Original Article

Comparative Analysis of Rhizospheric Bacteria Associated with Four Medicinal Plants

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ABSTRACT

Rhizosphere microbes contribute greatly to the growth, development and functioning of plants and trees. Microbes living in the rhizospheric area depend on the soil conditions and the products released by the plants. The objective of this work is to study microbes present in the rhizospheric region of Amla (*Phyllanthus emblica* L.) soil. A comparative study was also conducted among Amla (*Phyllanthus emblica* L.), Guava (*Psidium guajava* L.), Jamun (*Syzygium cumini* (L.) Skeels) and Neem (*Azadirachta indica* A. Juss.) growing on the same soil. The work indicates that the plant and its rhizosphere may have some effect on microbial diversity. The study on the rhizosphere of amla plant soil was undertaken to have a better understanding of the microbes present on the rhizosphere. Different microbes present in the *Phyllanthus emblica* (Amla) rhizospheric region. A large variation can be found in many other bacterial species. Details study in this regard will bring many interesting facts about plant-microbe interaction.

Key words: *Azadirachta indica*, Bacteria, Microbial, *Phyllanthus emblica, Syzygium cumini,* Rhizospheric.

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INTRODUCTION

A highly dynamic narrow region of soil which is directly associated with abiotic factors and soil microorganisms is known as rhizosphere.[1] In the rhizosphere area plant roots have strong effects on the abiotic environment. Plant soil provides the necessary constituents for microbes to thrive which shows that solute activity is higher in rhizospheric region. Increased interactions of microorganisms affect the soil structure of rhizosphere. These microbes perform oxidation reduction reactions in soil. Exudates, secondary metabolites, lysates, mucilages, mucigel are plant derived compounds which are responsible for providing additional carbon to the soil which helps the rhizosphere region to hold large variety of microorganisms. Microscopic techniques reveal different types of organisms present in the rhizopheric region. Most commonly found microorganisms associated with the rhizospheric region are bacteria, fungi, actinomycetes, algae, archae and protozoa. Microbes in the rhizospheric region provide many benefits such as nitrogen fixation, increased phosphorus uptake, defence against pathogen, providing essential macronutrients, chemical and physical modifications affecting plants, promote plant growth, root growth stimulation, abiotic stress control and control of diseases.[2] The soil samples collected from horticulture, farm forest and cropland revealed that among three soil types farm forest soil had higher soil organic carbon, micronutrient cations (Zn, Fe, Cu, Mn) and different microbial properties.[3,4] Rhizospheric microbes also known as biocontrol agents are effective, safety, and eco-friendly, manage plant diseases, boost plant growth due to which their demand gradually increases.[5] Rhizosphere soil collected from different planted species shows that number of bacteria and fungi increases with increase in the age of dump.[6] Plant and its rhizosphere contain a vast majority of microbial diversity which has many

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interesting facts about plant microbe interaction. The study on rhizospheric soils of Amla, Guava, Jamun and Neem were undertaken to have a better understanding on the microbes present in its rhizospheric area.

MATERIALS AND METHODS

Rhizospheric soil samples were collected from the experimental site (Campus garden, Department of Life sciences, Ramadevi Women's University, Bhubaneswar, Odisha).

Geographical Location of Experimental Site: Ramadevi Women's University is located at 20.29263 degree N 85.841589 degree E. It has 28 acres campus. It is situated in Bhubaneswar, Capital of Odisha. Bhubaneswar is located in the district of Khordha (Swain *et al*. 2018). It belongs to the eastern coastal plains, along the Eastern Ghats Mountains central line. Topographically, western uplands and eastern lowlands are separated from it.[7]

Climate Conditions of Experimental Site: Bhubaneswar has a tropical climate which lies on 45m above sea level. 27.4°C (81.3 °F) is the average mean temperature. The average annual rainfall is 1505 mm (59.3 inch).[8]

Soil Type of Experimental Area: Bhubaneswar has 65 percent laterite soil, 25 percent alluvial soil and 10 percent sandstone. Khordha district has three types of soils Alfisols, Ultisols, Entisols. Alfisols include alluvial and red loamy soil having pH from 6.5 to 7.3. Ultisols include laterite, red and yellow soils having pH 4.5 to 6.0. Entisols include coastal alluvial soils.[9]

