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Isolation and purification of *Rhizobium* from French bean (*Phaseolus vulgaris* L.) root nodules

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ABSTRACT

Six rhizobial isolates were collected from root nodules of *Phaseolus vulgaris* plants and isolates were designated as Rb1. the majority of the isolates were rapid growers within the medium and growth obtained on 3- 5 days of the incubation whereas a few of the isolates were slow growers and growth obtained on 3-4 days. Rhizobial colonies in Yeast Extract Mannitol Agar (YEMA) medium were round mucoid and white. Under microscopic examination, bacteria appeared as non-motile, gram negative rod shaped. The Rhizobial isolates was characterized based on the growth behavior in the medium of Congo red, Hofer's alkaline broth and polyhydroxy butyrate staining. All of these tests confirmed that findings of the present study have been isolated *Rhizobium* species.

Key words: Root, Plants, Rhizobial, Medium, Gram Negative

1. INTRODUCTION

Bacteria which can stimulate plant growth by interacting with plants are called plant growth promoting bacteria (PGPB). They effect the plant growth and development directly or indirectly either through releasing plant growth regulators (PGRS). Substances' enhancing availability and uptake of nutrients through fixation and mobilization, reducing harmful effects of pathogenic microorganisms on crop yielding plants. The direct mechanisms of plant growth promotes growth-promoting mechanisms involve fixation of nitrogen, solubilization of phosphorus, sequestering of iron by siderophores production, phytohormones production such as auxins, gibberellins, cytokinins, and lowering of ethylene concentration (1). According to previous studies (2-4), the indirect mechanism of plant growth promotion includes reduction of iron from the rhizosphere, antibiotic production, antifungal metabolites synthesis, and lysing enzymes of fungal cell wall production. Many PGPR have various plant growth-promoting attributes that influence plant growth at different developmental stages. A plant growth-promoting bacterium (PGPB) may lower the ethylene levels preventing root inhibition on seed germination. PGPR may stimulate cell separation through phytohormones (5). Further plant growth may be enhanced by PGPR by provide enough quantity of iron and phosphorus from the earth (6, 7). Soil nutrient fortification by nitrogen fixing symbiotic bacteria present in legumes has been known for centuries (8-10). Scientific expression of symbiosis's between plant and bacteria was started in 19th century (1). French beans (*Phaseolus vulgaris*) are the most widely cultivated bean around the world. Which are usually well-known Rajmash are used abundantly by the common people, in view of the fact that an alternative diet of protein. It is very nourishing and contains protein, fat, carbohydrate and a large number of minerals such as calcium, phosphorus, and iron (11). Hence, it is proposed to study the isolation and purification of *Rhizobium* from *Phaseolus vulgaris* root nodules.

2. MATERIALS AND METHODS

2.1. Sample collection

Phaseolus vulgaris (French bean) varieties were collected from local seed house at Guntur district of Andhra Pradesh,

India sample collection method was followed (12).

2.2. Collection of nodules

The French bean plants were uprooted and loosely adhering soil was removed by gentle shaking. The mature nodules along with roots were washed in running water until the removal of soil particles adhering. The collected nodules were kept in polythene sheet and transported to the laboratory for further analysis.

2.3. Isolation and purification of Rhizobium

The collected nodules were washed five to six times with distilled water. They were surface sterilized using 0.1% mercuric chloride solution for 1 min, 70% ethanol for 4-5 min and washed in distilled water; it was transferred to 70% ethanol for 2 min and finally washed in distilled water to remove all the traces of sterilants. The sterilized root nodules were crushed with pestle and mortar by adding small aliquots of sterile water, which was 10^{-7} dilution. This suspension was in order diluted up to 10^{-7}. The diluted suspensions 10^{-5}-10^{-7} were selected and 0.1 ml of suspension was inoculated in Petri plates containing sterile YEMA (Yeast Extract Mannitol Agar) medium with congored. The inoculated plates were incubated at 29± 2°C for 3 days. At the incubation period ending, the rhizobial colonies appeared white, translucent and elevated. They were picked out using a sterile inoculating loop and uniformly streaked on YEMA medium. The isolates were purified, subcultured and stored for further studies.

