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Isolation of bacteria from rhizosphere soil of pulses with antagonistic activity against soil borne pathogenic fungi

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Rhizosphere microflora of three different pulses namely, black gram (*Phaseolus mungo*), green gram (*Phaseolus aureus*) and soybean (*Glycine max*) were studied by soil dillution and plating method. Results showed that the total number of microorganisms increased markedly with the age of soil. Bacteria showed a significant increase in number in rhizosphere soil in comparison to control soil. Bacterial isolates were predominant over fungi in rhizosphere of all the three pulses. Two strains of *Bacillus* species (B_3 and B_{12}) were isolated from the rhizosphere soil of *P. aureus* and were evaluated from their efficacy as biological control agents against a range of soil borne fungi. *In vitro* studies of antagonists were performed using dual culture technique. B_3 and B_{12} isolates inhibited mycelial growth of *Fusarium* sp. and *Macrophomina* sp. by 66% and 62% and 54% and 46% respectively. Whereas only B_3 isolate exhibited a clear zone of inhibition towards mycelial growth of *Pythium aphanidermatum.* Both the strains failed to inhibit *Sclerotium rolfsii.* Results showed the potential of these bacterial antagonists to act as successful biological control agents against soil borne pathogenic fungi in the field.

Key words: Rhizosphere, pulses, *Bacillus*, soil borne pathogens, biocontrol agents.

INTRODUCTION

In the light of present day constraints on plant disease control practices, especially those imposed on the use of chemical pesticides and fungicides, biological control methods of plant pathogens offer powerful alternatives to synthetic chemicals and are successfully exploited nowadays in modern agriculture. Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents since the rhizosphere provides the frontline defense for roots against attack by pathogens (Weller, 1988). Among the various antagonists, rhizobacteria play a vital role in biological control of soil borne plant pathogens. Bacteria which have been used for the management of plant diseases occur in many genera including *Azotobacter* (Meshram and Jager, 1983), *Bacillus* (Broadbent and Baker, 1971; De-Jensen *et al.,* 2002); *Enterobacter* (Kageyama and Nelson, 2003), *Pseudomonas* (Marimuthu *et al.,* 2002;) Hultberg *et al.,* 2000) *Rhizobium* (Chakraborty and Purkayastha, 1984), *Streptomyces* (Sabaratnam and Traquair, 2002) and *Xanthomonas* (Chen and Hoitink, 1987). These beneficial bacteria suppress the plant pathogens by producing one or more of a variety of mechanisms and metabolites that include antibiotics (Keel *et al.,* 1992), Siderophores (Kloepper *et al.,* 1980; Schippers *et al.,* 1987), parasitism and lysis (Ordentich *et al.,* 1987), volatile substances such as cyanide (Voisard *et al.,* 1989) and induced systemic resistance or ISR (Van Loon *et al.,* 1998). Activity is not restricted to only one of these mechanisms, and indeed an efficient biocontrol agent may affect pathogens by a combinations of mechnisms, and indeed an efficient biocontrol agent may affect pathogens by a combinations of mechanisms (Chet, 1987).

The aims of this study were to isolate bacterial species from rhizosphere soil of pulses and to screen these isolates *in vitro* for antagonism against soil borne plant pathogens.

MATERIALS AND METHODS

Isolation of rhizosphere microflora

Seeds of pulses were sown in earthenware pots (20 cm diameter) containing soil. One set of pots was kept as control with only soil. The plants were kept under ordinary conditions of day-light, temperature and humidity of the experimental garden. The soil was collected from the rhizosphere regions of the leguminous plants up to 60 days at 30 days interval. For isolation, basic serial dilution and plating procedure of Johnson and Curl (1972) was followed with modifications. The serial dilutions (upto 10^4) were prepared from 1 g of soil. After this 1 ml of each dilution was plated on Petriplates containing potato dextrose agar (PDA) medium. The Petriplates were incubated at 30°C temperature for 72 hours and after this each microbial colony was transferred to PDA slants and nutrient agar (NA) slants.

Identification and preliminary characterization

Identification of fungal spores was done by colony characteristics, growth and morphology study under microscope and was subsequently confirmed after consulting relevant literature by Gilman (1959). The morphological and biological characteristics of bacterial isolates were studied by means of conventional methods (Sneath, 1986).

