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RESEARCH ARTICLE

Effect of *Rhizobium* on Seed Germination and Growth of Plants

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Abstract

Rhizobium species are group of bacteria that fix atmospheric nitrogen symbiotically and stimulate the growth of plants. In the present study, root nodulating bacteria were isolated from root nodules of Soybean (Glycine max L.) on yeast extract mannitol agar (YEMA) medium. The isolates were identified by cultural, morphological and biochemical characterization. *Rhizobium* isolates were found to be rod shaped, gram negative, acid and mucous producing. Optimum temperature and pH of the *Rhizobium* was found to be 36°C and 7.0 respectively. While evaluating antibacterial and antifungal activity of *Rhizobium* against rhizospheric bacteria and pathogenic fungi (*Aspergillus niger* and *Fusarium oxysporum*), it was found that *Rhizobium* did not inhibit the rhizospheric bacteria but inhibited the growth of pathogenic fungi which indicates that *Rhizobium* may secrete antifungal compounds. In pot assays of *Rhizobium*, seed inoculation was more effective than soil inoculation. In case of seed germination on the growth of Bengal gram (*Cicer arietinum*), Moth beans (*Vigna aconitifolia*), Green gram (*Phaseolus aureus* Roxb.) and Peas (*Pisum sativum*) crop plants, it was noted that enhanced growth rate was obtained; hence *Rhizobium* can be used as bioinoculant.

Keywords: *Rhizobium*, soybean, rhizospheric bacteria, antibacterial, antifungal, pot assay, seed germination.

Introduction

Soybean (Glycine max L.) also known as golden bean is the prominent oilseed crop in world accounting for more than 50% of the world oilseeds production (Pawar *et al*., 2011). Amongst the soil bacteria, *Rhizobium* which belongs to the *Rhizobiaceae* family (Deshwal and Chaubey, 2014) has a beneficial effect on the growth of plants (Shahzad *et al*., 2012). *Rhizobium* species can exist as free-living soil saprophytes or as nitrogen fixing endo-symbionts of legume host plants that is within the root nodules of host legumes or in close association with the plant roots (Gothwal *et al*., 2008; Shamseldin *et al*., 2008). The rhizosphere or the zone of influence around plant roots possess various microorganisms such as bacteria, actinomycetes, fungi and algae which affect the physical, chemical and biological properties of soil (Gothwal *et al*., 2008). Rhizobia infect the roots of legumes and induce formation of nodules, where nitrogen fixation takes place (Beattie and Handelsman, 1989). The enzyme system of bacteria supplies constant source of reduced nitrogen to the host plant and the plant in turn provides nutrients and energy for the activities of the bacteria (Singh *et al.,* 2008). *Rhizobium* increases plant growth by various ways such as production of plant growth hormones, vitamins, siderophores, by solubilisation of insoluble phosphates, induction of systemic disease resistance and enhancement in stress resistance (Hussain *et al.,* 2009). This interaction reduces the need of nitrogenous fertilizers during the growth of leguminous crops, which are herbaceous woody plants that produce seeds and are a good source of dietary protein for consumption by humans (vegetable oil) and animals (animal feed) (Singh *et al.,* 2008; Shahzad *et al.,* 2012). *Rhizobium* also has been found to effectively control various soil-borne plant pathogenic fungi such as *Fusarium oxysporum* (Arfaoui *et al.,* 2006). So rhizobial inoculants have been frequently applied as biofertilizers having antagonistic activity (Gachande and Khansole, 2011). Increased cultivation of legumes is essential for the regeneration of nutrient-deficient soils and providing needed nutrients to humans and animals (Shahzad *et al.,* 2012). In the present study, *Rhizobium* was isolated and characterized from the root nodules of soybean and its antagonistic activity was studied against rhizospheric bacteria and pathogenic fungi such as *Aspergillus niger* and *Fusarium oxysporum*. Seed germination and pot trial were conducted to assess the potential of isolated *Rhizobium* for improving the growth and yield of local crops.