2.4. Rhizobium Cultural characteristics

Rhizobium cultural characteristics are size & shape, Gram's staining, YEMA medium with congo red, Staining of polyhydroxy butyrate test (PHB) and Hoffer's alkaline broth test (HAB)

2.4.1. Size and shape

The Rhizobium isolates were grown on YEMA medium. After 24h the colony shape and size of the Rhizobial isolates were measured and recorded.

2.4.2. Gram's staining

A skinny smear of rhizobial isolates were individually prepared on a glass slide and heat fixed. Afterward the smear was stained via crystal violet for one minute and then washed with H_2O, flooded with Gram's iodine. After 1 min the glass slide washed again with water and decolorized by alcohol. The glass slide was washed and dried through air. Finally the glass slide was observed under microscope.

2.4.3. YEMA medium with Congo red

The Yeast Extract Mannitol Agar medium was prepared in adding solution of Congo red (2.5 ml/1000 ml). The isolates of rhizobia were inoculated in the medium and incubated at 28± 2°C for 48 h.

2.4.4. Staining of polyhydroxy butyrate test (PHB)

A loopful culture of Rhizobium and spread over the microscopic slide and air dried. 1 ml of carbolfuschin solution was added and allowed to stand for 30 minutes the slide was washed in running water, air dried and it was observed under the microscope.

2.4.5. Hoffer's alkaline broth test (HAB)

1ml of rhizobial culture was transferred to 250 ml of conical flask containing 100 ml of Hoffer's alkaline broth [at pH 11]. The flask was incubated at the temperature 28± 2°C for 48 h; the growth rate was measured in turbidity meter.

3. RESULTS AND DISCUSSION

The morphological characteristics of *Rhizobium* were shown in

Table 1. 6 rhizobial strains were isolated from nodules French bean, every rhizobial isolates showed dominant growth on yeast extract mannitol agar medium and 6 rhizobial isolates were named as Rb1 to Rb 6. At 3-5 days of the incubation, the rhizobial growth obtained, some of the isolates were slowly grows and growth obtained on 5th day (Figure 1). On YEMA medium the morphology was mostly mucoid colony, and translucent. *Rhizobium* Rb 6 colony was appear as less mucoid while the remaining isolates in colonies are mucoid and highly mucoid on 3 days of incubation at room temperature 28±2oC (

Table 1 and Figure 3). The diameter of the isolated Rhizobia ranged from 0.4-9± 1.5 – 4.0 µm (Figure 2). All the isolates were gram negative, non-motile, and rod shaped bacteria, observed under the light microscope.

