-fixing bacteria. The high number of isolates from the rhizosphere indicates that rice soils have numerous nitrogen-fixing bacteria that can enhance plant growth.

In vitro Screening of Rhizobacteria for their Growth-Promoting Activities (GPA)

Indole-3-acetic acid production

All bacterial isolates produced IAA indicating that they have the ability to convert tryptophan into IAA (Table 1). IAA production was manifested by the change in color of the broth culture from pink to red. The intensity of color also served as a basis in estimating the amount of IAA produced, i.e. yellow means no IAA production and intense pink or red means high IAA produced. In terms of intensity of IAA production, 19 were low, 19 were medium and 17 were high.

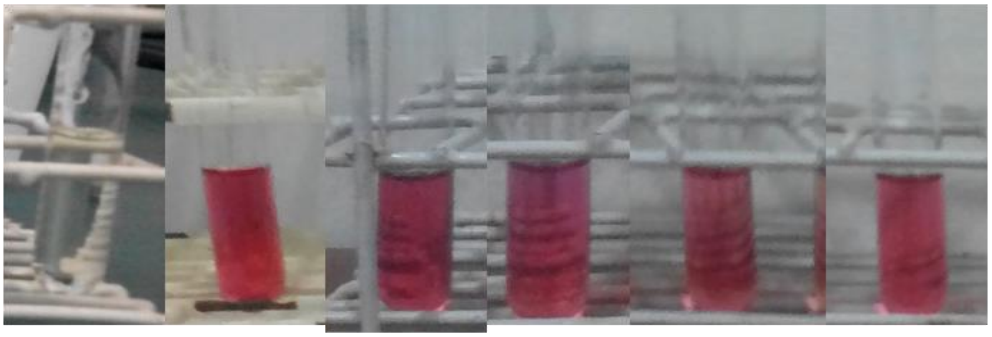

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Figure 1. Indole-3-acetic acid (IAA) production in NFB with Salkowski's reagent (A. control; B. N144-44; C. N300-21; D. N300-41; E. N450-22 and F. N455-11 with red-high IAA production)

Phosphate Solubilization

Twenty-three out of fifty-five isolates were positive in phosphorus solubilization (Table 2). Figure 2 presents phosphorus solubilization activity of the bacterial isolates. Phosphorus solubilization was manifested by the

formation of clearing zones on Pikovskaya's medium. The formation of clearing zone may be attributed to the production of organic acids by the bacteria. These observations imply that rice soils have bacteria that can help increase P availability in the soil and can enhance plant growth.

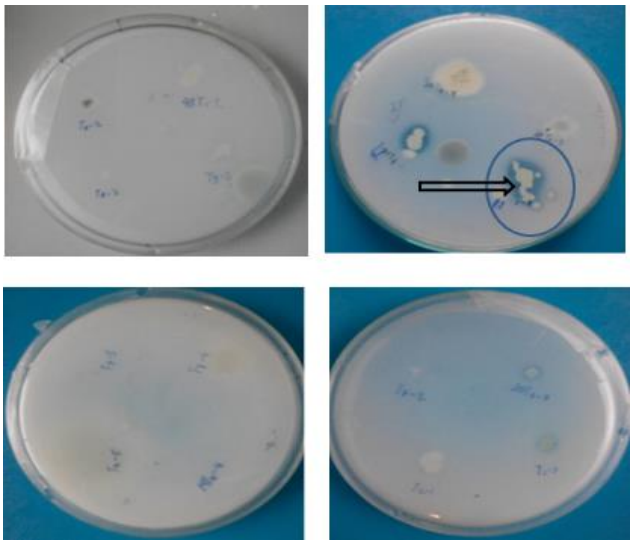

Figure 2. Phosphate solubilization on Pikovskaya's agar medium.

Starch Hydrolysis

Five of the 55 isolates screened were able to hydrolyze starch (Table 1). This test is used to identify bacteria that can hydrolyze starch using the enzymes α-amylase and oligo-1, 6-glucosidase. It is often used to differentiate species from the genera *Clostridium* and *Bacillus*.

A B C D E F

Figure 3. Starch hydrolysis assay on starch agar medium by 6 isolates (A. N146-41; B. N300-31; C. N303-22; D. N305-12; E. N302-22 and F. 30T1-31. A-E (+) can hydrolyze starch and F (-) cannot hydrolyze starch)

Table 1. Growth-promoting activities of the fifty-five bacterial isolates.