Material and methods

Isolation of Rhizobium from soybean root nodules: Soybean plants were uprooted carefully so that intact roots can be obtained. Healthy soybean nodules were detached from the root and further isolation of root nodulating rhizobia was carried out (Vincent, 1970). The detached root nodules were washed in tap water to remove the adhering soil particles from nodule surface. Fresh root nodules from soybean were collected and surface sterilized with 70% ethanol and

0.1% mercuric chloride and washed thrice with sterile distilled water. Root nodules were crushed in saline solution. *Rhizobium* was isolated by spreading 0.1 mL crushed root nodule suspension on YEM (Yeast extract mannitol, pH.7.0) agar plate and incubated at 36°C. *Rhizobium* colonies observed in 2-3 d (Singh *et al.,* 2008) were further used for morphological and biochemical characterization. To check the antibacterial activity, rhizospheric bacteria were isolated by serial dilution of soil. All the experiments were carried out in three replicates.

Morphological characterization: Morphological characters such as size, shape, colour, elevation, margin, opacity, bacterium shape and gram staining were performed for identification of the bacteria (Gachande and Khansole, 2011).

Biochemical characterization: All the tests were carried out with 3 replicates. For characterization of bacteria, the acid production test (Jordan, 1984), oxidase test (Kovaks, 1956), catalase test (Graham and Parker, 1964), methylene blue test (Gao *et al.,* 1994), starch hydrolysis test (Oliveira *et al.,* 2007), growth on glucose peptone agar (Kleczkowska *et al.,* 1968), lactose assay (Shahzad *et al.,* 2012; Vishal and Abhishek, 2014), gelatin hydrolysis test (Sadowsky *et al.,* 1983), citrate utilization test (Koser, 1923), growth in the presence of KNO_3 (El-Idrissi *et al.,* 1996), growth in NaCl (Sadowsky *et al.,* 1983), H_2S (Zobell and Feltham, 1934), urea hydrolysis test (Lindstrom and Lehtomaki, 1988), precipitation in calcium glycerophosphate (Hofer, 1941), Gram staining and motility (Arora, 2003) were performed.

Temperature and pH tolerance: Effect of different physical parameters on the growth of *Rhizobium* was studied by keeping plates at different temperatures and preparing YEM medium of different pHs. Differences in the range of growth temperatures were investigated by incubating bacterial cultures in YEM agar at 32, 34, 36, 38 and 40°C. Differences in pH tolerance were tested in YEM agar by adjusting the pH to 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. All the plates were incubated at 36°C for 72 h (Singh *et al.,* 2011).

Antibacterial and antifungal activity: The antibacterial and antifungal effect of *Rhizobium* was evaluated against rhizospheric bacteria and pathogenic fungi (*Aspergillus niger* and *Fusarium oxysporum*) by well diffusion method. Ampicillin antibiotic was used as a positive control and saline as negative control. The plates were incubated for 16-18 h at 37°C for antibacterial and 3-4 d at 28°C for antifungal activity and the zone of inhibition were recorded (Arfaoui *et al.,* 2006).

Effect of Rhizobium on seed germination: Isolated rhizobial colonies were inoculated in nutrient broth and allowed to grow overnight. Different seeds namely Bengal gram (*Cicer arietinum*), Moth beans (*Phaseolus trilobus*), Green gram (*Phaseolus aureus* Roxb.), Peas (*Pisum sativum*) were surface sterilized by 70% ethanol and then treated with 1% sodium hypochlorite for 2 min followed by repeated washing with sterile water. After this, the seeds were soaked in the rhizobial culture broth, while seeds which were soaked in normal nutrient broth kept as a control. Ten seeds of each treatment were kept equidistance in sterilized petriplates containing moist filter paper and the petriplates were incubated at 30°C. Seed germination and percent seedling emergence was calculated using following formula (Mia *et al.,* 2012):

$$\% \text{ Emergence} = \frac{\text{Number of emerged seedlings}}{\text{Number of seeds sown}} \times 100$$

Effect of Rhizobium on growth of plants by pot assay: In first treatment, twenty five seeds of Moth beans were treated with *Rhizobium* and then sown in pots containing 45 g soil for 8 d. In second treatment, three different soils such as autoclaved, unautoclaved and unautoclaved inoculated with *Rhizobium* were used as a potting mixture for sowing (Hussain *et al*., 2009). In third treatment, both soil and seeds were treated with culture of *Rhizobium*.