Sterilisation: This process starts with cleaning of equipments. Equipments such as beaker, conical flask, petriplates, measuring cylinder, test tubes were cleaned with surf water and washed with water. 3:1 ratio of hydrochloric acid and nitirc acid was mixed for preparation of aqua regia. Hence, for preparation of 48 ml of aqua regia, 36 ml of hydrochloric acid was mixed with 12ml of nitric acid. The washed materials were rinsed with aqua regia with careful precautions and again rinsed with water. Then the equipments were rinsed with distilled water and kept in dry oven for 2 hr at 160 degree Celsius. Autoclaving process was done. Firstly, autoclave machine was preheated and the equipments were tightly covered by paper and non-absorbent cotton using rubber so that moisture could not enter into the materials. If the moisture enters into the materials it was not properly sterilised. Then the equipments were kept in autoclave machine at 0-15 lb pressure for 15-20 min. On the mean time, laminar air flow was cleaned with ethanol and UV radiation was switched on for 15 min and then air flow was on for 10-15 min. Ethanol acts as a disinfectant and therefore used on hands for transferring the equipments from the autoclave machine into a clean plate to prevent contamination. The equipments were then carefully transferred to the laminar air flow.

Collection of Soil Sample: The soil sample was collected from Ramadevi Women's University campus garden. The soil around the roots of the plant was dig to 2 feet depth using a tool. 100gm of wet soil was collected on a paper from 2 feet depth. Then the collected soil was kept in an open space under the fan for drying. The collected soil was dried to become moisture free for 30 min. The debris present in the collected dry soil was separated. Fine soil was filtered out from the dry soil with the help of filter paper. In a weight machine, 1g of fine soil was measured using aluminium foil.

Preparation of Solid Media by Nutrient Agar: Commercial nutrient agar was taken. Nutrient agar was supplied by HIMEDIA (REF-M001-100G). In a weight machine, 7gm of nutrient agar was measured using aluminium foil. 250ml of distilled water was taken in a conical flask and to it nutrient agar was added and mixed thoroughly. The mixture was heated in a heater to dissolve the medium completely. Then the agar mixture undergoes autoclaving process for sterilisation. After completion of autoclaving, the mixture was transferred to laminar flow.[10]

Preparation of Petri plate: Melted agar placed in laminar flow was allowed to cool down. Agar mixture poured into petri plates until its temperature falls to about 45-50 degree Celsius. The mixture was poured in petriplates. The plates filled with agar were swirl so that it completely covers the bottom. Agar plates were allowed to cool and set. Then the lid was placed on top and once it sets it was allowed for storage. Agar plates were incubated in a inverted position by placing the lid at the bottom. This prevent water condensation as water can flow down to the agar surface and mix with the bacteria, which may allow the organisms movement from one colony to another and individual colony growth may not be observed.

Serial Dilution: Soil samples collected from the site was mixed thoroughly to make a composite sample for microbiological analysis. Sterile flask and sterile test tubes were taken and labelled. Sterile petriplates were labelled as 10-1, 10-2, 10-3, 10-4 and 10-5 with a wax pencil. 10g sample of finely pulverized, air dried soil was added into the flask containing 90 ml distilled water. The dilution was vigorously shaken for 20-30 min on a magnetic shaker to obtain microorganisms uniform suspensions. 9ml of distilled water was poured on each test tube. 9ml of distilled water was taken in test tube 1

and to it 1ml of suspension was added from flask under aseptic conditions by using sterile pipette to make 1:10 dilution (10-1) and shaken well for about 5 min. Another round dilution 1:100 (10-2) was prepared by transferring 1 ml suspension from test tube 1 into test tube 2 using a fresh sterile pipette and was shaken well. To make another dilution 1:1000 (10-3) again 1ml from test tube 2 was added to 9ml in test tube 3 by using another fresh pipette. Further dilution 10-4 to 10-5 was prepared as usual by using fresh sterile pipette. Then 1ml suspension was taken from 10-1, 10-2, 10-3, 10-4 and 10-5 test tubes and transferred to labelled petriplates. Then it was mixed gently by rotating on the agar petriplate. Upon solidification of the media, all the petriplates were placed in an incubator at 25 degree Celsius in an inverted position for 2-7 days.