Isolate	Iaa Production	Phosphorus Solubilization	Starch Hydrolysis
1. N140-31	+++	+	-
2. N140-41	+++	-	-
3. N140-61	+++	-	-
4. N142-11	+	-	-
5. N142-41	+++	-	-
6. N143-21	++	-	-
7. N143-31	+++	-	-
8. N144-41	+++	+	-
9. N145-21	+	-	-
10. N145-31	+	-	-
11. N146-31	++	+	-
12. N146-41	+++	-	+
13. N146-51	++	-	-
14. N146-51	+++	-	-
15. N147-1	+	+	-
16. N147-33	++	-	-
17. N147-41	+	-	-
18. N147-51	+++	-	-
19. N300-11	+	-	-
20. N300-21	+	-	+
21. N300-31	+	-	-
22. N300-41	+++	+	-
23. N301-11	+	+	-
24. N301-21	++	+	-
25. N301-22	++	+	+
26. N301-32	+++	-	-
27. N302-11	+++	+	-
28. N302-31	+	+	-
29. N303-21	++	+	-
30. N303-22	+	-	+
31. N303-31	++	+	-
32. N303-41	++	-	-
33. N304-21	+	+	-
34. N304-41	+++	-	-
35. N305-11	+	+	-
36. N305-12	+++	-	+
37. N306-14	+	-	-
38. N307-11	+	+	-
39. N307-21	+++	+	-
40. N307-31	+	-	-
41. N307-41	+++	+	-
42.N450-11	++	-	-
43.N450-22	+++	+	-
44.N450-31	+	-	-

Table 1. Continued

Isolate	Iaa Production	Phosphorus Solubilization	Starch Hydrolysis
45. N451-11	++	-	-
46. N451-21	++	+	-
47. N451-31	++	+	-
48. N453-11	++	+	-
49. N453-21	++	-	-
50. N454-11	+	-	-
51. N454-21	++	+	-
52. N455-11	+++	+	-
53. N455-21	++	+	-
54. N457-11	+	+	-
55. N457-21	++	+	-

Selection and Identification of Selected Bacterial Isolates

Five isolates were selected on the basis of their growth-promoting activities for further study (Table 2). These isolates were positive for both IAA production and phosphate solubilization. Similarly, they must be fast growers as indicated by the thickness of their growth after one to two days after incubation in N-free agar plates. The isolates selected were: N144-41, N300-21, N300-41, N450-22 and N455-11 with corresponding IAA produced of 0.2723, 0.8284, 0.8685, 0.4275 and 0.3343 ppm.

Based on BIOLOG GEN III Microbial ID System, probable identities of the four selected isolates are as follows: *Bacillus pumilus (*N450-22*), Bacillus coagulans (*N300-41*), Lactobacillus kefiri (*N144-41*)* and *Rothia amarae (*N455-11*)*. One isolate (N300-21) was not identified by the system used. Complete biochemical details of the four selected isolates are shown in Table 3.

Table 2. Growth-promoting properties of the five selected isolates.

Isolate	Iaa Production (Ppm)	Phosphate Solubilization	Starch Hydrolysis
N144-41 (*Lactobacillus kefiri)*	0.2723	+	-
N300-21 (unidentified)	0.8284	+	-
N300-41 (*Bacillus coagulans)*	0.8685	+	-
N450-22 (*Bacillus pumilus)*	0.4275	+	-
N455-11(*Rothia amarae)*	0.3343	+	-

Table 3. Biochemical details of the four selected isolates using BIOLOG GEN III System.

