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Isolation and characterization of rhizospheric microbes of maize plant

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Abstract
Plant growth promoting rhizobacteria (PGPR) exert beneficial effects on plant growth by inhibiting the area around plant roots. For sustainable crop production in communities that cannot easily afford chemical fertilizers can isolate culturable PGPR that can be used as biofertilizers. This study is aimed in isolation and characterization of rhizospheric bacteria from rhizospheres of maize plants. Rhizosphere and rhizoplane samples were obtained for characterization of isolates. Selected 10 isolates were Gram stained which showed that all the isolates were gram positive and morphological characteristics were also observed. Biochemical tests such as Catalase, Urease, Amylase and Indole test were performed to make the characterization of isolates easy. All the ten isolates (ABRSM20, ABRSM21, RKRSM3, ABRPM10, ABRSM19, RKRSM2, ABRSM17, ABRPM32, ABRSM11 and ABRSM44) exhibited plant growth promoting ability. These bacteria can be used to develop bio-fertilizer inoculants for improved soil fertility management and sustainable production of local cereals. Further extended research should be performed to confirm the reliability of the results of the current study and application of this research in agricultural productivity.

Keywords: Rhizoshpere, rhizoplane, plant growth promoting rhizobacter (PGPR), Rhizosperic microbes

1. Introduction
In the current cultivation process unselective use of fertilizers, particularly the nitrogen and phosphorus, has directed to substantial pollution of soil, air and water. Unnecessary use of these chemical exerts deleterious effects on soil microbes, moves the fertility status of soil and also contaminates environment. The use of fertilizers on a long term basis frequently leads to fall in pH and replaceable bases, thus construction them unattainable to crops and the productivity of crop failures. To avoid this problem the rhizosphere microbes are used as plant growth promoters instead of chemical fertilizer, which are environmental friendly. They are involved in several biotic actions of the soil ecosystem to make it active for nutrient turn over and maintainable for crop production [1]. In latest years great attention has been paid to PGPR to reduce agrochemicals (fertilizers and pesticides) for the plant growth elevation by a variety of appliances. An understanding of plant growth promoting *rhizobacteria* and their connections with biotic and abiotic factors is crucial in bioremediation techniques. Moreover plant growth promoting *rhizobacteria* can diminish chemical fertilizers application and economically, environmentally useful for lower production cost as well as identify the best soil and crop administration practices to reach more sustainable agriculture as well as fertility of soil [2].

Phosphorous is the second important key element after nitrogen as a mineral nutrient in terms of quantitative plant requirement. The use of phosphate solubilizing bacteria as inoculants simultaneously increase P uptake by the plant and crop yield. The principal mechanism for mineral phosphate solubiliztion is the production of organic acids, and acid phosphatases play major role in the mineralization of organic phosphorous in soil [3]. Plant growth promoting *rhizobacteria* have the aptitude to fix atmospheric nitrogen and run it to plants by two appliances: symbiotic and non-symbiotic. The microbes first go into the root and later on form nodules in which nitrogen addition occurs. Rhizobia are a massive group of *rhizobacteria* that have the ability to place symbiotic interactions by the colonization and formation of root nodules with leguminous plants, where nitrogen is fixed to ammonia and mark it existing for the plant [4]

