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Plant Growth Promoting Effect and Biocontrol Potential of *Rhizobium* spp. against *Macrophomina phaseolina*

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ABSTRACT

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Nitrogen fixation, siderophore production, phosphate solubilization and antimicrobial activity are some of the plant growth promoting effect of *Rhizobium* spp. associated with various leguminous plants but the subject needs to be discussed in more depth. Plant growth promoting effect and biocontrol potential of nitrogen fixing root nodule isolates from certain leguminous plants of Manipur was investigated. In present study, nodule isolates associated with ten leguminous plants from different districts of Manipur were selected. Indole acetic acid (IAA) production, phosphate solubilization and antagonistic activities of *Rhizobium* strains against *M. phaseolina* were evaluated using dual culture technique. Certain *Rhizobium* isolates performed moderately *in vitro*, for the production of phosphate solubilization and IAA production. *In vitro* study revealed that certain *Rhizobium* strains effectively reduced the growth of *M. phaseolina* by 50-88% inhibition zone as compared to control.

Introduction

Rhizobium promotes the growth of legumes through the formation of nitrogen-fixing nodules. Rhizobia can also associate with roots of non-legumes, without forming true nodules, and can promote their growth by using one or more of the direct or indirect mechanisms of actions. Phytohormone production (Atzorn *et al.*, 1988; Chabot *et al.*, 1996; Yanni *et al.*, 2001; Noel *et al.*, 1996;), secretion of other chemicals like lipo-chito-oligosaccharides (Miransari and Smith, 2009;) and lumichrome, solubilization of precipitated phosphorus (Chabot *et al.*, 1996; Yanni *et al.*, 2001; Peix *et al.*, 2001) and mineralization of organic P (Afzal, and Bano,

2008), improvement in uptake of plant nutrients by altering root morphology, production of siderophores (Antoun *et al.*, 1998; Arora *et al.*, 2001; Chabot *et al.*, 1996) to meet the iron requirements of the plant under iron-stressed conditions and lowering of ethylene level through ACC deaminase enzyme, are some examples of the rhizobial mechanisms with direct positive effects on non- leguminous plant growth. Indirectly, rhizobia improve the growth of plants through biocontrol of pathogens via antibiosis, parasitism or competition with pathogens for nutrients and space, by inducing systemic resistance in the host plant and through

increasing root adhering soil by releasing exopolysaccharides which regulate the water movement and facilitate the root growth. Some *Rhizobium* spp. have shown antimicrobial activities towards *Pseudomonas savastanoi*, (Kacem *et al.*, 2009) *Aspergillus niger* (Yuttavanichakul *et al.*, 2012) *Rhizoctonia solani*, *Fusarium oxysporum* and *F. solani* (El-Batanony *et al.*, 2007), *Pythium* sp (Bardin *et al.*, 2004; Huang and Erickson, 2007) *Phytophthora cinnamomi* (Malajczuk *et al.*, 1984) *Fusarium solani* f. sp. *Phaseoli* (Buonassisi *et al.*, 1986) *Fusarium oxysporum* f. sp. *Lentis* (Essalmani and Lahlou, 2003) with varying degree of growth inhibition. Studies on numerous plant-microbe interactions 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 by induction of systemic resistance against plant diseases. The potential use of *Rhizobium* spp. due to their multifaceted beneficial activities is likely to play an important in modern high intensive agricultural practices. In light of the above fact it is proposed to undertake “Plant growth promoting effect and biocontrol potential of *Rhizobium* spp. against *Macrophomina phaseolina*” in Manipur.

Materials and Methods

Survey, collection and preservation of nodule isolates

An intensive survey was conducted at several places of Imphal East District (Andro, Kakwa, Kyamgei, Naharup, Kongba, Moirang Kampu, Jiribam), Imphal West (Manipur University Campus, Chajing, Changangei, Mayang Langjing, Samurou), Thoubal District (Leishangthem, Kakching), Bishnupur District (Khoijuman, Moirang, Nambol Kangmong), Chandel District,

Senapati District (Mao), Ukhrul District (Kachai), Tamenglong District for collection of root nodules from certain leguminous plants viz., *Psophocapus tetragonolobus*, *Cajanus cajan*, *Canavalia gladiata*, *Sesbania sesban*, *Lablab purpureus*, *Pisum sativum*, *Vicia faba*, *Vigna umbelata*, *Phaseolus vulgaris*, *Phaseolus lunatus* found in Manipur.

Isolation and characterization

Soil was caring and gently removed from root materials of the plant. Nodules were then washed with running tap water to remove any soil and plant debris. Nodule shape, size, type, number and location of the nodules on the root system of the host plants were recorded. Healthy and intact nodules were selected randomly and immersed in glass beakers containing 95% ethanol (or isopropanol) for 5-10 sec to break surface tension and to remove air bubbles from nodule tissues. They were then transferred to a 3-5% (v/v) solution of sodium hypochloride for 5 min and washed 4-5 times in sterile distilled water.