Results and discussion

Cultural, morphological and biochemical characters: Colonies of *Rhizobium* were obtained on YEM agar after incubation for 2 d at 36°C (Fig. 1). The colonies were entire, opaque with regular margin, milky white, translucent, circular in shape, shiny, raised (convex), sticky consistency, musky odour of the colony and 2-4 mm in dia. These characteristics are similar with the standard characteristics of the *Rhizobium* which indicates that the isolated microorganisms are *Rhizobium* species. The isolated bacterium was aerobic, non spore forming, pink coloured gram negative rods and motile (data not shown). *Rhizobium* showed negative chemical reaction for indole, methyl red, Voges-Proskaur, hydrogen sulphide production, gelatin hydrolysis and citrate utilization (Table 1).

Fig. 1. *Rhizobium* colonies observed on YEMA.

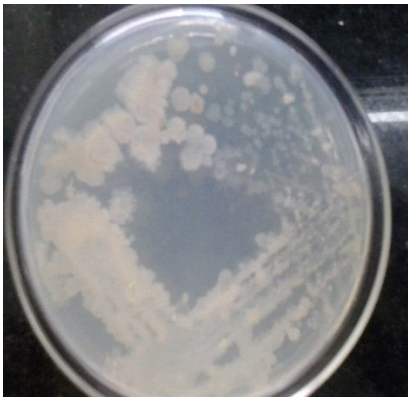


Table 1. Biochemical characteristics of the isolated microorganism.

Test	Remark
Acid production test	+ve
Catalase test	+ve
Oxidase test	+ve
Methylene blue test	-ve
Starch hydrolysis test	+ve
Growth on glucose peptone agar	+ve
Gelatin hydrolysis test	-ve
Citrate utilization test	-ve
Growth in the presence of KNO_3	+ve
Growth in NaCl	+ve
H_2S	-ve
Urea hydrolysis test	+ve
Precipitation in calcium glycerophosphate	+ve
Indole	-ve
Methyl red	-ve
Fluorescent assay	+ve
Ammonia production from peptone and urea	+ve
Gram staining	Gram negative rods
Motility	Motile

Previously, Sadowsky *et al.* (1983) commented the same results. While it showed positive reaction for catalase, oxidase test, starch hydrolysis assay, growth on GPA, urea hydrolysis, precipitation in calcium glycerophosphate, growth on 2% NaCl, citrate production, fluorescent assay and ammonia production from peptone and urea which is the characteristic of *Rhizobium* (Gachande and Khansole, 2011; Shahzad *et al*., 2012; Vishal and Abhishek, 2014). The bacterium was unable to grow on the media containing 0.1% methylene blue which acts as an agent against the growth of microorganisms. Also rhizobial cells were able to grow on GPA media which indicated that it could utilize glucose as the carbon source which is the confirmatory test for *Rhizobium.* Similar results were also observed by Singh *et al*. (2008).

Temperature and pH tolerance: The optimum temperature was 36°C. Decrease in temperature tolerance started at 38°C, growth was totally absent at temperature 40°C (Fig. 2). Optimum pH range for rhizobia was between 6.5 to 7.5. No growth was observed in the medium with pH 3.5 (Fig. 3). At pH 4, minimum growth was observed. Similar results were recorded by Sing *et al.* (2011).

Antibacterial and antifungal activity: Isolated *Rhizobium* inhibits the growth of *Aspergillus niger* and *Fusarium oxysporum* which is pathogenic fungi and affects on the yield of crop plants (Fig. 4 and 5). The zone of inhibition (in cm) recorded was 1.1±0.3 for *A. niger* and 0.4±0.2 for *F. oxysporum. Rhizobium* has also been reported to produce toxic metabolites, enzymes or volatile compounds which have inhibitory effects on soil-borne pathogens.

