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## Biocontrol of Fusarium wilt by *Bacillus pumilus*, *Pseudomonas alcaligenes*, and *Rhizobium* sp. on lentil

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**Abstract:** The present study examined the effects of *Bacillus pumilus*, *Pseudomonas alcaligenes*, and *Rhizobium* sp. on wilt disease caused by *Fusarium oxysporum* f. sp. *lentis* and on the growth of lentil. Inoculation with *F. oxysporum* caused significant wilting, and reduced plant growth, the number of pods, and nodulation. Inoculation with *B. pumilus* together with *P. alcaligenes* caused a greater increase in plant growth, number of pods, nodulation, and root colonization by rhizobacteria, and also reduced *Fusarium* wilting to a greater degree than did individual inoculation. Use of *Rhizobium* sp. resulted in a greater increase in plant growth, number of pods, and nodulation, and reduced wilting more than *B. pumilus* did. Combined application of *B. pumilus* and *P. alcaligenes* with *Rhizobium* sp. resulted in the greatest increase in plant growth, number of pods, nodulation, and root colonization by rhizobacteria, and also reduced wilting in *Fusarium*-inoculated plants.

**Key words:** *Bacillus*, biocontrol, *Fusarium*, *Pseudomonas*, *Rhizobium*, wilting

### Mercimeklerdeki *Fusarium* solma hastalığının *Bacillus pumilus*, *Pseudomonas alcaligenes* ve *Rhizobium* sp. ile biyokontrolü

**Özet:** Mercimek büyümesinde *Fusarium oxysporum* f. sp. *Lentis*' in neden olduğu solma (wilt) hastalığı üzerine *Bacillus pumilus*, *Pseudomonas alcaligenes* ve *Rhizobium* sp. türlerinin etkisi çalışılmıştır. *F. oxysporum* inokülasyonu önemli ölçüde solmaya neden olur, bitki büyümesini, pod sayısını azaltır ve nodülasyona neden olur. *B. pumilus* ile birlikte *P. alcaligenes* inokülasyonu ise bitki büyümesinde, pod sayısında ve nodülasyonda büyük artışa neden olur, rihizobakterlerle kök kolonizasyonu artar, tek başına inokülasyon ile mukayese edildiğinde *Fusarium* solması azalır. *Rhizobium* sp. bitki büyümesinde, pod sayısında, nodülasyon çok fazla artışa neden olur. *B. pumilus* 'e göre solma oldukça azalır. *B. pumilus* ile birlikte *P. alcaligenes* ve *Rhizobium* sp.' nin inokülasyonu bitki büyümesinde pod sayısında, rihizobakterler ile kök kolonizasyonunda çok fazla artışa neden olur ve *Fusarium* ile inoküle edilmiş bitkilerde solmayı azaltır.

**Anahtar sözcükler:** *Bacillus*, biyokontrol, *Fusarium*, *Pseudomonas*, *Rhizobium*, solma

### Introduction

Lentil (*Lens culinaris* Medik.) is a valuable pulse and is grown in India. *Fusarium* wilt, caused by *Fusarium oxysporum* Schlecht. Emend Snyder &

Hansen f. sp. *lentis* Vasudeva and Srinivasan is one of the most important diseases of lentil worldwide, and is a major factor that limits the successful cultivation of this important crop (1,2).

Some bacteria are associated with the roots of crop plants, have beneficial effects on their host, and are referred to as plant growth-promoting rhizobacteria (PGPR) (3). The rhizosphere is subjected to dramatic changes and the dynamic nature of the rhizosphere creates interactions that lead to biocontrol of diseases (4,5-8). PGPR are free living bacteria that may have beneficial effects on plants, viz. seedling emergence, colonizing roots, stimulating overall plant growth, mineral nutrition, and water utilization, as well as disease suppression. The manipulation of the crop rhizosphere with PGPR for the biocontrol of plant pathogens has shown considerable promise (9). Similarly, the presence of rhizobia in the rhizosphere may also protect host roots from damage caused by pathogens (10-12).

