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Biofertilizer Production from Nitrogen Fixing Bacteria Isolated from Root Nodules of Leguminous Plants

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Abstract : *Biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants uptake of nutrients by their interactions in the rhizosphere when applied through seed or soil. They accelerate the extent of availability of nutrients in a form easily assimilated by plants. Use of biofertilizers is one of the important components of integrated nutrient management, as they are cost effective and can replace the use of chemical fertilizers for sustainable agriculture. In the present study, the isolation of nitrogen fixing bacteria from root nodules of Arachis hypogaea was performed for the production of the biofertilizers. The result showed that the Rhizobium biofertilizer was successfully produced from nitrogen fixing bacteria which was isolated from root nodules of Arachis hypogaea.*

Keywords: *Biofertilizers, Rhizosphere, Assimilated, Integrated, Sustainable.*

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Introduction :

Biofertilizers are fertilizers that are derived from soil and applied after mixing with carrier materials to the soil or seed to provide natural food and improve the fertility of the soil (Gomare et al. 2013). These fertilizers help in enhancing the nutrient quality of soil by their interaction with the rhizosphere roots of plants when they are applied both on top soil and seed treatment (Temam *et al*. 2017). Microbes can be considered as engineers of soils and many ecosystem services that are linked to terrestrial ecosystems, including plant production, safeguarding of drinking water or carbon sequestration, are closely linked to microbial activities and their functional traits (Vatsayan and Ghosh, 2013). The beneficial microbes are fascinating, versatile and capable of growing on a wide range of substrates and carry out extremely useful processes that cannot be achieved by other physical and chemical means (Paul *et al*. 2014).These fertilizers might help in up

taking of the plant nutrients through their activities in the soil or rhizosphere and makes them available to the plants on the soil. Biofertilizers are important as because they help in reducing the use of synthetic fertilizers (Amutha *et al.* 2014).

Nitrogen fixing free-living microorganisms have frequently been reported as plant growth promoters (Datta *et al*. 2015). Biological nitrogen fixation can be an important source of nitrogen for supporting aquatic primary productivity. Bacteria known collectively as the "*Rhizobia"* are famous for their ability to induce nodules on the roots (and occasionally, stems) of legume plants. Within these nodules, the differentiated, "bacteroid" forms fix atmospheric nitrogen and the resultant ammonia being used as a source of fixed nitrogen (Jayantha *et al*. 2016). This symbiosis provides the bacteria with an exclusive niche and, in return, the plants obtain a personalized nitrogen source. They occur in the so-called free-living forms, for example, aerobic *Azotobacter*, anaerobic *Clostridia* or in symbiosis with certain higher plants, for example, *Rhizobia* with legumes, *Anabaena* with *Azolla* (Chowdhury *et al*. 2017). There exist associative symbiosis in which nitrogen fixing prokaryotes (for example, *Azospirillum*, *Azotobacter*, *Enterobacter* species) have been foundto occur in rhizosphere of different plants such as sugarcane, maize, wheat, rice, grasses and others. *Azotobacter spp.* strains present in neutral or alkaline soils and are the most commonly occurring species in aerable soils (Chowdhury *et al.* 2015).

Materials and Methods :

Root nodules of groundnut plant were collected from different agricultural land of Patna region in sterilized polythene bags and then they were further processed within 24 hours of procurement.

Isolation of Nitrogen fixing bacteria: The serial dilution of the root nodule extract was prepared with 0.1gm of sample in 10 ml of sterilized normal saline. For isolation of *Rhizobium,* YEMA media (Yeast extract,1g; Mannitol, 10 g; Dipotassium Phosphate, 0.5g; Magnesium Sulphate, 0.2g; Sodium Chloride, 0.1g; Agar 20g; Distilled Water, 1000 ml; pH 6.8) with 1% congo red(0.025g) was prepared (Datta *et al*. 2015). Media was poured into sterilized petriplates under aseptic condition. Plates were left for solidification and incubated for 24-48 hours at 37°C.

Maintenance of isolates: Out of the different colonies obtained on the medium, the colonies of *Rhizobium* was selected based on the cultural characteristics and were streaked on Nutrient agar plates and maintained for further use.

Characterization of isolates: The bacterial isolates from root nodules were identified on the basis of morphological characteristics.

Morphological characteristics: Further the isolated colonies were identified on the basis of the Gram's staining (Hans Gram) and other colony characteristics like color, margin, texture, and elevation.

Biochemical characterization: They were subjected to different biochemical tests including indole production test, urease test, starch hydrolysis test, gelatin hydrolysis test, MR-VP test, citrate utilization test, nitrate reduction test and catalase test (Vatsayan and Ghosh, 2013).