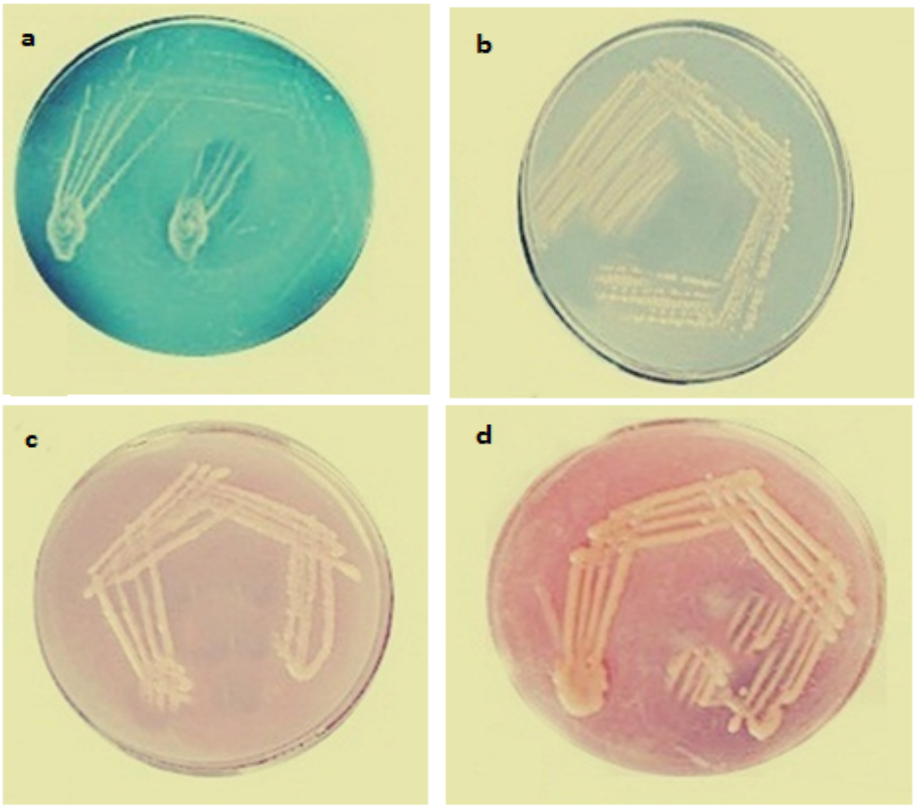

Figure 1. Rhizobial colonies on Yeast Extract Mannitol Agar (YEMA) medium (a, b.YEMA medium without congored), (c, d.YEMA medium with congored)

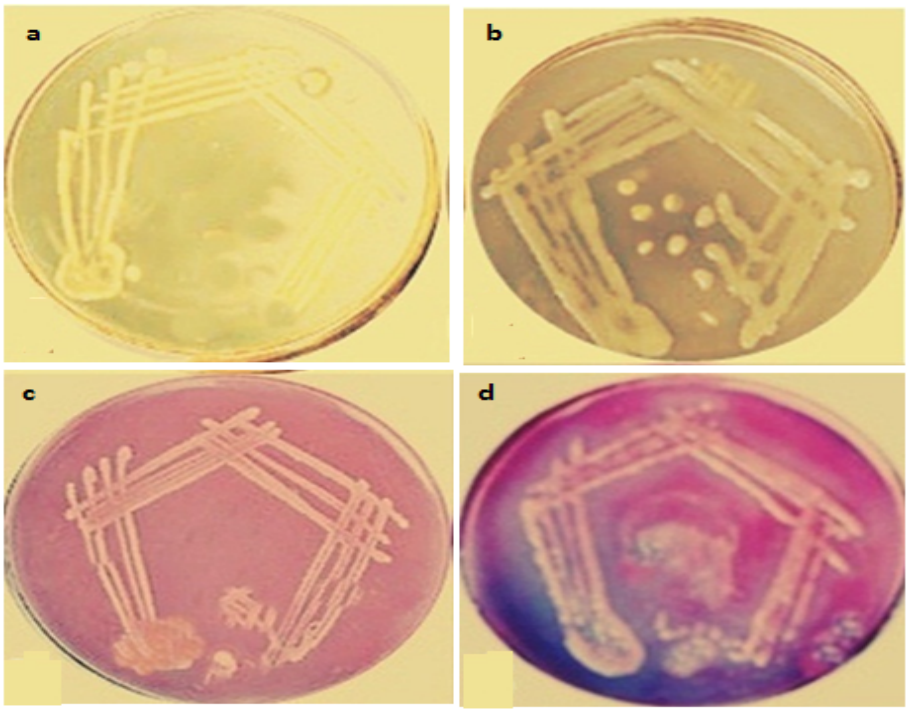

Figure 2. Rhizobial colonies on Yeast Extract Mannitol Agar (YEMA) medium (a, b.YEMA medium without congored), (c, d.YEMA medium congored)

Every identification tests are used for the distinguishing *Rhizobium* from French bean. Within medium of congo red, rhizobia visible as clear white, glistening, prominent and moderately smaller colonies in contrast to stained bacteria colonies. At pH 11 the Rhizobial isolates growth was not showed in Hofer's alkaline broth. Bacteria growth at elevated pH levels, while rhizobia are unable to grow at high pH. Hofer's alkaline broth test useful for distinguishing the two related genera.