Fig. 2. Effect of temperature on the growth of *Rhizobium.*

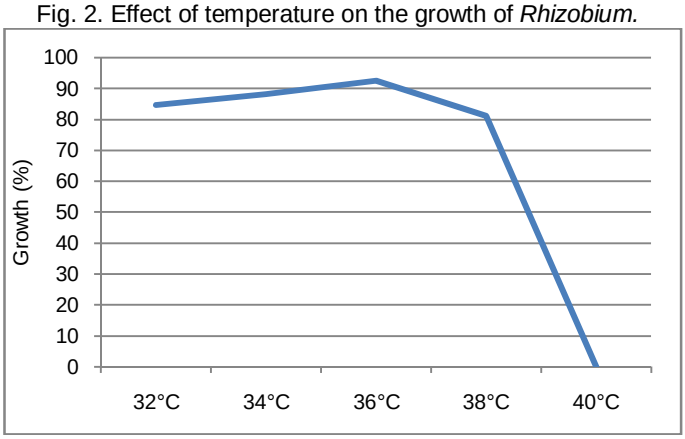


Fig. 3. Effect of pH on the growth of *Rhizobium.*

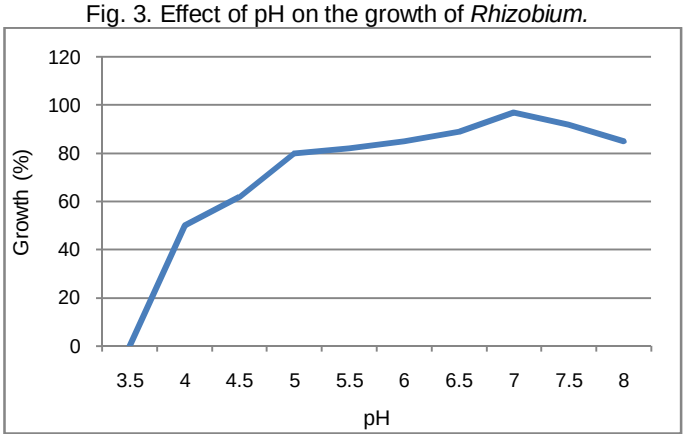
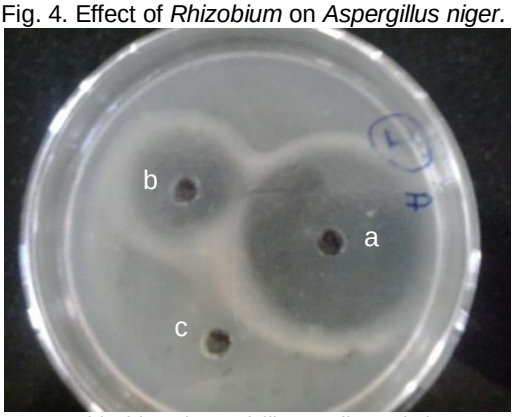
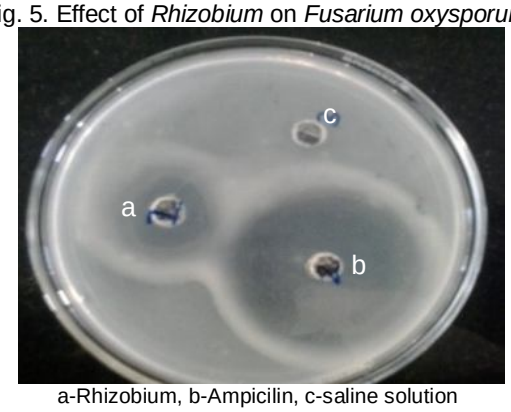