Inoculation by Streak Plate Method: All the plates were labelled at the bottom. 10-5 petriplate was taken for pure culture process. The colony on the parent petriplate was taken for subculture. Then pure culture was done by using newly fresh colony of subculture petriplates under aseptic conditions. The pure culture was done in the following manner. The agar plate was hold containing the agar medium and to it sterilised loop was introduced. One loopful of culture was withdrawn using the sterile loop. Petriplate was hold at an angle of 60 degree. The loop containing the droplet of culture was placed on the agar surface at the edge and was streaked across the surface in parallel lines. The loop was reflamed and allowed to cool down to avoid contamination. The petriplate was turned 90 degree. The inoculation loop was touched to a corner of the culture and streaked across the agar surface. The loop should be avoided to touch that area or else it can lead to contamination. At last, complete streaking was done on the rest of the agar surface. After completion of streaking, the petriplate lid was replaced and the loop was sterilised. All the plates were placed in the incubater in an inverted position at 25 degree Celsius for 48-72 hr.

Staining Procedure: For primary staining, a clean glass slide was taken, washed and dried. Bacterial smear from the culture was prepared. The smear was then allowed to dry. It was then fixed by heat so that the cells can stick to the slide. The smear was then added by crystal violet for the designated period. The stain was poured off and the smear was washed gently and blot dried. Same procedure was repeated for other cultures. For gram staining procedure, a new glass slides was taken and on it bacterial culture thin smear was withdrawn. It was allowed to dry and heat fixed. The smear was hold and to it crystal violet was added. The slide was washed gently with distilled water. Iodine solution was added to the smear. It was then washed gently with 95% ethyl alcohol. Then, the smear was treated with Ethyl alcohol drop by drop, until no more colour flows from the smear. Distilled water was used to wash the slides. Then, the smear was treated with safranin. This is known as counter staining. The slide was washed and blot dried. The stained slide was allowed to air dried.

Preparation of Nutrient Broth: Commercial broth was taken. Nutrient broth was supplied by HIMEDIA (REF-M002-100G). In a weight machine, 3.25gm of nutrient broth powder was measured and was mixed with 250 ml of distilled water in a conical flask. The mixture was heated in a heater to dissolve the medium completely. The broth mixture undergoes autoclaving for sterilisation and then transferred to laminar air flow.

Inoculation for Broth Liquid Culture: The broth culture was thoroughly mixed by thumping the bottom of the tube. Both the culture and the broth tube were hold in the left hand. The loop was sterilized by holding in the right hand then both plugs were removed from these tubes by grasping them between the fingers of right hand. Alternatively, the mouth of both tubes was flamed. A loop full of culture was transferred into the sterile broth with the sterile loop. Alternatively, the mouth of both tubes was flamed and both caps were replaced to respective tubes. The inoculating loop was sterilized by flaming immediately after transfer. Each organism was inoculating into a separate tube of sterile nutrient broth. All the tubes were inoculated, including control, in an incubator at 30 degree Celsius for 24 hr.[11]

Fermentation Test- 10 g of Trypticase, 5 g of Fructose, 15 g of Sodium chloride, 0.018 g of Phenol red was mixed with 1000ml of Distilled water by maintaining pH of 7.3 to prepare fermentation medium. Then the broth fermentation medium was taken into separate test tubes and Durham's tube was dropped inside it. Then it undergoes autoclaved method at 12lb pressures. Fermentation tubes were labelled. The sugar fermentation broths were inoculated with each bacterium. One inoculated tube of each fermentation broth was prepared as a comparative control. Then all the tubes were kept in the incubator for 24-48 hr at 35 degree Celsius.

Catalase Test- 15 g Trypticase, 5 g Phytone, 5 g Sodium chloride, 15 g Agar was mixed with 1000ml of Distilled water to prepare Trypticase soy agar slants having pH 7.3. The prepared medium was transferred into culture tubes and was allowed for sterilisation by autoclaving process. Inoculation of trypticase soy agar slants was done. For control, an uninoculated trypticase soy agar slants was prepared. The cultures were allowed for incubation at 35 degree Celsius. 3-4 drops of hydrogen

peroxide was taken and was allowed to pour on the each slant culture growth.[12]

RESULTS

This study deals with Rhizospheric bacteria isolation and characterization from the rhizospheric soil of *Phyllanthus emblica* L. and their different biochemical test were done.