Table 1. Morphological characteristics of *Rhizobium* isolates of French bean

S.No	Rhizobium isolates	Colony nature	Shape of cell	Gram's staining	Cell motility	Growth obtained (days)
1	*Rhizobium* 1	mucoid	Rod	Gram negative	Negative	4days
2	*Rhizobium* 2	mucoid	Rod	Gram negative	Negative	3-5 days
3	*Rhizobium* 3	Highly mucoid	Rod	Gram negative	Negative	5 days
4	*Rhizobium* 4	mucoid	Rod	Gram negative	Negative	4 days
5	*Rhizobium* 5	mucoid	Rod	Gram negative	Negative	3-5 days
6	*Rhizobium* 6	Less mucoid	Rod	Gram negative	Negative	5 days

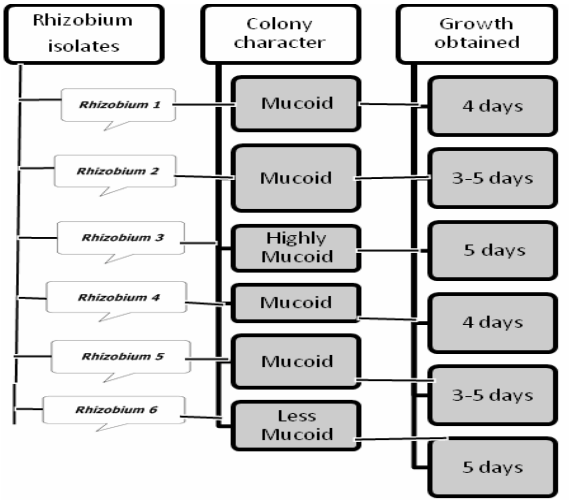

Figure 3. Morphological characteristics of isolates

In congored test and Hoffer's alkaline broth test negative results were observed, red color cells were observed in polyhydroxy butyrate strain test indicated the positive results. The isolates were represented as Rhizobia based on colony characteristics, morphology of cell and cultural characteristics. Elevated levels of mucus were identified in 20% of the isolates, 15% were white and 35% isolates were mucoid, thick colonies. All isolates are gram negative, rod shaped and non-motile. The *Rhizobium* grown on Yeast Extract Mannitol Agar medium and produced little to average sized colonies reported (12). These results were supported by Bergey's Manual (13). In present study all the isolates from French bean plants are not grow in Hofer's medium. Untainted *Rhizobium* isolates are unable to grow on lactose (14). Rhizobial colonies are white, mucoid and transparent, *Rhizobium* showed red colour in stain of PHB under microscope. Growth reactions of *Rhizobium* have been investigated by different workers (15). *Rhizobium* nodulation specificity an interest of identification of chemical signals that are exchanged between plant and bacterium during the symbiosis process. These are consideration to conclude nodulation specificity studied (16). Similar results were obtained (17) used for the characterization of *Rhizobium* isolates from various species and confirmed to those organisms, the present study are used to identification of *Rhizobium*.

4. CONCLUSION

Every one of the *rhizobium* isolates were suitable PGPR for the growth promotion of *Phaseolus vulgaris*. Considering the promoting abilities of plant growth isolates for bioinoculant preparation is possible. This study illustrate that these isolates having best characteristics of plant growth promoting (PGPR)that assist in the seed germination, root and shoot length and also enhance the biomass of the plant *Phaseolus vulgaris*.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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