Carbon Source	Selected Isolate			
	N144-41	N455-11	N450-22	N302-11
Dextrin	-	-	(+)	-
D-Maltose	-	-	-	+
D-Trehalose	-	-	+	+
D-Cellobiose	-	-	+	(+)
Gentiobiose	-	-	+	-
Sucrose	-	-	+	(+)
D-Turanose	-	-	-	-
Stachyose	-	-	(+)	-
D-Raffinose	-	-	(+)	-
α-D-Lactose	-	-	-	-
D-Melibiose	-	-	(+)	(+)
β-Methyl-D-Glucoside	-	-	+	-
N-Acetyl-D-Glucosamine	-	-	+	(+)
N-Acetyl-β-Mannosamine	-	-	-	(+)
N-Acetyl-D-Galactosamine	-	-	-	-
N-Acetyl Neuraminic Acid	-	-	-	-
α-D-Glucose	-	-	(+)	+
D-Mannose	-	-	(+)	+
D-Fructose	-	-	+	+
D-Galactose	-	-	+	(+)
3-Methyl Glucose	-	-	-	-
D-Fucose	-	-	(+)	-
L-Fucose	-	-	-	-
L-Rhamnose	-	-	-	-
Inosine	-	-	(+)	-
D-Sorbitol	-	-	-	-
D-Mannitol	-	-	+	-
D-Arabitol	-	-	-	-
myo-Inositol	-	-	-	-
Glycerol	-	-	+	(+)
D-Glucose-6-PO_4	-	-	-	-
D-Fructose-6-PO_4	(+)	+	(+)	-
D-Aspartic Acid	-	-	+	-
D-Serine	-	-	-	-

+ positive; - negative; (+) mismatched positive; (-) mismatched negative

Table 3. Continued

Carbon Source	Selected Isolate			
	N144-41	N455-11	N450-22	N302-11
Gelatin	-	-	(+)	-
Glycyl-L-Proline	-	-	(+)	-
L-Alanine	-	-	+	-
L-Arginine	-	-	+	-
D-Saccharic Acid	-	-	-	-
p-Hydroxyl-Phenylacetic Acid	-	-	-	-
Methyl Pyruvate	-	-	+	-
D-Lactic Acid Methyl Ester	-	-	-	-
L-Lactic Acid	-	-	(+)	-
Citric Acid	-	-	+	-
α-Keto-Glutaric Acid	-	-	(+)	-
D-Malic Acid	-	-	(+)	-
L-Malic Acid	-	-	+	-
Bromo-Succinic Acid	-	-	(+)	-
Tween 40	-	-	(+)	-
y-Amino-Butyric Acid	-	-	+	-
α-Hydroxyl-Butyric Acid	-	-	-	-
α-Hydroxyl-D,L-Butyric Acid	-	-	-	-
α-Keto-Butyric Acid	-	-	(+)	-
Acetoacetic Acid	(+)	+	(+)	-
Propionic Acid	-	-	(+)	-
Acetic Acid	-	-	(+)	-
Formic Acid	-	-	-	-
Identity	*Lactobacillus kefiri* (28%)	*Rothia amarae* (24%)	*Bacillus pumilus* (65%)	*Bacillus coagulans* (35%)

+ positive; - negative; (+) mismatched positive; (-) mismatched negative

Evaluation of the Effectiveness of Selected Bacteria in Enhancing Rice Growth

Effectiveness of the selected isolates in enhancing the oven dry weight (ODW) and root length of rice seedlings at 14 DAS are shown in Figure 4. Inoculation with N300-21 and *Lactobacillus kefiri* significantly increased the oven dry weight by 100% relative to the uninoculated treatment. However, inoculation with *Rothia amarae, B. pumilus,* and *B. coagulans* showed no significant difference to the control. In terms of root length, inoculation with *L. kefiri,* increased the root length by 24% relative to uninoculated treatment.

Based on IAA production, *B.coagulans* produced the highest (0.8685 ppm) amount of IAA among the five selected isolates. The low performance of *B. coagulans* in terms of enhancing the ODW and root length of seedlings may not be related to its IAA production but may be attributed to the ability of the isolates to solubilize phosphorus. However, the amount of phosphorus solubilized by each isolate was not quantified in this study.