In vitro test for antagonistic activity of rhizobacteria

The antagonistic effects of two isolates of *Bacillus* sp. (isolate III-B_3 and isolate XII-B_{12}) were evaluated by the dual culture test. Agar discs (5 mm) carrying actively growing mycelium of different fungi were placed on one side of the Petripate and cells of bacteria were streaked on the opposite side of the same plate. The plates were incubated at 30°C for 7 days. The growth of mycelium (radial colony growth) on control plates was taken as reference for computing antagonistic activity of bacteria with the following equation :

$$\text{Reduction in mycelial growth percentage (\%)} = \frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100$$

Treatment = mycelial growth of fungus in plate with streaked bacteria

Control = mycelial growth of fungus in plate without bacteria.

RESULTS AND DISCUSSION

A number of viable propagules (bacterial and fungi) were obtained from rhizosphere soil of three different pulses by soil dilution and plating method. The total number of microbial colonies was significantly higher in rhizosphere soils in comparison to nonrhizosphere control soil ($P<0.05$). Among the three legumes, highest number of microbial colonies were noted in rhizosphere soil (2-month-old) of *G. max* followed by *P. aureus* and *P.mungo* (Table 1, Fig. 1). Rhizosphere effects were found to be more pronounced with bacteria (including filamentous forms) than that of fungi. Bacteria showed a significant increase in number ($P<0.05$) in rhizosphere soil in comparison to control soil. Results of dilution plate counts

Table 1. Rhizosphere microflora of different pulses.

Soil sample	Average number of microorganisms* [a](c.f.u./g soil $\times 10^4$)			
	Bacteria		Fungi	
	30 days	60 days	30 days	60 days
Non-rihizosphere soil (control)	14.66 ± 2.33	20.33 ± 2.85	1.33 ± 0.33	1.66 ± 0.66
Rhizosphere soil				
P. aureus	27.33 ± 4.98	36.99 ± 3.53*[b]	1 ± 0.00	1 ± 0.00
P. mungo	30.99 ± 4.26*[b]	33.66 ± 2.9*[b]	1 ± 0.00	1.33 ± 0.33
G. max	33.3 ± 2.08*[c]	39.66 ± 5.49*[b]	1 ± 0.00	1.33 ± 0.33

** Average of microorganisms in three replicates of Petriplates;*[b] : $P<0.05$; *[c] : $P<0.01$

c.f.u : Colony forming units; incubation period : 72 hrs; Temperature : 30 ±2°C.

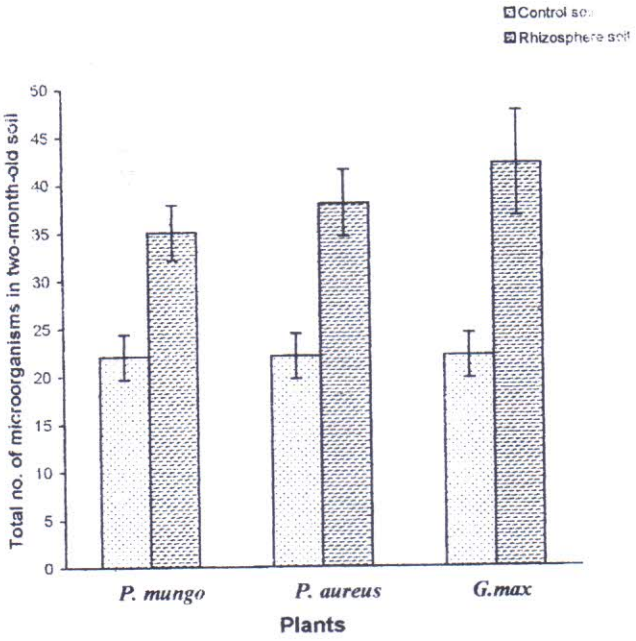

Fig. 1 Total number of microorganisms in control soil and rhizosphere soil of three different pulses.

showed that number of fungal colonies was much less than those of bacteria. This may be due to the reason that fungi are more difficult to assess than bacteria because of their filamentous nature and the fact that the serial dilution and plating procedure favours isolation of abundantly sporulating species. A number of fungal species including *Aspergillus* sp., *Cladosporium* sp., *Rhizopus* sp. and *Penicillium* sp. were isolated from rhizosphere soil of pulses.