Several plant progression stimulating *rhizobacteria* can create cytokinins or gibberellins or both can create either cytokinins or gibberellins or both for plant growth promotion. However it seems that plant growth promoting bacteria produce lower cytokinins levels compared to phytopathogens so that the effect of the plant growth promoting *rhizobacteria* on plant growth is stimulatory while the consequence of the cytokinins after pathogen is inhibitory. The continual and careless applications of chemical P fertilizers, leads to the harm of soil fertility by disturbing microbial multiplicity, and consequently falling yield of crops. Phosphate solubilizing microorganisms PSM through various appliances of solubilization and mineralization are capable to convert inorganic and organic soil P correspondingly. This has lead to increased interest in the harnessing of microorganisms to support P cycling in agro-ecosystem [5]. *In vitro* study of PGPRs showed two most powerful strains that were able to promote the plant growth by two times. Interestingly they also denoted that when individually a strain is inoculated into the plants the effect of the strain is lower as in the un-inoculated plant. Another thing that came in the study as a novel happens, that the effect of the strains on plant growth was also dissatisfying it's real work just because of the absence of the green house. It was justified the experiment in the theory that was made in the green house and put the both together, and was checking the effect, though they were succeeded to found the promoted growth by means of two times as the phosphate solubilizing appeared more than that of the individual inoculants but not out of the green house. It was also seemed that the production of nodule fresh weight, nodule number and shoot nitrogen contents were much higher than the other treatments [6]. PGPR influenced the plant growth by direct or indirect modes [7]. Direct modes are production of growth stimulators, improvement in plant nutrient status (release of phosphates and micronutrients from insoluble sources), lowering of the ethylene level in plant, and induction of systemic resistance. Indirect modes of action are production of antibiotics, production of biocontrol agents and degradation of xenobiotics [8]. Phosphorus, the second major plant nutrient is an integral part of plants generally deficient in soils due to its speedy fixation [9]. Phosphate anions (H2PO4 -, HPO4 2-) are extremely reactive and form metal complexes with Ca in calcareous soils and Fe3+ and Al3+ in acidic soils [10]. These metal ion complexes precipitated the 80% of added P fertilizer. Phosphate solubilizing microorganisms (PSM) have attracted the researchers to exploit their potential to utilize phosphate reserves in semi arid regions and to enhance the crop yields. Phosphate solubilizing microorganisms have established their role for optimum growth of plants under nutrient imbalance conditions [11].

Material and Methods

Sample collection

The maize plant rhizosphere soil samples were collected from Abbottabad and Rawlakott agricultural sites. The samples were brought immediately to laboratory following standardized procedures.

Isolation of Rhizospheric samples

One gram of weighted sample of soil was taken and dissolved in tube having 9ml distilled water and mixed thoroughly. After that 1ml of dilution was transferred to another 9ml water tube to make 10^{-4}, 10^{-5} and 10^{-6}.

Rhizoplane Sample

Root was shed to remove adhering soil and kept attached particles on root. One gram of root was dipped in 9ml sterilized distilled water and one ml from dilution was taken from it to prepare the dilution up to 10^{-6}. After serial dilution, bacterial isolation was done by taking aliquot of 0.1ml through micropipette from dilution number 10^{-4} to 10^{-6} and transfer on L.B media in the Petri plates and spread through glass spreader. Plates were incubated at 37ºC for 24 hour.

Pure cultures of isolated bacteria

Pure-culture of grown bacterial colonies was obtained by sub-culturing on agar plates multiple times and plates were incubated at 37 ^{0}C for growth in an incubator. The purified cultures were preserved at 4 ^{0}C.

Gram Staining

Gram stains separate bacteria into two groups, gram negative and gram positive based on cell wall staining. Thin bacterial smear is treated with crystal violet followed by treatment with iodine which works as mordant. For decolorizing primary stain slide is then washed with 95% ethanol. Finally, the smear was counter stained with a basic dye safranin. Gram-positive bacteria preserved the crystal violet-iodine complex when washed with the decolorizer, however gram-negative bacteria dropped their crystal violet-iodine complex and developed colorless.

Isolation of Bacteria from Maize (*Zea mays*)

After the collection of maize sample, the bacteria were isolated according to the serial dilution method. Saline was made by dissolving 0.85gm of NaCl in distilled water and autoclaved for 15 mins at 121 °C and then cooled at room temperature. From this saline solution, 9mL each was transferred to autoclaved test tubes. Then 1gm of soil sample was suspended in the first test tube. Further dilutions ranging from 10^{-1} to 10^{-8} were made. On overnight prepared LB agar (Appendix I) plates, 100 µL of each dilution was spread. The petri dishes were incubated for 24 hrs, at 37 °C. Different bacterial colonies that appeared after 24 hrs, of incubation were picked. These colonies were then repeatedly sub cultured on LB agar plates to get single pure colony of bacteria. The pure colonies obtained were stored in 20% glycerol stock.

Results

Morphological Characterization

For colony morphology LB agar was used. Single bacterial colony were streaked on media and incubated for 24 hrs. at 37 °C. After the incubation, colony morphology of bacterial strains was studied including color, colony shape, edge shape and texture diameter.

Gram Staining

Gram stains separate bacteria into two groups, gram negative and gram positive based on cell wall staining. Isolates obtained were gram positive.