Each surface sterilized nodule from different legumes was crushed with a sterile glass rod in a test tube containing 1 ml distilled water with 0.5 % NaCl. One loopful of the nodule suspension was streaked on Petri plates containing Yeast extract mannitol agar (Congo red/Bromothymol Blue as an indicator) to obtain a pure isolated colony and incubated at 28 °C for 7-10 days and make daily observations for the appearance of typical colonies of rhizobia.

The purity of the isolates were checked by microscopic examination and authenticated by testing the capacity of typical isolates to form nodules on roots from which the isolates were obtained. Pure cultures of the Rhizobia were stored on YEMA (Yeast Extract Mannitol Agar) slants for further use.

Phosphate solubilization by test bacteria

The *Rhizobium* strains were tested for their ability to dissolve phosphate under *in vitro* conditions using NBRIP media (Glucose 10g, Ca₃ (PO₄)₂ 5g, MgCl·6H₂O 5g, KCl 0.2g, (NH₄)₂SO₄ 0.1g, MgSO₄·7H₂O 0.25 g, Agar 15g, distilled water 1 lt). The strains were spot inoculated on the medium, and P solubilisation was checked by measuring the zone of clearance.

Assay for indoleacetic acid (IAA) production

Quantitative IAA production in liquid culture was determined colorimetrically. 72 hr old cultures of *Rhizobium* strains grown in 100 ml YEM for 72 h at 30°C at 120 rpm were centrifuged at 5000 rpm for 30 min. The supernatant (50 µl) was mixed with 450 µl phosphate buffer. Development of pink colour indicates IAA production. From this mixture 60 µl was added to 440 µl of phosphate buffer in a tube containing 500 µl of Salkowski reagent (12 g of FeCl₃ per litre of 7.9 M H₂SO₄). Red color formation was quantified as the absorbance at a wavelength of 540 nm in a spectrophotometer. The amount of IAA produced per milliliter of culture was estimated using a standard curve.

Fungal isolates

Macrophomina phaseolina culture was provided by Indian Institute of Agricultural Research, New Delhi. The culture was maintained on Potato Dextrose Agar at 27° ±1°C. The stock culture of the fungus was kept at for long term storage at 4°C.

Screening and bioassay for antagonistic activity of effective rhizobial strain

In vitro antifungal assay was performed using the dual culture techniques. The bacterial isolates tested for their antifungal activity

were fully grown in the YEM broth media. Test fungi were grown on Potato Dextrose agar plates (PDA). 5 days old mycelial discs (5mm dia.) of *Macrophomina phaseolina* were placed at 2 sides from the center on PDA. Exponentially grown Rhizobial isolate was spot inoculated at the center. The distance between the fungus and the Rhizobia was kept at 2.5 cm. Inhibition radial growth of test fungus was observed daily. Culture plates with the test fungus only served as control. In each case three (3) replicates were taken. The petriplates were incubated at 26±1°C in B.O.D. incubator for five days. The diameters of the colonies were measured after five days and average values compared with control were taken as a measure of fungitoxicity. Growth inhibition (%) of test fungus was determined by using the formula quoted by Pani and Patra (1997).

$$\text{Growth Inhibition percentage} = \frac{\text{Control} - \text{Test} \times 100}{\text{Control}}$$

Results and Discussion

Isolation and characterization of nodule isolates

Physical and biochemical characterization of nodule isolates from different leguminous plants was performed. The rhizobia isolated from *Psophocapus tetragonolobus*, *Cajanus cajan* and *Lablab purpureus* exhibited slow growth rate and showed white colour colony on YEMA (BTB indicator) indicating as non-*Rhizobium* colony. However, the nodule isolates from *Canavalia gladiata*, *Sesbania sesban*, *Pisum sativum*, *Vicia faba*, *Vigna umbelata*, *Phaseolus vulgaris* and *Phaseolus lunatus* exhibited a fast growing yellow colour colony with thick mucous which indicated as *Rhizobium* colony. All the nodules isolates were Gram negative, motile, non-spore forming, non-capsulated, rod shaped and aerobic but could grow under anaerobic condition. The cells were between

0.9 - 1.2 µm in diameter and 2-3 µm in length. Growth was evident in temperatures between 10 - 42°C and pH 6 -10; optimum growth was observed in 28 - 36°C and pH 7 - 8. The nodule isolates were authenticated by reinoculation of the Rhizobial isolates with the host legumes. The rhizobial isolates were inoculated with the surface sterilized host legumes seed and grown in Leonard's Jar and inside plant growth chamber.

Phosphate solubilisation and Indole acetic acid (IAA) production

The rhizobial strains were determined for phosphate solubilizing and IAA production in the culture media (Table 1). Among 10 (ten) rhizobial strains tested only 2 (two) strains were found to exhibit negative result for phosphate solubilisation in the culture media. The remaining 8 (eight) rhizobial strains showed positive result with various degree of phosphate solubilisation in the NBRIP media. However positive result of IAA production in the culture media were observed in all 10 (ten) rhizobial strain tested.