Two isolates of *Bacillus* sp. (B_3 and B_{12}), from rhizosphere soil of *P. aureus,* showed clear antagonism against three phytopathogenic soil borne fungi *in vitro.* The bacterial isolates were preliminary characterized by means of conventional methods. Results revealed that the isolates were gram positive, endospore forming, motile, catalase positive. Moreover they were capable to catabolism starch, casein and gelatin (Table 2). In terms of antagonism between the isolates of *Bacillus* sp. and soil borne pathogenic fungi, it was found that both B_3 and B_{12} isolates inhibited mycelial growth of *Fusarium* sp. by 66% (Fig. 2) and 62% and *Macrophomina* sp. by 54% and 46% respectively. Whereas only B_3 isolate exhibited a clear inhibition (50%) towards mycelial growth of *P. aphanidermatum.* Both the isolates failed to suppress the mycelial growth of *Sclerotium rolfsii in*

Table 2. Characteristics of isolated *Bacillus* strains (B_3 and B_{12})

Morphological characteristics	B_3	B_{12}
Shape	Rod	Rod
Gram straining	Positive	Positive
Endospore form	Round, terminal	Elongated, intercalary
Motility	Present	Present
Biological characteristics		
Catalase	+	+
Starch catabolic ability	+	+
Caesin catabolic ability	+	+
Gelatin catabolic ability	+	+

Table 3. Antagonistic of isolated *Bacillus* sp. (B_3 and B_{12})

Name of test organisms	Reduction in mycelial growth percentage (%)	
	Bacillus sp. (isolate III-B_3)	*Bacillus* sp. (isolate XII-B_{12})
Fusarium sp.	66	54
Macrophomina sp.	62	46
Pythium aphanidermatum	50	0
Sclerotium rolfsii	0	0

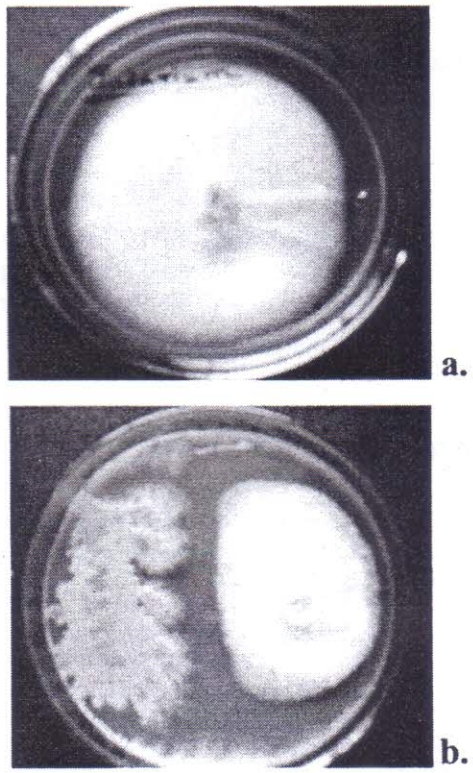

Fig. 2 : (a & b) : Dual culture test showing the reduction of mycelial growth of *Fusarium* sp. B_3 isolate of *Bacillus* sp. a) Control b) Treated with B_3 isolate of *Bacillus* sp.

vitro (Table 3).

A number of gram-positive *Bacillus* species have been shown to be potential biocontrol agents against fungal pathogens. The antagonists studies have been mainly *B. subtilis* (De Jensen *et al.,* 2002) and occasionally *B. mojavensis* (Bacon and Hinton, 2002), *B. pumilis* (Yan *et al.,* 2002), *B. polymyxa* (Hyun *et al.,*1999) and *B. cereus* (Silo-suh *et al.,* 1994). *Bacillus* spp. are appealing candidates for biocontrol because they produce endospores that the tolerant to heat and desiccation (Weller, 1988).

Kim *et al.* (2003) isolated strains of *Bacillus* sp. (GB017 and GB0356) from soil which were characterized to be gram positive, motile and were found to inhibit *Fusarium* sp., *Pythium* sp. *Rhizoctonia* sp. *in vitro.* Culture filtrates of rhizosphere fungi and bacteria were shown to inhibit radial colony growth of *F. udum,* the causal organism of wilt disease of pigeon pea. Among *Bacillus* species isolated, *B. licheniformis* (strain 2042) showed marked inhibition (>50%) against test pathogen (Singh *et al.,* 2002).

The most crucial outcome of this brief investigation is the isolation of two strains of *Bacillus* sp. from rihizosphere of *P. aureus* which markedly inhibited the mycelial growth of soil borne fungal pathogens. Further research is needed to evaluate the efficacy of these bacterial isolates against the pathogenic fungi under field conditions. The challenge is to develop widely adapted biocontrol agents with broad-spectrum activity.

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