Fig. 4. Effect of *Rhizobium* on *Aspergillus niger.*



a-Rhizobium, b-Ampicilin, c-saline solution

Fig. 5. Effect of *Rhizobium* on *Fusarium oxysporum.*

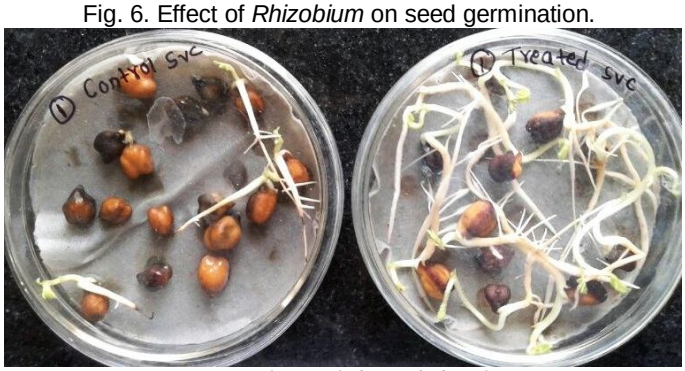


a-Rhizobium, b-Ampicilin, c-saline solution

Table 2. Effect of *Rhizobium* on seed germination.

Seed	Day 1 (24 h)		Day 2 (48 h)		% seed germination	
	Control	Treated	Control	Treated	Control	Treated
Bengal gram (*Cicer arietinum*)	0.35±0.05	1.34±0.39	1.5±1.6	2.56±0.057	71	83
Moth beans (*Vigna aconitifolia)*	1.558±0.25	2.05±0.432	3.0±0.408	4.12±0.478	62	77
Green gram (*Phaseolus aureus* Roxb.)	1.225±1.35	1.825±1.59	3.65±0.58	4.37±0.25	69	83
Peas (*Pisum sativum*)	0.733±0.84	1.8±1.0	1.67±0.28	2.833±0.38	85	99

Fig. 6. Effect of *Rhizobium* on seed germination.



a. Bengal gram (*Cicer arietinum*)

b. Moth beans (*Vigna aconitifolia)*

c. Green gram (*Phaseolus aureus* Roxb.)

d. Peas (*Pisum sativum*)

Rhizobium does not inhibit the growth of bacteria from rhizosphere which indicate that *Rhizobium* inhibits the growth of harmful microorganism but not useful microorganism. This gives clue about communication and cell signalling in *Rhizobium* occurs by quorum sensing mechanism which encourages the growth of each other.

Effect of Rhizobium on seed germination: All the seeds showed maximum germination after 48 h. In comparison of the control (non-treated seeds) and test (seeds coated with *Rhizobium* culture), highest seed germination was obtained in the test. The number and length of the sprouts were significant in the test as compared to the control (Table 2 and Fig. 6a-d).

Fig. 7. Effect of *Rhizobium* on growth of plant by pot assay.

a. Autoclaved soil

b. Unautoclaved (normal) soil

c. Unautoclaved soil + inoculated with *Rhizobium*

Pot assay: In case of pot assay, best results were obtained when both seed and soil were treated with *Rhizobium*. Seed coating gave good results as compared to soil inoculation. The growth obtained in pot assay was: Seed and soil both treated with *Rhizobium* culture > Seed coated with *Rhizobium* > Soil inoculated with *Rhizobium*. The comparative growth rate for three different soils was: Unautoclaved soil inoculated with *Rhizobium* > Unautoclaved soil (normal) > Autoclaved soil (Fig. 7a-c).

Conclusion

Isolated bacterium was proved as *Rhizobium* based on their cultural, morphological and biochemical characteristics. While studying growth of bacteria at different temperatures and pHs, the optimum was found to be 36°C and 7.0 respectively. In antibacterial and antifungal activity of *Rhizobium*, it inhibited the growth of soil-borne pathogenic fungi but does not affect on the growth of rhizospheric bacteria. Seeds dressed with *Rhizobium* and plant grown in soil inoculated with *Rhizobium* showed high seed germination and stimulatory growth over the control. These results summarized that *Rhizobium* can be effectively used as a bioinoculant or biofertilizer to enhance the yield of crops.

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