In general, a single biocontrol agent is used for biocontrol of a plant disease caused by a single pathogen (13). This may sometimes account for the inconsistent performance, because a single biocontrol agent is not active in all soil environments or against all pathogens that attack the host plant. On the other hand, mixtures of biocontrol agents with different plant colonization patterns may be useful for the biocontrol of various plant pathogens via different disease suppression mechanisms (13). Moreover, mixtures of biocontrol agents with taxonomically different organisms that require different optimum temperatures, pHs, and moisture conditions may colonize roots more aggressively, improving plant growth and the efficacy of biocontrol agents. An increase in suppression and enhanced consistency against multiple cucumber pathogens were observed using strain mixtures of PGPR (14).

The present study aimed to examine the effects of *Bacillus pumilus* and *Pseudomonas alcaligenes*, alone and in combination with *Rhizobium* sp., on plant growth, number of pods, number of nodules per root system, colonization of roots by rhizobacteria, and on wilting caused by *F. oxysporum* f. sp. *lentis*.

## Materials and methods

### Preparation and sterilization of soil mixture

Sandy loam soil (pH 7.2) collected from a field belonging to Aligarh Muslim University (A.M.U.) Botany Department was passed through a 10 mesh

sieve. This soil, river sand, and organic manure were mixed in the ratio of 3:1:1 and 9-cm diameter clay pots were filled with 1 kg of the mixture. Water was poured into each pot just to wet the soil surface before sterilization at 137.9 kPa for 20 min at 121.9 °C. Sterilized pots were allowed to cool at room temperature before use.

### Raising and maintenance of test plants

Lentil (*Lens culinaris* Medik.) cv. K-75 seeds were surface sterilized with 0.1% sodium hypochlorite for 2 min and rinsed 3 times with sterile water. Five seeds were sown in steam-sterilized soil in 9-cm diameter clay pots; 1 week after germination thinning was performed to maintain 1 seedling per pot. Two days after thinning the seedlings received the treatments listed in Table 1 and un-inoculated plants served as controls. The plants were watered as needed and kept in a glasshouse at  $20 \pm 2$  °C.

### Preparation of fungus inoculum

*Fusarium oxysporum* f. sp. *lentis* was isolated from infected lentil roots obtained from Chherat and Aligarh (CA) was maintained on potato dextrose agar (PDA). *F. oxysporum* f. sp. *lentis* CA was deposited at the culture collection of the Department of Botany, A.M.U. Inoculum of this fungus was prepared by culturing the isolate in Richard's liquid medium (15) for 15 days at 25 °C. Mycelium was collected on blotting paper, and excess water and nutrients were removed by pressing between 2 folds of blotting paper. Mycelium (100 g) was macerated in 1 L of distilled water and 10 mL of this suspension containing 1 g of fungal mycelium was poured around the roots, as described in the inoculation technique.

### Rhizobacterial inocula

*Bacillus pumilus* (MTCC No. 1640) and *Pseudomonas alcaligenes* (MTCC No. 493) were obtained from the Microbial Type of Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. These bacteria exhibited biocontrol activity against a root-knot nematode in our previous study (16). These isolates were subcultured on nutrient broth (Hi-Media Laboratories, Mumbai, India), and incubated at  $37 \pm 1$  °C for 72 h. One milliliter of nutrient broth suspension contained about  $1.5 \times 10^7$  CFU/mL of each isolate; 10 mL of this suspension was poured into each pot around the lentil seedling.

### **Rhizobium inoculum**

A commercial charcoal culture of *Rhizobium* sp. (Lentil strain AQ07) (100 g) was obtained from Quarsi Agricultural Farm, Aligarh, India. The charcoal culture was suspended in 1000 mL of distilled water and 10 mL (equivalent to 1 g of inoculum) was inoculated around the rhizosphere in each pot after thinning.