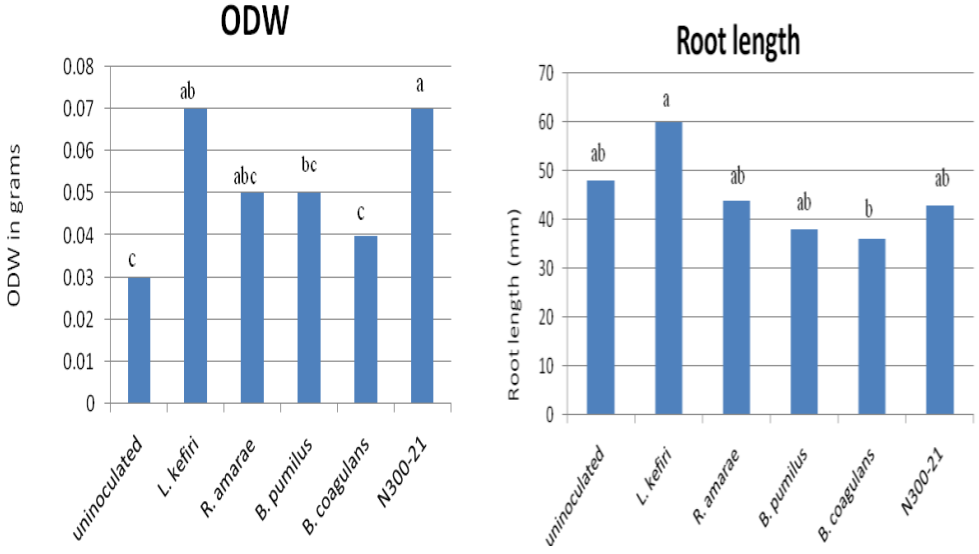

Figure 4. Oven dry weight and root length of 14 day-old rice seedlings as affected by the different bacterial isolates.

Discussion

In this study, isolates were considered nitrogen-fixers due to their ability to grow in Burk's medium which is selective for diazotrophs. Nitrogen-fixing bacteria are able to fix atmospheric nitrogen and grow when cultured on this nitrogen-free medium (HiMedia, 2016). The fifty-five isolates were found also to be IAA producers. These IAA producers are potential plant growth stimulants provided that tryptophan, the precursor of IAA, is available

(Marumo, 1986). The appearance of pink color in the broth culture was due to the complex produced by Fe-H_2SO_4 solution and IAA (Ali *et al*., 2013). Many microorganisms produce indole, which might have been responsible for the positive findings in colorimetric tests (Ahmad *et al.*, 2005).

In terms of phosphate solubilization, twenty-three isolates were found positive. It is generally accepted that the mechanism of mineral phosphate solubilization is associated with the release of low molecular weight organic acids, which, through their hydroxyl and carboxyl groups, chelate the cations bound to phosphate, thereby converting it into soluble forms (Kannapiran and Sri Ramkumar, 2011). Kannapiran and Sri Ramkumar (2011) added that phosphate solubilizing bacteria have the ability to release organic acids such as citric, glyoxalic, malic, ketobutyric, succinic, fumaric, and tartaric. The growth of phosphate-solubilizing bacteria (PSB) often causes soil acidification, which leads to phosphorus solubilization (Zhu *et al.*, 2011).

In starch hydrolysis 5 out of fifty-five isolates can hydrolyze starch. The iodine must be added to the starch agar in order to interpret the results of the starch hydrolysis test. Iodine reacts with the starch by forming a dark brown color. Consequently, hydrolysis of the starch will create a clearing zone around the bacteria (Robyt, 1998). The bacterial isolates are non-starch hydrolysing. This could be due to the low exoenzyme-producing ability of the isolated bacteria, making the bacteria unable to split starch into smaller subunits.

Five potential bacterial isolates were selected based on the screening results. The BIOLOG GEN III Microbial ID System is a new breakthrough in bacterial identification which enables testing of gram-negative and gram-positive bacteria in the same panel. This involves testing of bacterial isolates using 74 organic compounds as C-source. The gram-negative characteristics are common among nitrogen-fixing bacteria (Dhevendaran *et al*., 2013). Current evidence suggests that certain components present in these organic acids are involved in a variety of functions including the modulation of nutrient availabilities, increased tolerance to heavy metals, or attraction of rhizobacteria (Carvalhais *et al*., 2011).

The present results indicate that rhizosphere could be a good source of potential plant growth-promoting bacteria. Several bacteria were isolated and proved to be promising plant growth-promoters based on the series of screening conducted. Growth-promoting activities of these bacteria include IAA production, phosphate solubilization and starch hydrolysis. These characteristics are known to positively affect plant growth by various direct and indirect mechanisms. Furthermore, identified bacteria could be useful in the formulation of new inoculant in agriculture. However, further evaluation of the

identified isolates under screenhouse and field conditions is recommended to uncover their efficacy as effective microbial inoculant.

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