Production of biofertilizer: The broth medium was prepared for the inoculum production and they were inoculated with the different strains and incubated at 37°C for 3-4 days. After this, the preparation of carrier material was done for the production of biofertilizer. Two carrier materials were selected namely clay and wheat bran. The carrier was sterilized and mixed with the prepared inoculum culture in a ratio of 3:1 where 300ml of inoculum culture was mixed with 1kg of carrier material and then it was left for air drying in a closed room (Bhattacharjee and Dey, 2014). The prepared biofertilizer was applied to the selected plants to study the growth of the plant which was compared with the control plant.

Results and Discussion :

The present study encompassed the production of biofertilizers from root nodules of leguminous plant.

Isolation of Nitrogen fixing bacteria: The isolates of Rhizobium were obtained from root nodule of leguminous plant. These isolates are maintained for sub culturing.

Characterization of isolates: From the sample of root nodule the Rhizobium was isolated. The potential isolates were identified on the basis of cultural, morphological and biochemical characteristics (Tables 1 and 2).

Morphological characteristics: The bacterial isolate showed the following characteristics under the light microscope at 100X magnification as gram negative rods (Fig.1).

Biochemical characterization: Different biochemical tests were performed for the identification of Rhizobium which are further discussed in Table 2. The present study encompassed the production of biofertilizers from root nodules of leguminous plant. From the biochemical analysis it was found that Rhizobium spp. showed positive results for starch hydrolysis, urease test, MR test, indole production test, catalase test and nitrate reduction test which was found to be similar with the result of Datta et al. (2015)

Production of biofertilizer: The biofertilizer from root nodules of leguminous plant was successfully produced. Rhizobium biofertilizers are recommended for grain legumes to improve the productivity status. Biofertilizer was mixed with suitable carrier such as wheat bran and clay. However, Chowdhury et al. (2017) used lignite and peat as carrier material and confirmed that carrier plays an important role in maintaining sufficient shelf life.

For the pot cultivation Mung bean (Vigna radiata) was selected. The growth of the plant with the different isolates was compared to the control after 15 days of sowing (Fig. 2 and Table 3). The Rhizobium that was used for the production of biofertilizer showed an enhanced effect on the shoot length (Fig. 3), number of leaves (Fig. 4), and number of branches (Fig.5) of selected plant. On comparing the result with the study of Zaidi et al. (2003) who worked on *Rhizobium* strain, he concluded that *Rhizobium* species are used in leguminous crop production for their ability to fix atmospheric nitrogen enhance legume growth, nodulation.

Statistical analysis: On the basis of statistical analysis by Chi-square test showed that there was an association between the biofertilizer used and the growth of the plant.

Conclusion :

In the present study biofertilzer production by nitrogen fixing bacteria was done. Biofertilizer was produced using *Rhizobium.* For the isolation of *Rhizobium* root nodule of *Arachis hypogaea* (Ground nut) was used. The identification and characterization of these microorganisms was done on genus level on the basis of morphological and biochemical characteristics, however 16S rRNA sequencing is required for identification.

Using this nitrogen fixing bacteria, biofertilizer was successfully produced, wheat bran and clay was used as carrier and its effect was studied on a selected plant namely *Vigna radiata* (Mung bean). In a normal environmental condition the overall increase in the growth of plant was observed after a time period of 15 days. Increase in shoot (cm), number of leaves, number of branches was being observed and recorded. These results indicated a significant increment in all the growth parameters of plants treated with biofertilizer as compared to control. The use of biofertlizers is desirable as they are natural, biodegradable, organic and mostly cost effective then chemical fertilizers. It is necessary to continue researching in this field as it has the potential to be highly profitable for the farmers as well as provides a way to a more sustainable future.

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Table 1. Cultural and morphological characteristics of the bacterial isolates

Colour	Texture	Margin	Elevation	Gram's Reaction	Shape
Orangish red and glistering	Smooth	Regular	Convex	Gram negative	Rod shaped
Orangish red and glistering	Smooth	Regular	Convex	Gram negative	Rod shaped
Orangish red and glistering	Smooth	Regular	Convex	Gram negative	Rod shaped

Table 2. Biochemical characteristics of the selected isolate

S. No.	Biochemical test	Selected isolate
1.	Urease test	+ve
2.	Citrate utilization test	-ve
3.	Starch hydrolysis test	+ve
4.	Gelatin hydrolysis test	-ve
5.	Indole production test	+ve
6.	Catalase test	+ve
7.	Nitrate reduction test	+ve
8.	MR test	+ve
9.	VP test	-ve

Table 3. Average growth characteristics of Mung bean after 15 days of application with *Rhizobium spp*. biofertilizer with different carrier materials

Plant No	Carrier used	Shoot length (cm)	Number of leaves	Number of branches
1.	Control	15	11	4
2.	Wheat bran+ *Rhizobium spp*	19	15	6
3.	Clay + *Rhizobium spp.*	22.2	18	6