Morphological characterizatics

Strain ID	Form	Color	Margin	Elevation
ABRSM21	Punctiform	Off white	Erose	Flat
RKRSM3	Punctiforn	Off white	Undulate	Flat
ABRPM10	Circular	Milky	Entire	Flat
ABRSM19	Punctiforn	Milky	Erose	Flat
RKRSM2	Irregular	off white	Erose	Flat
ABRSM20	Punctiforn	Off white	Erose	Flat
ABRSM17	Punctiforn	off white	Undulate	Flat
ABRPM32	Circular	Brown	Undulate	Convex
ABRSM11	Irregular	Light yellow	Undulate	Convex
ABRSM44	Rhizoid	Milky	Filamentous	Flat

Biochemical Testing

Biochemical tests were performed for differentiating the bacterial population on the basis of their biochemical activities. Biochemical tests performed are:

Catalase Activity

Obligate aerobes and facultative anaerobes usually have the enzymes superoxide dismutase, which catalyzes the damage of superoxide, and either catalase or peroxidase, which catalyze the damage of hydrogen peroxide. Catalase production and activity can be distinguished by addition of the substrate H_2O_2 to an appropriately incubated culture. Due to presence of catalase, released of free O_2 gas is observed in the form of bubbles which symbolize a positive catalase test; absence of bubble formation is a negative catalase test.

Urease Activity

Some bacteria are capable of producing an enzyme called Urease, using Urea establishing the end products ammonia, CO_2, and water. Urease activity is noticed by growing bacteria in a medium containing urea and using a pH indicator such as phenol red. Once urea was hydrolyzed, it resulted in pH change. This increased in pH caused the indicator to change medium to deep pink or purplish red and was a positive test for urea hydrolysis. No change in color was observed as a negative test.

Amylase (Starch Hydrolysis)

Many bacteria yield enzymes called hydrolases. The starch molecule contains two constituents' amylose and amylopectin. Both amylopectin and amylose are quickly hydrolyzed by definite bacteria, using their amylases; to yield dextrins, maltose, and glucose. Gram's iodine can be used to point out the presence of starch. When it links starch, it forms a blue to brown complex. Hydrolyzed starch does not create a color change. A clear zone formed after adding Gram's iodine to a medium containing starch and bacterial growth, amylase has been formed by the bacteria. No clear zone demonstrated that starch had not been hydrolyzed.

Indole Test

The bacteria contains the enzyme tryptophanase can hydrolyze tryptophane to its products, namely, Indole, pyruic acid, and ammonia. The bacteria use the pyruic acid and ammonia to satisfy nutritional need indole is not used for the same purpose and accumulates in the medium. The presence of indole can be detected by the addition of Kovacs reagent. Bacteria produced red layer after addition of Kovacs reagent to the medium.

Biochemical Test Results

Strain ID	Catalase	Ammonia	Pectinase	Amylase	Indole
ABRSM21	-ve	+ve	-ve	-ve	+ve
RKRSM3	+ve	-ve	-ve	+ve	+ve
ABRPM10	+ve	-ve	-ve	-ve	-ve
ABRSM19	-ve	-ve	-ve	+ve	+ve
RKRSM2	+ve	+ve	-ve	+ve	-ve
ABRSM20	-ve	+ve	-ve	-ve	-ve
ABRSM17	-ve	-ve	-ve	-ve	+ve
ABRPM32	+ve	+ve	-ve	-ve	-ve
ABRSM11	-ve	-ve	-ve	-ve	+ve
ABRSM44	+ve	+ve	-ve	+ve	+ve

Discussion

As the above conducted study is showing the different features among the different strains that were isolated from the rhizoplane and rhizosphere of the plant. Specificity in the selection of the area and the strains has plant growth promoting ability. Characterization of the bacteria based on biochemical activities creates multifunctional beneficial and mutualistic relationship between the plants and roots. Morphological characterization can be used to harness the harmful and dangerous or decomposing minerals that interlink during metabolic process form the soil. Shapes and size of the bacterial colonies plays an important role in selection of the right discipline from the soil that can if not be positive never could be defective.

Conclusion

This study showed that selected 10 strains (ABRSM20, ABRSM21, RKRSM3, ABRPM10, ABRSM19, RKRSM2, ABRSM17, ABRPM32, ABRSM11 and ABRSM44) have plant growth promoting ability. Further extended research should be performed to confirm the reliability of the results of current study.

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