

## Antimicrobial activity of *Rhizobium japonicum* and *Bradyrhizobium japonicum* on different plant pathogenic fungal strains

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### ABSTRACT

Symbiotic nitrogen fixation is a key to the nitrogen nutrition to the legumes. The most important agents for the symbiotic nitrogen fixation are the bacteria of the genus *Rhizobium*, which invade the root hairs of leguminous plant and develop nodules on the roots, where nitrogen fixation occurs. *Rhizobium* promotes growth of plants by fixing nitrogen from the atmosphere and is also a biocontrol agent which inhibits growth of pathogens. The biocontrol effect is due to the secretion of secondary metabolites. The present study describes the physiological, biochemical characterization and antagonistic activity of *Rhizobium* species were isolated from root nodules of leguminous plant. The *Rhizobium spp.* were rod shaped, gram negative and mucous producing. Antifungal activity of *Rhizobium spp.* isolates were tested against three fungi which are potential phytopathogens on legumes. Inhibition zones were observed, hence *Rhizobium spp.* can be used as biocontrol agent.

**KEY WORDS:** ANTAGONISTIC ACTIVITY, ANTIFUNGAL ACTIVITY, BIOCONTROL EFFECT, INHIBITION ZONE, *RHIZOBIUM SPP*

### INTRODUCTION

Chemicals used to control plant diseases contaminate the soil environment, degrade its fertility and also defile underground water, causing health risk. Thus, biocontrol agents emerge as an alternate to those antifungal

chemicals, these are inexpensive, eco-friendly and have no harmful effects on human, animals and plants (Deshwal *et al.*, 2003). Legumes establish a symbiotic interaction with soil bacteria termed as Rhizobia. These bacteria in association with legumes can fix atmospheric N and through this feature. Hence, they are introduced

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into agricultural systems to improve soil fertility, plant growth and limit the use of chemical fertilizers (Ouma *et al.*, 2016; Srinivasan, 2017; Yassine, 2018).

Rhizobia not only fix nitrogen from atmosphere and supply it to plants but also promote the growth of plants. Rhizobia synthesize phyto-hormones and solubilization of minerals act as a biocontrol agent and can inhibit the growth of pathogens. Due to the secretion of secondary metabolites such as antibiotics and HCN by rhizobia, they have the capacity to restrict the growth of fungal pathogen. In iron stress conditions in rhizobia, siderophore production provides an added advantage, resulting in the exclusion of pathogen due to iron starvation. Rhizobacteria possessing all these features are also referred as plant growth promoting rhizobacteria (PGPR). Not only this but these bacteria also protect plants against flooding, drought, salt, flower wilting, metals, organic contaminants, and both bacterial and fungal pathogens, (Glick, 2014; Subramaniam *et al.*, 2015; Srinivasan, 2017 and Yassine, 2018).

Legumes fix atmospheric nitrogen only in association with a bacterial symbiont of the genus *Rhizobium*. Rhizobia have been arbitrarily divided into two classes: fast growing and slow growing. Normally, commercial cultivars of the soybean *Glycine max* are nodulated only by slow-growing *Rhizobium japonicum* (Keyser *et al.*, 1982). The isolate appeared to be effectively nodulate only the wild soybean *Glycine soja* and an unbred soybean cultivar from China (*G. max* cv. Peking) *Rhizobium japonicum* 191 is a member of a salt-tolerant group of *R. japonicum* recently described by (Keyser *et al.*, 1982).

*Bradyrhizobium japonicum* is a Gram negative bacterium belonging to rhizobia group associated with roots of soybean and have the capacity to fix N<sub>2</sub> in the presence of nitrogenase enzyme (Baoling *et al.*, 2007). This bacterium and nitrogenase enzyme both are very sensitive for the environmental conditions. The commercially introduced strains must compete with highly adapted indigenous rhizobia for legume nodulation under specific physiological, biological and environmental soil conditions. Soil acidity limits symbiotic nitrogen fixation by limiting *Rhizobium* survival in soils, as well as reducing nodulation (Ibekwe *et al.*, 1995). Chakraborty and Purkayastha, (1984) reported that some strains of *Bradyrhizobium japonicum* produce rhizobitoxine which can protect soybean crops from the infection of *Macrophomina phaseolina*, which is a charcoal rot fungus of leguminous crops. *Rhizobium* has shown to reduce root-rot of soybeans caused by *Phytophthora megasperma*. Rhizobial mechanism such as improvement in intake of plant nutrients by altering root morphology, production of siderophore (Antoun *et al.*, 1998; Arora *et al.*, 2001; Chabot *et al.*, 1996; Srinivasan, 2017) to meet the iron requirement of the plant under iron stressed condi-

