Isolation of siderophore-producing strains of *Rhizobium meliloti* and their biocontrol potential against *Macrophomina phaseolina* that causes charcoal rot of groundnut

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Of the 12 isolates of root-nodulating bacterium, *Rhizobium meliloti* isolated from the medicinal plant, *Mucuna pruriens*, only two were able to produce siderophores. The two isolates (RMP_3 and RMP_5) were able to inhibit a widely occurring plant pathogen, *Macrophomina phaseolina* that causes charcoal rot in groundnut. Further, there was a marked enhancement in percentage seed germination, seedling biomass, nodule number and nodule fresh weight of *M. phaseolina*-infected groundnut plants inoculated with the strains RMP_3 and RMP_5, compared to uninoculated and uninfected controls. Thus plant growth promotory nature of both the rhizobial strains was confirmed.

EXTENSIVE use of chemicals to control plant diseases has disturbed the delicate ecological balance of the soil, leading to groundwater contamination, development of resistant races of pathogen and health risks to humans[1]. Reluctance of most companies to test newer chemicals due to the financial and registration difficulties has further aggravated the problem[2]. However, considerable attention has been paid to plant growth-promoting rhizobacteria (PGPR), as the best alternative to chemicals, to facilitate eco-friendly biological control of soil and seed-borne pathogens[3].

Most of the microorganisms which exhibit PGPR activity, belong to the Gram-negative group and among these fluorescent Pseudomonads are the most widely studied. The siderophores of fluorescent Pseudomonads and their introduction into the rhizosphere have been widely explored. Siderophore production in iron stress conditions confers upon these organisms an added advantage, resulting in exclusion of pathogens due to iron starvation. However, use of antagonistic rhizobia has an added advantage in that they have also the ability to fix nitrogen. Different strains of rhizobia have now been reported to produce siderophores. Strains of root-nodulating bacteria have also been reported to produce phytohormones like indole acetic acid (IAA)[4], and antibacterial compounds like rhizobiotoxin[5] and bacteriocins[6]. This ability also confers upon nodule bacteria a selective advantage and may lead to both direct and indirect control of plant pathogens.

Macrophomina phaseolina (Tassi) Goid, is one of the most destructive plant pathogens causing charcoal rot, dry-root rot, wilt, leaf blight, stem blight and damping-off diseases in a wide range of host plants[7,8]. *M. phaseolina* has a very wide host range and therefore, it is not easy to attain host resistance/tolerance. Chemical management is often uneconomical and not feasible, because the pathogen is both seed- and soil-borne[8]. Biocontrol can thus offer a very good alternative for management of this pathogen.

Unfortunately, most of the biocontrol agents perform *in vitro* but fail to control the pathogens in field conditions. The present investigation is aimed at assessing the antagonistic potential of *Rhizobium meliloti* isolates, both *in vitro* and *in vivo*, against *M. phaseolina*, that causes charcoal rot of groundnut.

The root-nodulating bacterium isolates from *Mucuna pruriens* were morphologically, biochemically and physiologically tested according to Holt *et al.*[9] and were identified to be *Rhizobium meliloti*[10].

The pathogenic strain of *M. phaseolina* was isolated from the diseased seeds of groundnut by the blotter technique[11]. The pathogen was identified using standard mycological literature.

Siderophore production by the isolates was tested by chrome azurol S (CAS) assay[12]. Siderophore production was also checked by the top layer method. The strains were spread over YEM agar and incubated for 48 h at 30°C. After incubation, a thin layer of CAS reagent[12] in 0.7% agar was spread on the bacterial growth and plates were again incubated for 24 h at 30°C. Formation of yellow-orange halo around the colonies indicates siderophore production[13]. To determine the type of siderophore, culture supernatants were used. The presence of catechol-phenolic-type siderophores was tested according to Arnow[14] and Rioux *et al.*[15], taking 2,3-

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dihydroxybenzoic acid as the standard. The presence of hydroxamate siderophores in the supernatant was checked according to Gibson and Magrath[16], taking the absorption spectrum of supernatant in the visible range in a Shimadzu UV-VIS spectrophotometer (model UV-1601).

Rhizobial isolates were checked for the ability to produce hydrocyanic acid (HCN) and IAA. HCN production was determined on tryptic soya agar[17]. Production of IAA was determined according to the modified method of Gupta *et al.*[18].

The bacterial isolates were screened for ability to inhibit *M. phaseolina* on agar plates. Rhizobial strains were cultured in YEM broth. *M. phaseolina* was also raised on YEM agar. A 6 mm mycelial disc of *M. phaseolina* was centrally placed on YEM agar plates and exponentially grown rhizobial strain (24 h) was spotted on two sides of the mycelial disc. The plates were incubated at 30°C for 5 days, to measure inhibition of radial fungal growth as a clear zone between fungal and bacterial colonies. Per cent inhibition was determined by the reduction in fungal growth compared to control.