Amla Plant (*Phyllanthus emblica* L.): It is also known as Indian gooseberry. It is a deciduous tree belongs to Phyllanthaceae family. It is usually 1-8m in height and can reach upto 26ft 3in height. The plant grows best in well drained, light and medium heavy soils which is rich in organic matter. The tree can be grown in moderate alkaline soils. The pH ranges between slightly acidic 6.5 to slightly alkaline 8.5.[5]

Colony morphology and Gram staining of Amla: Rhizospheric bacteria isolated from Amla root region were inoculated and tested for cultural and biochemical reaction (Figure 1-4). The petriplates were taken out of the incubator and the colony morphology was studied. The results showed that 10-1 and 10-2 petriplates shows too many colonies which was unable to count. 10-3 shows some of the colonies but it was not suitable for identifying specific bacteria. 10-4 petriplate show two white colonies. Shape of the white colony was spherical raised while 10-5 petriplate show white large colony. The shape and edge of the colony was smooth. There was a transparent zone around white colony. No yellow colony appeared. In both the petriplate, the colony growth was abundant and showed opaque density. The results from the simple staining from 10-5 petriplate showed that rod shaped bacillus was present in both parent culture and pure culture and Gram staining showed gram positive bacteria in both parent and pure culture (Table 1).

Figure 1: Isolation of Rhizospheric Bacteria.

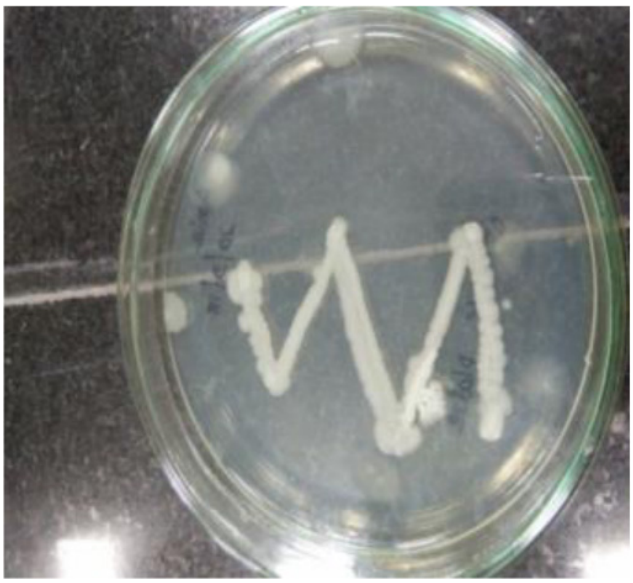

Figure 2: Pure Culture.

Figure 3: Nutrient broth Culture.

Liquid Culture Characterization and Gram Staining of Amla: It was observed that in liquid culture, there was no surface growth and no sediment on conical flask. The liquid appears as slight cloudy in the conical flask. Rod shaped bacillus were observed from primary

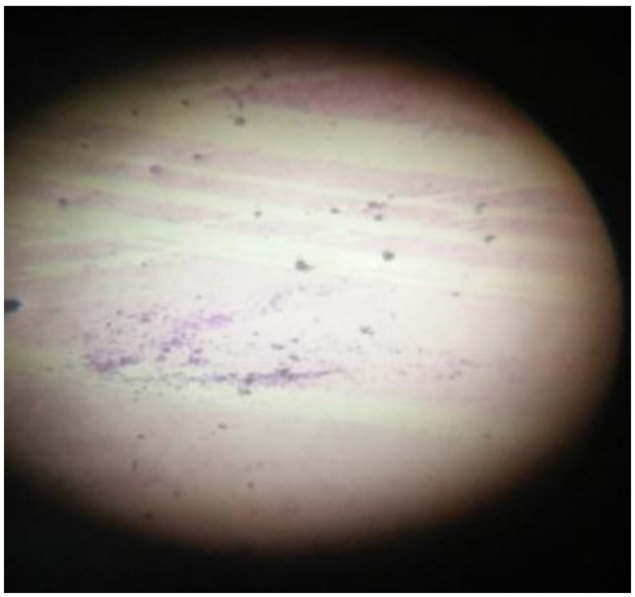

Figure 4: Microscopic view of Rhizospheric bacteria.