tions and lowering of ethylene through ACC deaminase enzyme are some example with direct positive effects on non leguminous plant growth. *B. japonicum* strain inhibit the growth of seven pathogenic microorganisms causing disease in soybean was studied by (Balasundaram and Sarbhoy 1998) The fast growing rhizobial strains were found to completely inhibit the growth of white sclerotia of *S. rolfsii*. Different strains of *Rhizobium* and *Bradyrhizobium* have been reported to inhibit the growth of *M. phaseolina* (Deshwal *et al.*, 2003; Arora *et al.*, 1998). The rhizobia having antagonistic property showed more competency in root hair infection in host plants as compared to non- biocontrol rhizobia. Rhizobia also appear to influence the plant defense mechanism by stimulating the production of phytoalexins by plants. Antibiotics produced by rhizobia have been found to play an important role in disease control. HCN, a secondary metabolite produced by several microorganisms, has deleterious effect on the growth of some microbes (Knowles, 1996).

Studies conducted on numerous plant microbe interaction have shown that such antagonistic rhizobacteria could function by competition and antibiosis i.e. by producing antimicrobial compounds like bacteriocin (Rodelas *et al.*, 1998; Joseph *et al.*, 1983) but also indirectly induces systemic resistance against plant diseases. The enzyme system of bacteria during nodulation in the roots 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). It has also been evaluated that *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; Yassine, 2018).

Some *Rhizobium spp.* have shown antimicrobial activities towards *Pseudomonas spp.* (Kacem *et al.*, 2009) *Aspergillus niger* (Yuttavanichakul *et al.*, 2012). In the present study *Rhizobium* was isolated from the root nodules of soybean and its antagonistic activity was studied against pathogenic fungi such as *Aspergillus niger* and *Fusarium oxysporum*.

## MATERIAL AND METHODS

**a) Isolation of nitrogen fixing bacteria from soybean root nodules:** Soybean plants were uprooted carefully from the soil so that intact roots can be obtained without destroying the nodules. Healthy soybean nodules were detached from the root and further isolation of root nodulating rhizobia was carried out (Vincent, 1970).

The separated root nodules were washed in tap water to remove the adhering soil particles from nodule sur-

face. 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. Test isolate was isolated by spreading 0.1ml crushed root nodule suspension on YEM (Yeast extract mannitol, pH.7.0) agar plate and incubated at 36°C. Colonies of test isolate were observed in 2-3 days (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 triplicates. *Bradyrhizobium japonicum* (RJ(s)TAL102) was collected from M.P. State Agro Industries, Bhopal.

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

**Biochemical characterization:** All the tests were carried out with triplicates. For characterization of bacteria, the acid production test (Jordan, 1984), oxidase test (Kovaks, 1956), catalase test (Graham and Parker, 1964), methylene blue test, starch hydrolysis test (Oliveria *et al.*, 2007), glucose peptone agar test (Kleczkowska *et al.*, 1968), gelatin hydrolysis test (Sadowsky *et al.*, 1983), growth in NaCl (Sadowsky *et al.*, 1983), citrate utilization test gram staining and motility were performed.

**c) Temperature tolerance:** Effect of different physical parameters on the growth of test isolate was studied by keeping plates at different temperatures. Differences in the range of growth temperatures were investigated by incubating bacterial cultures in YEM agar at 32°,34°,36°,38° and 40°C.

**d) Antifungal activity:** The antifungal effect of test isolate were evaluated against pathogenic fungi (*Aspergillus niger*, *Alternaria alternata* and *Fusarium oxysporum*) by filter paper method. Fluconazole antifungal was used as a positive control and saline as negative control. Bacterial suspension was made and filter paper was dipped into it and placed on the surface of assay plates labelled as Control. The plates were incubated for 24 - 48 hour at 28°C to observe antifungal activity and the zone of inhibition were recorded (Arfaoui *et al.*,2006).