### **Inoculation technique**

One-week-old healthy lentil seedlings were used for inoculation. For inoculation with *F. oxysporum*, *B. pumilus*, *P. alcaligenes*, and *Rhizobium* sp. the soil around the roots was carefully moved aside without damaging the roots. The inoculum suspensions were poured around the plants and the soil was replaced. An equal amount of sterile water was poured on the controls.

### **Experimental design**

The experiment was carried out in a completely randomized block design, with 2 main blocks: A) without fungus and B) with fungus. Each main block was tested with and without *Rhizobium*, i.e. a) without *Rhizobium* b) with *Rhizobium* ( $2 \times 2 = 4$ ). Each sub-block was inoculated separately with the following 4 treatments: 1) control (C); 2) *B. pumilus* (Bp); 3) *P. alcaligenes* (Pa); 4) Bp + Pa ( $4 \times 4 = 16$  treatments). Each treatment was replicated 5 times.

### **Observations**

Plants were harvested 90 days after inoculation. Plant length, plant dry weight, number of pods, number of nodules, colonization of roots by rhizobacteria, and the wilting index were recorded. To determine dry weight (g), shoots were dried in a hot air oven at 60 °C for 48 h.

Lentil roots inoculated with rhizobacteria were partially collected 1 month after sowing. One gram of roots was surface sterilized and crushed in sterile normal solution (NSS), and 0.1 mL of serially diluted extract was placed on nutrient agar plates and incubated at  $37 \pm 1$  °C for 72 h. These plates were placed on a Quebec colony counter to count the bacterial colonies. Colonies falling within the 30-300 range were selected and multiplied by the reciprocal dilution factor to obtain the number of bacterial colonies (17) represented as colony forming units (CFU) per gram of root. The wilting index of plants

inoculated with *F. oxysporum* f. sp. *lentis* was recorded by scoring disease severity on a scale of 0-5, where 0 is no wilting and 5 is severe wilting. Wilting was observed on the basis of the percentage of necrosis in the xylem as follows: 0 is no necrosis; 1 is up to 20% necrosis; 2 is 21%-40% necrosis; 3 is 41%-60% necrosis; 4 is 61%-80% necrosis; 5 is more than 80% necrosis.

### **Statistical analysis**

All data were statistically analyzed as a 3-factor experiment (fungus  $\times$  *Rhizobium*  $\times$  PGPR), and least significant differences were calculated at  $P = 0.05$  (18). Tukey's test was performed to denote significant differences between treatments.

### **Results**

Inoculation with *B. pumilus* and *P. alcaligenes* caused a significant increase in the shoot dry weight of plants without fungus, as compared to un-inoculated plants, and the inoculation with both caused a greater increase in shoot dry weight than that caused by inoculation of each inoculum alone (Table 1). Inoculation with *Rhizobium* sp. caused a greater increase in shoot dry weight than did *B. pumilus*; however, the increase in shoot dry weight of plants without fungus caused by *P. alcaligenes* was statistically similar to that caused by *Rhizobium* sp. Inoculation with *Rhizobium* sp. and *B. pumilus* plus *P. alcaligenes* caused the greatest increase in shoot dry weight of plants without fungus (Table 1).

Inoculation of plants with *F. oxysporum* caused a significant reduction in shoot dry weight, as compared to un-inoculated plants (Table 1). Inoculation of pathogen-inoculated plants with *B. pumilus*, *P. alcaligenes*, or both caused a significant increase in shoot dry weight, as compared to plants inoculated with *F. oxysporum* alone. Inoculation of pathogen-inoculated plants with *Rhizobium* sp. caused a greater increase in shoot dry weight than that caused by *B. pumilus*; however, the increase in shoot dry weight of pathogen-inoculated plants induced by *P. alcaligenes* was similar to that induced by *Rhizobium* sp. The use of *Rhizobium* sp. with *B. pumilus* plus *P. alcaligenes* resulted in the greatest increase in shoot dry weight in pathogen-inoculated plants (Table 1).

Table 1. The effects of *Bacillus pumilus*, *Pseudomonas alcaligenes*, and *Rhizobium* sp. on the growth of lentil.