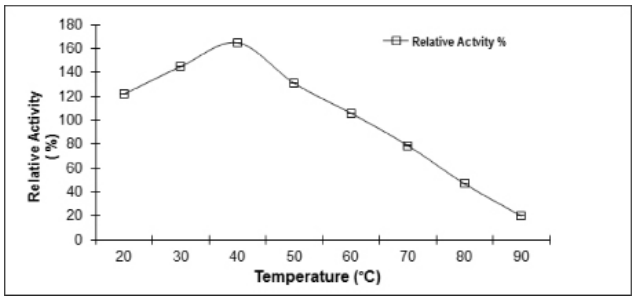

Figure 5: Fermentation Test.

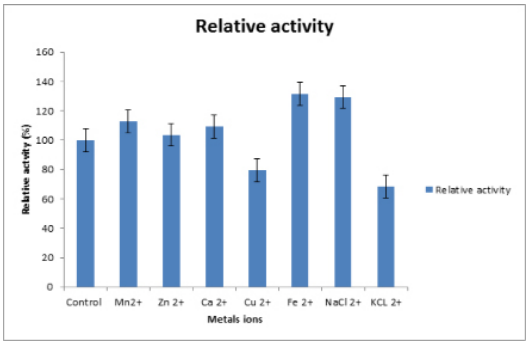

Figure 6: Catalase Test.

Table 1: Colony morphology and Gram staining analysis of isolated Rhizospheric bacteria from Amla Rhizospheric soil in two different culture mediums.

Culture Medium	Colony Morphology	Cell Shape	Gram Staining
Nutrient Agar	Spherical raised white Colony with clear transparent zone	Rod Shaped Bacillus	Gram +ve
Nutrient Broth	No surface growth, No sediment, Slight cloudy	Rod Shaped Bacillus	Gram +ve

Table 2: Biochemical characterization of isolated Rhizospheric bacteria of Amla Soil.

Isolates	Biochemical Test	
	Fructose Fermentation	Catalase Test
Rod Shaped Gram Positive Bacillus	+	+

staining of broth culture. Gram staining of broth culture revealed gram positive bacteria (Table 1).

Biochemical Test of Amla: Fermentation test showed gas in the Durham's tube (Fermentation tube) inside the labelled test tube. It was concluded from the fermentation test that *Phyllanthus emblica* produces gas which means it shows positive towards acid production (Figure 5). On the other hand, catalase test observed that bubbles were coming out from the hydrogen peroxide slant culture (Figure 6). This shows bubbles of free oxygen gas were coming out vigorously. Hence, concluded that bacteria have catalase activity (Table 2).

Overview

1. Morphology- Shape- Rod shaped bacillus
   Arrangement- Single
   Gram stain- positive
2. Culture Characteristics- Colonies- White large with clear zone
   Temperature- Medium temperature
   Growth- Abundant
   Form- Circular and irregular
   Margins- Entire
   Elevation- Raised
   Density- Opaque
3. Broth Media Surface Growth- None
4. Broth Media Clouding- Slight
5. Broth Media Sediments- None
6. Primary Staining- Rod Shaped Bacillus
7. Gram Staining- Gram Positive Bacteria
8. Fermentation Test- Acid Production
9. Catalase Test- Bubbles of Free Oxygen Gas