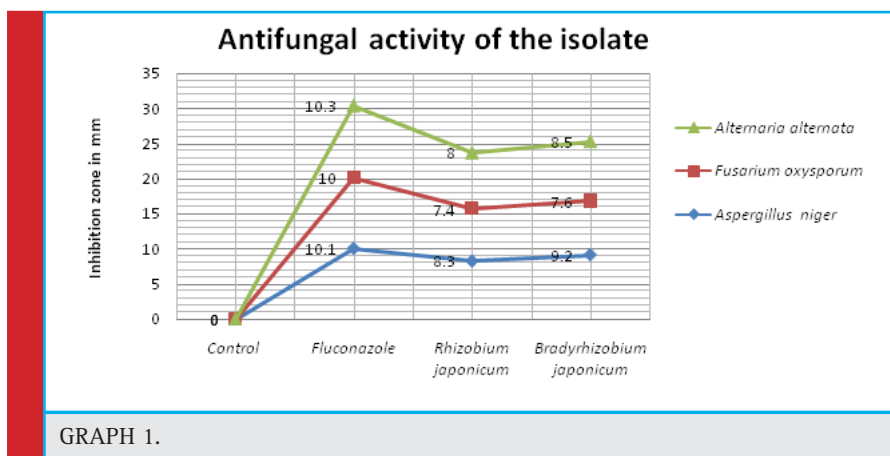
## RESULTS AND DISCUSSION

The colonies isolated from roots of soybean were entire, opaque with regular margin, milky white, translucent, circular in shape, shiny, raised (convex), sticky consistency, musky colour of the colony and 2-4mm in diameter. The isolated bacterium was aerobic, non spore forming, pink coloured gram negative rods and motile. *Rhizobium* showed positive results for acid production test, catalase test, oxidase test, starch hydrolysis test, glucose peptone agar test and NaCl test. Also the nega-

Table 1. Biochemical characteristics of the isolate		
TEST	REMARK	
	<i>Rhizobium japonicum</i>	<i>Bradyrhizobium japonicum</i>
Acid production test	+ve	+ve
Catalase test	+ve	+ve
Oxidase test	+ve	+ve
Methylene blue test	-ve	+ve
Starch hydrolysis test	+ve	+ve
Glucose peptone agar test	+ve	+ve
Gelatin hydrolysis test	-ve	+ve
NaCl test	+ve	+ve
Gram staining	Gram negative rod shaped	Gram negative rod shaped

Table 2. Antifungal activity of the isolate				
S. no.	Treatments	<i>Aspergillus niger</i>	<i>Fusarium oxysporum</i>	<i>Alternaria alternata</i>
1.	<i>Rhizobium japonicum</i>	8.3	7.4	8.0
2.	<i>Bradyrhizobium japonicum</i>	9.2	7.6	8.5
3.	Fluconazole	10.1	10.0	10.3
4.	Control	0	0	0

\*Note: Inhibition zone measured in mm



GRAPH 1.

tive result were seen for gelatin hydrolysis and methylene blue test. The optimum temperature was 28°C. There is a gradual decrease in a colony count was observed after 40°C and growth was totally absent at temperature 44°C in broth.

Both the isolates of *Rhizobium* inhibited the growth of *Aspergillus niger*, *Alternaria alternata* and *Fusarium oxysporum* which is pathogenic fungi and affects on the yield of crop plants. The zone of inhibition (in mm) was recorded and measured.

The test isolates were identified as *Rhizobium japonicum* and *Bradyrhizobium japonicum* by morphological and biochemical characterization. These characteristics were found to be similar with the standard characteristics of the *Rhizobium* and thus this indicates that the isolated microorganisms are *Rhizobium japonicum* and *Bradyrhizobium japonicum*.

The zone of inhibition (in mm) recorded was 8.3 for *Aspergillus niger*, 7.4 for *Fusarium oxysporum* and 8 for *Alternaria alternata* from *Rhizobium japonicum* and for *Bradyrhizobium japonicum* the zone of inhibition was recorded 9.2 for *Aspergillus niger*, 7.6 for *Fusarium oxysporum* and for 8.5 *Alternaria alternata*. Rhizobia have been reported to inhibit significantly the growth of *Fusarium spp.* and *Aspergillus spp.* (Srinivasan, 2107). Antifungal activity of *Bradyrhizobium japonicum* against *Fusarium oxysporum* has been reported by Mariastuti *et al.*, (2018) that inhibition of *Fusarium oxysporum* by *Bradyrhizobium japonicum* ranged from 19% to 54.9%. Thus, indicating *Rhizobium japonicum* and *Bradyrhizobium japonicum* as a biocontrol agent.

## CONCLUSION

The aim of the study was screening of *Rhizobium spp.* (*Rhizobium japonicum* and *Bradyrhizobium japonicum*) and determine its antifungal activity against *Aspergillus niger*, *Alternaria alternata* and *Fusarium oxysporum*

causing various root rot diseases. In our investigation the antifungal activity of *Rhizobium spp.* were found to inhibit fungal growth showing inhibition zone, suggesting production of certain antifungal metabolites. *Rhizobium spp.* could be effectively used as a biocontrol agent in the form of bio-inoculant against fungal pathogen but enhancement in its antifungal properties would prove to be more efficacious. Therefore efforts are required to understand biocontrol mechanism of rhizobia against fungi. Genetic engineering approach can also be used to introduce the genes coding for the synthesis of antifungal and antimicrobial metabolites into rhizobial strains selected for use in biocontrol.

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