Earthen pots (24 × 12 × 12 cm) were filled with garden soil. Mycelia of *M. phaseolina* were inoculated in pre-sterilized oat grains in one-litre capacity flasks and incubated for 5 days at 30°C, to prepare the culture for soil infestation. The grain-based culture of *M. phaseolina* was mixed in both the sterile and non-sterile soil, so as to make the inoculum level of approximately 10^5 cfu g^{-1} soil. Rhizobial strains RMP_3 and RMP_5 were grown in YEM broth up to log phase (10^8 cells ml^{-1}). The cells were harvested by centrifugation and coated (approximately 10^8 cfu per seed) on the surface-sterilized groundnut seeds by the slurry method[19]. After drying for 3–4 h, the seeds were sown in the pots. The experiment was designed with the following treatments with groundnut: (i) soil (control); (ii) soil + *M. phaseolina*; (iii) soil + RMP_3; (iv) soil + RMP_5; (v) soil + RMP_3 + *M. phaseolina*; (vi) soil + RMP_5 + *M. phaseolina*. Five replicates of each treatment were taken. The plants were watered with tap water whenever required. The disease incidence was noted as percentage of the plants showing charcoal rot after 60 days. Plants were harvested to measure seedling biomass and nodule fresh weight of a local variety of *Arachis hypogaea*, and compared with controls.

Root colonization study was carried out in earthen pots filled with garden soil. The soil was infested with *M. phaseolina* and surface-sterilized seeds, bacterized with the isolates RMP_3 and RMP_5, resistant to ampicillin (10 μg) were sown in respective pots. Controls were also maintained as described earlier. Plants were uprooted carefully along with the adhering rhizosphere soil. The soil was removed by shaking the roots gently. One gram of the rhizosphere soil was serially diluted in sterile distilled water for determining cfu g^{-1} soil. Cfu counts of both rhizobia and *M. phaseolina* were determined on YEM agar by the spread plate technique. The plates were incubated at 30°C for 48 h and 5 days for rhizobia and fungi, respectively. For isolating and identifying rhizobial colonies, YEM agar was supplemented with 10 μg ampicillin and actidione (to control fungal growth).

Of the 12 rhizobial isolates screened for siderophore production on CAS agar, only 02, RMP_3 and RMP_5 showed orange colour production and yellow-orange coloured halo around the colonies, on CAS reagent overlaid on YEM agar. Larger halo was formed around the colonies of strain RMP_5 in comparison to those of strain RMP_3, after 24 h of incubation. The 48-h-old supernatant of culture broth of both the strains showed a major peak at 400 nm, which corresponds to hydroxamate-type of siderophore. The culture supernatant of none of the strains produced phenolate–catechol siderophore. In rhizobia, the ability to synthesize siderophore is restricted to a limited range of strains, rather than a wide distribution[13]. There are now, however, a wide range and type of siderophores reported in rhizobia[20]. Hydroxymate-type of siderophores have also been reported in different rhizobial strains[13,21].

None of the isolates produced hydrocyanic acid. Earlier studies have also reported a very low incidence of cyanogens in rhizobia and in other PGPR[22,23]. In fact, it has been reported that production of HCN proved to be deleterious to the plant[22]. However, all the twelve strains produced IAA. Production of IAA is reported to be more common in rhizobia. Prevost *et al.*[24] reported that 96% of the rhizobial isolates produced IAA, whereas Antoun *et al.*[22] observed IAA production by 56% strains of *R. meliloti*.

Of all the isolates only the two siderophore-producing strains, RMP_3 and RMP_5, showed strong antagonism against *M. phaseolina* (Figure 1). Strains RMP_3 and RMP_5 showed 72% and 77% inhibition of fungal growth, respectively after 5 days of incubation, compared to control. There was a gradual increase in fungal inhibition with incubation time and strain RMP_5 was found to be more effective than RMP_3 (Figure 2). The

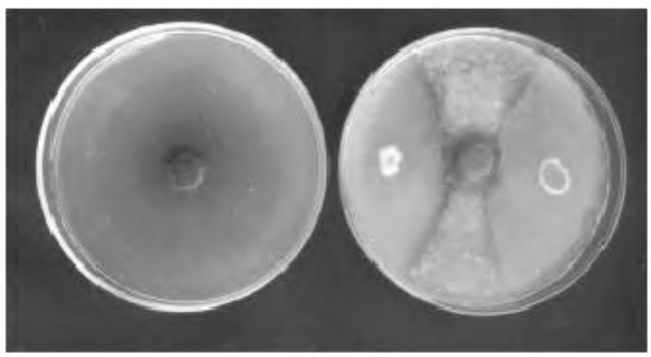

Figure 1. Antagonistic effect of strain RMP_5 on *M. phaseolina* on YEM agar.