DISCUSSION

The relation between plant–microbes diversity fluctuates with biodiversity loss, poor nutrients and environmental change. Microbial richness and their relationship with plant diversity is get affected by environmental change and loss of biodiversity. The experimental study shows that in the bacterial community the plant–microbes relationship is much stronger.[13] Various studies showed that the rhizosphere holds many microbial species including multiple bacterial species which inspire plant growth and helps in preventing pathogens infection and increases the nutrients and water uptake. Non-pathogenic rhizospheric bacteria such as *Bacillus* spp. and *Pseudomonas* spp.., provide protection to host plants against some pathogenic fungi. Environmental change, addition of chemical manures, heavy metals leads to destruction of both plant and microbial organisms as these species are very sensitive towards heavy metal lethality. Therefore, studies on rhizosphere helps in understanding the problems such as global warming, manures used in agriculture, climate change, soil poor nutrients and set up treatments and strategies management to maintain the soil productivity and microbial diversity.[14] Bacteria are considered as beneficial microbes in the rhizosphere which act as first line of defence against pathogenic bacteria, fungi and other parasites. Beneficial bacteria include *Pseudomonas* species acts as protective bacteria which repress disease such as root-fungus diseases; *Streptomyces diastatochromogenes* species restrain bacteria growth which causes potato scab disease by antibiotic production and *Bacillus thuringiensis* guard the plants from fungus gnats. Research on mechanisms of microbial protection and rhizosphere personation can lead to agriculture development by microbes.[15] Work done on Amla rhizospheric soil showed rod shaped gram positive bacillus bacteria having different biochemical activities. These rod shaped gram positive bacillus bacteria helps in the growth of the plants and protect the plant against various pathogenic micro-organisms. Simultaneously, other works were also carried out in the laboratory on microbial diversity of plant Guava (*Guajava psidium*), Jamun (*Syzygium cumini*) and Neem (*Azadirachta indica*). These plant soils were collected from the same experimental site near the amla (*Phyllanthus emblica*). A comparative study between rhizosphere microbes of Amla, Guava, Amun, Neem on the same soil was observed (Table 3). These samples were also undergoes the same above procedure and results were as follows:

Table 3: Biochemical Characterization of Isolated Rhizospheric Soil Bacteria.

Plant Soil	Isolates	Biochemical Test	
		Fructose Fermentation	Catalase Test
Guava	Spherical shaped gram positive bacillus	+	-
Jamun	Spherical shaped gram positive coccus	+	+
Neem	Round shaped gram positive coccus	+	+

Guava (*Psidium guajava* L.).

Small round white flat colony having smooth edge, large white raised colony with rough edge and yellow round flat colonies with smooth edge were present on agar plates. It showed abundant growth and opaque density. White and yellow colony was taken for pure culture technique. Rod shaped bacteria were observed from white colony and coccus bacteria were observed from yellow colony in Primary staining procedure. Gram positive bacteria were observed from both the white and yellow colony gram staining procedure. There was no surface growth and no sediment on broth media. There was slight clouding. Gas was produced in fermentation test which shows acid production. Free oxygen gas bubbles were not observed during catalase test.

Jamun (*Syzygium cumini* (L.) Skeels).

Large yellow round smooth edge colony and small white raised rough edge colony were present on agar plates. It showed abundant growth and opaque density. White and yellow colony was taken for pure culture. Yellow colony primary staining reveals spherical shaped bacteria coccus while white colony staining shows rod shaped bacteria bacillus. Gram staining test shows result that yellow colony have gram positive bacteria and white colony have gram negative bacteria. It also shows no surface growth and no sediment on broth media. Slight clouding was observed in broth media. Gas production was observed in fermentation test which means acid production. Bubbles of free oxygen gas were produced during catalase test.

Neem (*Azadirachta indica* A. Juss.).

White flat smooth edged colony with clear transparent zone, yellow round raised colony and small round off white colony were present on agar plates. It showed moderate growth and opaque density. Yellow colony was taken for pure culture. Round shaped bacteria were observed in primary staining and gram positive coccus was observed in gram staining. No surface growth, no sediment and slight clouding were observed in broth

media. Production of gas occurred on fermentation test whereas bubbles of free oxygen gas were produced during catalase test.

CONCLUSION

Rhizosphere microorganisms have a great effect in the functioning of plants by influencing their physiology and development. Rhizobacteria inhibits the growth and activity of other competing microorganism by producing metabolites. The variety, age, health, soil texture and organic matter content of the plant play a crucial part in the rhizospheric microbial activity composition and dynamics. The present study observed rod shaped bacillus and showed positive towards production of alcohol and catalase test.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Author Contributions

SM and GM (equal contribution to both authors): Conceptualization and designing the research work; BJ: Experimental and field work; SM, GM and BJ: Writing of manuscript.

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