Treatments		Plant length (cm plant <sup>-1</sup> )		Plant dry weight (g plant <sup>-1</sup> )		No. of pods plant <sup>-1</sup>	
		Without <i>Rhizobium</i>	With <i>Rhizobium</i>	Without <i>Rhizobium</i>	With <i>Rhizobium</i>	Without <i>Rhizobium</i>	With <i>Rhizobium</i>
Without pathogen	C	52.6 kl	68.6 e	1.44 i	1.90 d	20 j	33 de
	Bp	63.8 gh	73.4 c	1.74 e	2.06 bc	26 gh	39 bc
	Pa	66.2 f	75.5 b	1.84 d	2.12 ab	29 fg	41 ab
	Bp + Pa	71.1 d	78.4 a	2.02 c	2.18 a	36 cd	44 a
With pathogen	C	43.6 m	56.6 j	1.12 k	1.46 i	14 k	24 hi
	Bp	52.4 l	60.2 i	1.34 j	1.58 gh	19 j	29 fg
	Pa	54.4 k	62.4 h	1.42 ij	1.64 fg	21 ij	32 ef
	Bp + Pa	58.3 j	65.2 fg	1.50 hi	1.70 ef	26 gh	34 de

C: Control; Bp: *B. pumilus*; Pa: *P. alcaligenes*; R: *Rhizobium* sp.

Values in the same column followed by the same letters are not significantly different (Tukey's test,  $P < 0.05$ ).

The number of pods per plant significantly declined when plants were inoculated with *F. oxysporum* (Table 1). When *F. oxysporum*-inoculated plants were treated with *Rhizobium* sp. + *B. pumilus* + *P. alcaligenes* a significant increase in the number of pods per plant was observed. Combined use of *Rhizobium* sp. with *B. pumilus* plus *P. alcaligenes* was more effective in increasing the number of pods per plant than their individual application (Table 1).

Inoculation with *Rhizobium* sp. caused a significant increase in the number of nodules per root system, both in pathogen-inoculated and un-inoculated plants (Table 2). No significant difference was observed in the number of nodules per root system between plants inoculated with *B. pumilus* and *P. alcaligenes* alone or in combination (Table 2). Use of *Rhizobium* sp. with *B. pumilus* or *P. alcaligenes* induced more root colonization than did the absence of *Rhizobium* with rhizobacterium, both in pathogen-inoculated and un-inoculated plants (Table 2). Plants inoculated with *F. oxysporum* alone had a wilting index of 4. The wilting index was reduced to 3 when pathogen-inoculated plants were treated with *B. pumilus* + *P. alcaligenes* and was reduced to 2 when pathogen-inoculated plants were treated with *Rhizobium* sp. With other treatments the wilting index was 1 (Table 2).

## Discussion

PGPR are effective root colonizers that survive and proliferate along with plant roots, resulting in enhanced plant growth (19,20). Egamberdieva (21) observed the positive effect of PGPR on the growth of wheat and pea, while seed inoculation improved seedling growth in maize (22). Our results are in agreement with the findings of earlier research (23-26).

The present study demonstrates that use of *B. pumilus*, *P. alcaligenes*, and *Rhizobium* sp. improved the growth of *F. oxysporum*-inoculated plants. These results may have been due to direct antagonism of pathogens, antibiotic production, or competition with pathogens for essential nutrients (27). *Bacillus* spp. are known to reduce the wilting index in *F. udum*-inoculated plants (23). Improvement in plant growth could be attributed to the inhibitory effects of *Bacillus* spp. on pathogens (28-30).

Use of *Bacillus* spp. resulted in rapid colonization of all tissues in tomato, including the vascular stele, and induced resistance against *F. oxysporum* (31). A reduction in root-rot disease of chickpea by *Bacillus* sp. (B22) in *Macrophomina phaseolina*-infected plants was also reported (32). Similarly, *Pseudomonas* spp. also have the ability to suppress parasitic root



Table 2. The effects of *Bacillus pumilus*, *Pseudomonas alcaligenes*, and *Rhizobium* sp. on nodulation, colonization of roots by rhizobacteria, and wilting index of lentil.