Antagonistic effect of rhizobial strains against *Macrophomina phaseolina*

Two hundred and ten (210) rhizobial strains were screened for the antagonistic effect

against *Macrophomina phaseolina*. Not all the rhizobial strain exhibited antagonistic effect against the test fungi. In the present study *Rhizobium* isolates associated with root nodules of *Cajanus cajan*, *Pisum sativum*, *Vicia faba*, *Vigna umbelata*, *Phaseolus vulgaris*, *Phaseolus lunatus* exhibited antagonistic effect against the test fungi.

In vitro antifungal bioassay of ten (10) most effective rhizobial strains was tested against *Macrophomina phaseolina* and different result were observed. None of the Rhizobia showed complete growth inhibition of the test fungi but exhibited significant growth reduction. The highest percentage of growth inhibition rate of *Macrophomina phaseolina* was observed from the nodule isolate RHIVUMU09 (88.86%) followed by RHIVUA24 (80.91%) and RHIPSMU76 (72.76%) (Table 2).

The symbiotic association between leguminous plant and the rhizobia is beneficial for both the host plant and the rhizobia. The rhizobia not only provide nitrogen source to the host plant but also promote the growth of the plant by producing plant growth hormone like IAA, inhibition of plant pathogen, making phosphate available to the plant, etc.

Table.1 Biochemical properties of the rhizobial strains

Nodule Isolate Code	Phosphate solubilization (mm)	Growth (OD at 540nm)	IAA (µg ml ⁻¹) production	Specific productivity (IAA production/ growth)
RHIPSMU76	3±0.04	1.92	129.2	67.29
RHIPSS	4±0.03	1.63	35.9	22.02
RHIPSK	3±0.02	1.90	96.1	50.58
RHIVUMU09	5±0.12	1.67	87.8	52.57
RHIVUA	5±0.04	1.82	52.5	28.84
RHICCMU	2±0.02	1.69	67.9	40.18
RHIVFK	-	1.68	101.2	60.23
RHIVFNK	-	1.49	69.3	46.51
RHIPVK	2±0.14	1.80	76.4	42.44
RHIPLMU23	4±0.09	1.72	90.7	52.73

Table.2 *In vitro* antifungal activity of effective rhizobial strains against *Macrophomina phaseolina*

Sl. No.	Nodule Isolates Code	Fungal colony diameter (cm)	Inhibition (%)
1	Control	8.70 ± 0.20	
2	RHIPSMU76	2.37 ± 0.21	72.76
3	RHIPSS21	2.68 ± 0.30	69.19
4	RHIPSK16	2.44 ± 0.16	71.87
5	RHIVUMU09	0.97 ± 0.12	88.86
6	RHIVUA24	1.66 ± 0.22	80.91
7	RHICCMU01	3.49±0.14	59.83
8	RHIVFK21	3.34± 0.14	61.66
9	RHIVFNK16	2.94± 0.17	66.23
10	RHIPVK13	3.22± 0.14	62.96
11	RHIPLMU23	4.10 ± 0.17	52.87
	SEM	0.16	
	CD	0.35	

The inhibition of fungal growth of the test fungi *in vitro* by certain of the rhizobia and formation of inhibition zones were presumably due to the metabolites released by the bacteria into the culture medium. These metabolites may include antibiotics and/or cell-wall degrading enzymes. Different studies have implicated antifungal secondary metabolites produced by *Rhizobium* spp. in the control of plant diseases caused by pathogenic fungi (Ehteshamul- Haque and Ghaffar, 1993; Siddiqui *et al.*, 2000). Previous studies suggest that rhizobia possessing the traits of volatiles, cyanide production, and phosphate solubilisation can increase plant growth (Glick *et al.*, 1995). Indeed, some P-solubilising organisms have been reported as plant growth promoters, but rigorous proof is lacking (De Freitas *et al.*, 1997; Whitelaw *et al.*, 1997). Different studies have indicated that phosphate solubilisation may play a key role in plant growth promotion (Chabot *et al.*, 1996). The strains of *Rhizobium leguminosarum* biovar *viceae* and *MesoRhizobium* sp. nodulating chickpea were the most effective solubilisers of phosphate (Halder *et al.*, 1990). Although the basic mechanisms behind such protection are not clearly defined, the possibility that

competition, antibiosis, direct parasitism and induced resistance by the antagonistic bacteria, may operate synergistically after inoculation with effective *Rhizobium* isolates cannot be ruled out.

The present study can be concluded as i) different isolates performed moderately *in vitro*, for the production of phosphate solubilization and IAA production and ii) certain nodules isolates were able to form inhibition zones *in vitro* against *Macrophomina phaseolina*, suggesting the production of antibiotics. The present study suggests that the effective rhizobia could be used effectively as biocontrol agents against *Macrophomina phaseolina* and they could be fielded within an integrated disease management package.

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