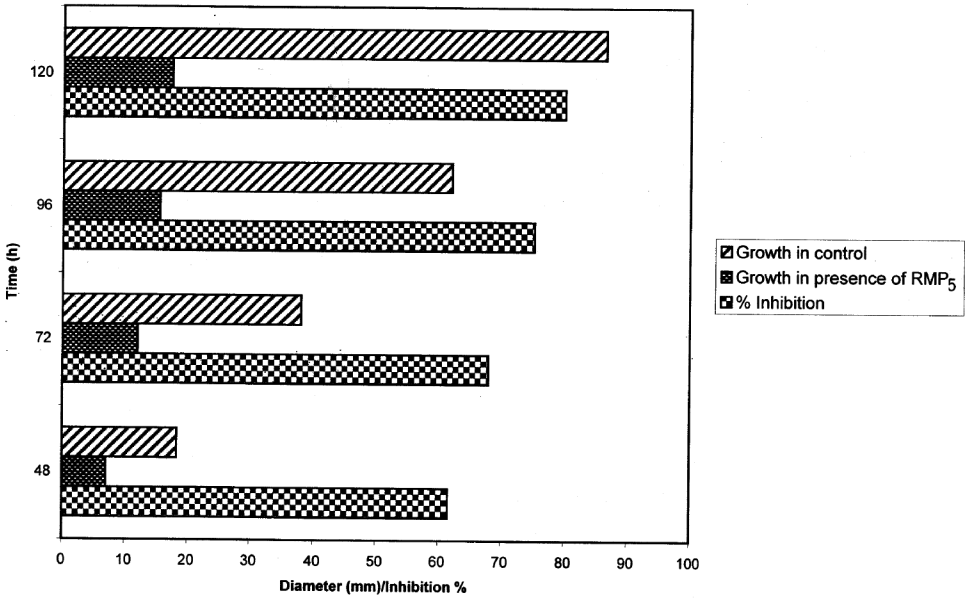

Figure 2. Inhibition of *M. phaseolina* by strain RMP_5.

Table 1. Effect of seed bacterization with rhizobial strains on percentage germination, seedling biomass and nodule fresh weight of groundnut in *M. phaseolina*-infested soil

Treatment	Seed germination (%)	Seedling biomass (g/plant)		Nodule fresh weight (mg/plant)	
		30 days	60 days	30 days	60 days
Control*	78.0	4.24	9.68	14.8	61.4
M. phaseolina•	58.0	2.94	3.24	9.6	24.2
RMP_3♦	88.8**,b	4.48	12.72**,b	20.2	88.7**,b
RMP_5♦	90.9**,b	4.72	13.21**,b	24.7	96.1**,a,b
RMP_3 + *M. phaseolina*	85.1**,a	4.31	11.28**,a	13.7	71.6**,a
RMP_5 + *M. phaseolina*	88.6**,b	4.45	11.78**,a	17.1	75.6**,a

*With no pathogen and rhizobia; •With no rhizobia; ♦Without *M. phaseolina*; Results are mean of five replicates ± SD.
**Highly significant at $P > 0.01$; [a,b]Means in the column followed by same letter are not significantly different.

formation of inhibition zone by the rhizobial antagonistic strains RMP_3 and RMP_5 against *M. phaseolina in vitro*, indicated secretion of certain metabolites by both the strains. Increase in inhibition percentage with incubation period, suggested release of metabolites with incubation.

Rhizobial isolates RMP_3 and RMP_5 did not show nodulation in groundnut plants, as evidenced by the absence of nodulation in sterile soil. Seed germination percentage of groundnut increased considerably in *M. phaseolina*-infested soil by seed bacterization with both the strains (Table 1). Strain RMP_5 showed 52.8% and RMP_3 46.7% increase in germination percentage over pathogen control (Table 1). The per cent germination of seeds coated with rhizobial cells was even better than the control. Bacterization with strains RMP_3 and RMP_5 increased the plant biomass and nodule fresh weight in comparison to pathogen-infested control. Seedlings (unbacterized) in *M. phaseolina*-infested soil developed symptoms of charcoal rot. The black spots were clearly visible on the stem of wilted plants. These plants showed poor nodulation as evidenced by about 61% decline in fresh nodule weight in comparison to control. On the other hand, plants raised from bacterized seeds were healthy and showed no signs of charcoal rot disease in *M. phaseolina*-infested soil. After 60 days, the disease incidence reached 84%. Disease incidence reduced to a mere 3.8% in RMP_5 and 7.9% in RMP_3-treated plants. Seed bacterization with RMP_5 and RMP_3 increased seedling biomass by 22% and 16.5% respectively, of control, in infested soil. Nodule fresh weight was also enhanced by seed bacterization with rhizobial strains (Table 1). The increment in seedling biomass and nodule fresh weight, by seed coating, was even more in non-infested soil. There was a 36% increment in seedling biomass and 56.5% in nodule fresh weight