Treatments		No. of nodules (root system <sup>-1</sup> )		Colonization of roots by rhizobacteria (CFU root g <sup>-1</sup> )		Wilting index	
		Without <i>Rhizobium</i>	With <i>Rhizobium</i>	Without <i>Rhizobium</i>	With <i>Rhizobium</i>	Without <i>Rhizobium</i>	With <i>Rhizobium</i>
Without pathogen	C	6 f	21 cd	—	—	—	—
	Bp	4 f	24 ab	1.7 × 10 <sup>4</sup>	2.1 × 10 <sup>4</sup>	—	—
	Pa	3 f	26 ab	1.9 × 10 <sup>4</sup>	2.3 × 10 <sup>4</sup>	—	—
	Bp + Pa	2 f	28 a	2.2 × 10 <sup>4</sup>	2.6 × 10 <sup>4</sup>	—	—
With pathogen	C	5 f	16 e	—	—	4	2
	Bp	4 f	18 de	1.4 × 10 <sup>4</sup>	1.6 × 10 <sup>4</sup>	3	1
	Pa	3 f	20 cde	1.7 × 10 <sup>4</sup>	1.9 × 10 <sup>4</sup>	3	1
	Bp + Pa	3 f	23 cd	1.9 × 10 <sup>4</sup>	2.1 × 10 <sup>4</sup>	1	1

C: Control; Bp: *B. pumilus*; Pa: *P. alcaligenes*; R: *Rhizobium* sp.

Values in the same column followed by the same letters are not significantly different (Tukey's test, P < 0.05).

pathogens (33,34) via the production of biologically active substances (27,34); they also synthesize the enzyme that modulates hormone levels, limit the available iron via the production of siderophores, and kill pathogens by producing antibiotics (9). Siddiqui et al. (35) reported that the wilting index is reduced by the application of fluorescent pseudomonads and *Bacillus* spp. in pigeon pea.

The main facets of altered host metabolism were the induction of a structural response at the sites of pathogen entry and abnormal accumulation of electron-dense substances in colonized areas. *Bacilli* are known to suppress diseases by the inhibition of pathogens via diffusible or volatile products, induction of resistance in plants, aggressive root colonization, and stimulation of plant growth (9,12,19). In addition, induced systemic resistance by PGPR is also considered a mechanism for the biocontrol of plant pathogens (36). The present study highlights the role of PGPR in the synthesis of enzymes that may modulate plant hormone levels, limit available iron by producing siderophores, and inhibit pathogens by producing antibiotics (9).

Inoculation with *Rhizobium* sp. alone resulted in better growth in both *F. oxysporum*-inoculated and

un-inoculated plants, because this root nodule bacterium fixes atmospheric nitrogen and is reported to produce toxic metabolites that inhibit many plant pathogens (37). Chakraborty and Purkayastha (38) reported that *Rhizobium* secretes rhizobitoxine, while *Rhizobium leguminosarum* is reported to produce increased levels of phytoalexin (4-hydroxy-2,3,9-trimethoxy pterocarpan) in pea (39). Roslycky (40) reported production of an antibiotic bacteriocin by rhizobia. All this suggests that use of rhizobia that increase the nitrogen content and plant growth can also reduce disease severity (10,23). More detailed investigations of the relationships in various pathosystems, and interactions between microorganisms and host plants are needed in order to develop biocontrol methods for the related diseases.

## Conclusion

The results of the present study suggest that greater emphasis on the development of mixtures of biocontrol agents is needed, because they may better adapt to environmental changes that occur throughout the growing season and protect against a broader range of pathogens. Multiple organisms may

enhance the level and consistency of biocontrol via a more stable rhizosphere community, and increase the effectiveness in a wide range of environmental conditions. The mixture of *B. pumilus*, *P. alcaligenes*, and *Rhizobium* was the most effective treatment against the wilt disease of lentil. Further studies are needed to confirm these results under field conditions.

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