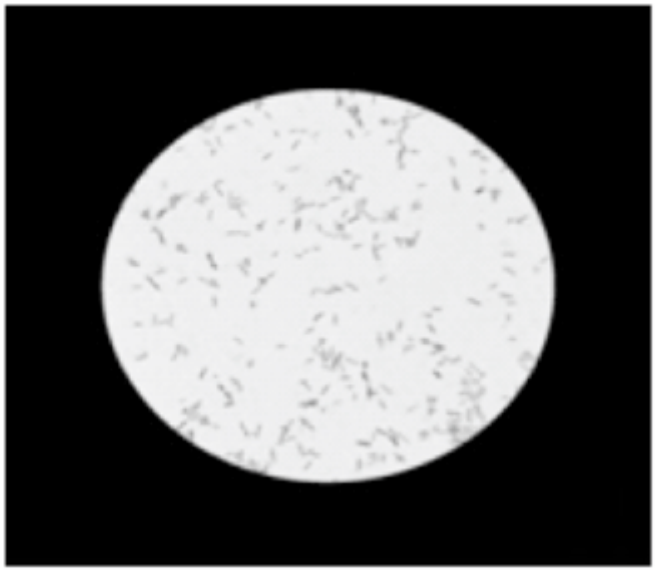

Fig. 1. Rhizobium observed as gram negative rods under 100 x magnification

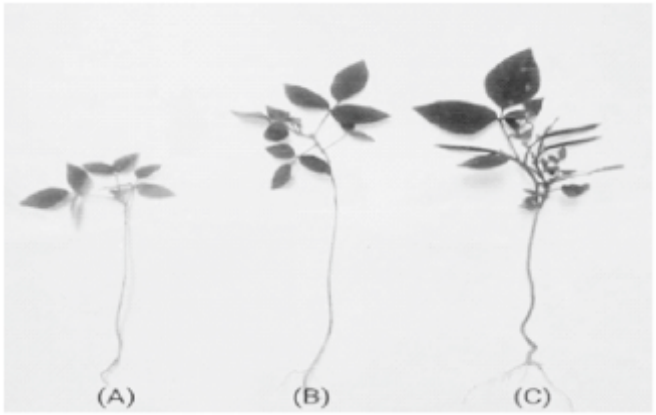

Fig.2. Growth of plant inoculated with Rhizobium biofertilizer (a)control (b) wheat bran as carrier (c) clay as carrier

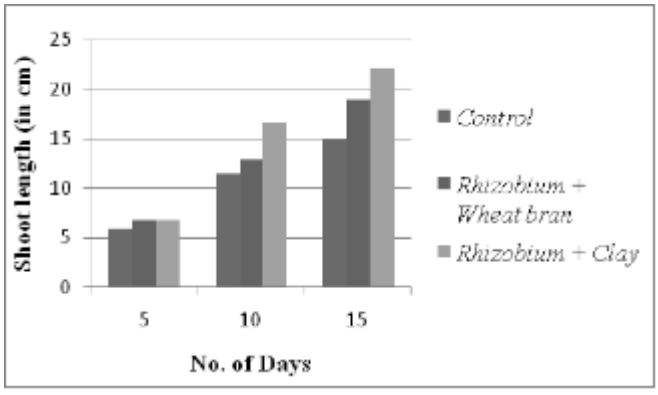

Fig.3. Average growth of shoot length of plant from a period of 1-15 days.

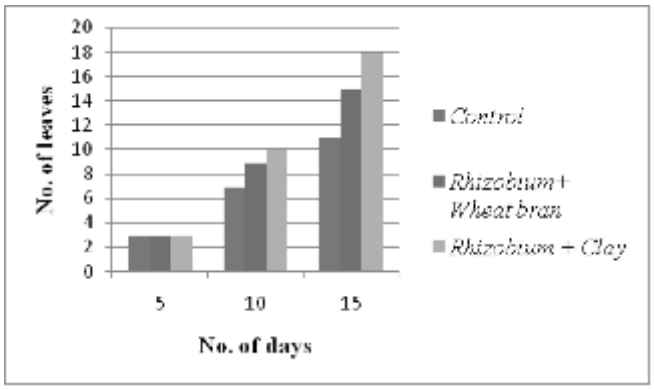

Fig. 4. Average growth in number of leaves of plant from a period of 1-15 days.

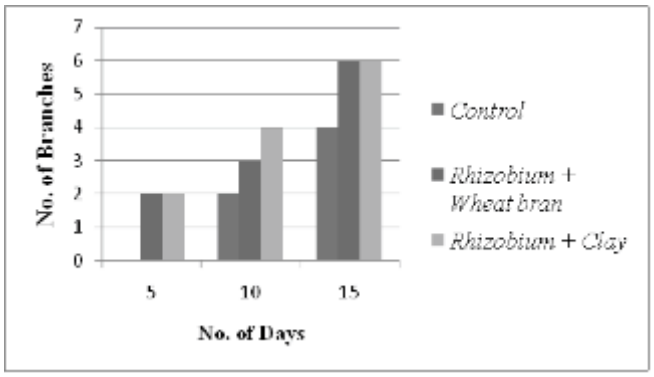

Fig. 5. Average growth in number of branches from a period of 1-15 days.

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