Table 2. *In vivo* population density of isolates RMP_3 and RMP_5 and fungal pathogen *M. phaseolina* in presence of each other

	Log cfu g^{-1} soil after time (days)				
	7	15	30	45	60
RMP_3*	7.16 ± 0.02	6.98 ± 0.019	6.48 ± 0.015	5.61 ± 0.018	5.00 ± 0.013
RMP_5*	7.38 ± 0.021	7.20 ± 0.021	6.77 ± 0.019	5.89 ± 0.017	5.37 ± 0.015
M. phasolina+	5.23 ± 0.013	5.52 ± 0.014	6.49 ± 0.012	6.56 ± 0.016	6.04 ± 0.014
RMP_3 + (*M. phaseolina*)	6.90 ± 0.024	6.67 ± 0.018	5.74 ± 0.017	5.07 ± 0.018	4.55 ± 0.015
RMP_5 + (*M. phaseolina*)	7.11 ± 0.019	6.86 ± 0.019	6.10 ± 0.021	5.42 ± 0.017	4.88 ± 0.011
M. phaseolina + (RMP_3)	5.08 ± 0.019	4.53 ± 0.018	3.31 ± 0.014	2.33 ± 0.014	1.18 ± 0.011
M. phaseolina + (RMP_5)	5.01 ± 0.017	4.41 ± 0.020	3.18 ± 0.022	2.11 ± 0.013	1.03 ± 0.009

*Without *M. phaseolina*; +Without rhizobia.
Results are mean of five replicates ± SD.

by strain RMP_5 in non-infested soil compared to control, which was slightly more than that of strain RMP_3 (Table 1).

Both RMP_3 and RMP_5 maintained high cfu g^{-1} up to 60 days in the presence of *M. phaseolina*, which was marginally lower than the population of both the isolates in soil without fungal infestation. The population density of RMP_5 was slightly higher than that of RMP_3 both in infested and non-infested soils. Both the strains strongly inhibited the *M. phaseolina* population in the rhizosphere. The population of the pathogenic fungus declined from 10^5 cfu g^{-1} to 67 and 62 cfu g^{-1} after 60 days by RMP_3 and RMP_5 bacterization, respectively (Table 2).

There was a considerable decrease in disease incidence, improvement in seedling biomass and fresh nodule weight by seed bacterization with the strains RMP_3 and RMP_5, in the presence of pathogen. Thus rhizobial strains not only acted as biocontrol agents against *M. phaseolina*, but also proved to be plant growth promotory in nature as evidenced by the increase in seedling biomass and fresh nodule weight over uninoculated controls. Siderophore and IAA have been reported to be responsible for biocontrol and plant growth promotory nature of various PGPR strains[22,25]. The root colonization data also supported the biocontrol ability and PGPR ability of both the rhizobial isolates. A steep decline in the rhizosphere population of *M. phaseolina* in the presence of rhizobial strains and a high population density of both the rhizobial strains in rhizosphere, indicated their association with groundnut roots. It has also been reported earlier that *Rhizobium* reduced charcoal rot disease caused by *Macrophomina* spp[26]. Strains of *R. japonicum* have been reported to protect soybean from *M. phaseolina* infection[5]. Rhizobia have earlier also been reported for their ability to act as PGPR for both legumes[27] and non legumes[22,28].

The isolates of *R. meliloti*, RMP_5 and RMP_3 have proved to be effective in promoting the growth of a non-host, besides inhibiting *M. phaseolina* causing charcoal rot disease in groundnut. Use of rhizobia has an added advantage over other organisms like Pseudomonads, in that they have the ability to fix nitrogen symbiotically with legumes and are eco-friendly and non-pathogenic to humans. Another benefit is that there is a better technical knowledge of inoculant production and application for rhizobia. Thus two isolates of *R. meliloti* (RMP_3 and RMP_5) appear to have a great potential in controlling soil- and seed-borne diseases caused by *M. phaseolina*.

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