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Beneficial Effects of Fluorescent Pseudomonads on Seed Germination, Growth Promotion, and Suppression of Charcoal Rot in Groundnut (*Arachis hypogea* L.)

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Rhizobacteria are used as inoculants to enhance crop yield and for biological control of fungal pathogens. Fluorescent pseudomonads isolated from the rhizosphere of groundnut showed suppression of the phytopathogen *Macrophomina phaseolina* that causes charcoal rot of groundnut, an economically important agroproduct. Two strains of fluorescent pseudomonads, designated as PS1 and PS2, were selected as a result of *in vitro* antifungal activity. After 5 days of incubation at 28±1°C, both PS1 and PS2 caused clear inhibition zones in dual cultures, restricting the growth of *M. phaseolina* by 71% and 74%, respectively. Both the strains were capable of producing siderophores, indole acetic acid, and hydrocyanic acid, and causing phosphate solubilization under normal growth conditions. These strains, when used as inoculants in groundnut, enhanced germination up to 15% and 30% with subsequent increase in grain yield by 66% and 77%, respectively. Conversely, when the pathogen alone was tested 57% decrease in yield was recorded. Thus the studies revealed the potential of the two pseudomonads not only as biocontrol agents against *M. phaseolina*, but also as a good growth promoter for groundnut.

Keywords: Pseudomonads, charcoal rot, groundnut, *Macrophomina phaseolina*, biocontrol, rhizobacteria

Macrophomina phaseolina (Tassi) Goid, is an important plant pathogen of groundnut, distributed worldwide. The fungus causes complex disease syndromes like charcoal rot of stem, root rot, seedling blight, foliage blight, tuber decay, dry rot, fruit rot, and pod and seed rot. In the absence of host plant, it survives over seasons, predominantly as small jet-black sclerotia in diseased plant parts or in the soil [9].

Although some chemical fungicides are known to be highly effective, such chemicals can be harmful for non-target microbes, plants, animals, soil, and environment. As a result, disease containment through an eco-friendly biocontrol approach, using natural antagonistic microflora, is becoming an inevitable component in the integrated management strategy of plant diseases. The rhizosphere is the first line of defense for roots, against attack by pathogenic fungi. Therefore, there is an excellent opportunity to find rhizosphere competent microorganisms that can act as potential biopesticides.

Fluorescent pseudomonads commonly isolated from the rhizosphere have been shown to be ideal biological control agents [6, 13, 17, 32–35] for the following reasons: (1) many of the secondary metabolites from pseudomonads inhibit other microorganisms, (2) a higher proportion of pseudomonads are inhibitory to a variety of microorganisms in comparison with other soil bacteria, (3) pseudomonads are selectively stimulated in the rhizosphere and when introduced *via* seed coating they can dominate the rhizosphere population, and (4) they extensively colonize the endorhizosphere [20, 26, 30, 33].

The beneficial effects of these bacteria are attributed to the production of diverse metabolites including siderophores, hydrocyanic acid (HCN), and phytohormones, and other associated activities such as greater phosphate solubilization, and competition in soil and root colonization. Application of fluorescent pseudomonads to seeds, seed pieces, and roots has resulted in increased plant growth and yield of over 100 percent. The full potential of rhizobacteria to promote plant growth can be achieved only when there is a better understanding of the factors controlling their ecology and establishment on roots.

The fungus *M. phaseolina* has a wide range, infecting about 500 plant species including peanut [33]. The charcoal rot in

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peanut causes considerable loss. In severe cases the whole plant becomes defoliated and results in complete loss of leaves.

In order to develop fluorescent pseudomonads as biocontrol agents for commercial use, a number of such organisms were isolated from the rhizosphere of groundnut (G) and sunflower (S) and assessed for their effectiveness against an important plant pathogen (*Macrophomina phaseolina*). An effort has been made to correlate the production of certain metabolites by pseudomonads in their effectiveness as biological control agents under laboratory as well as field conditions.

MATERIALS AND METHODS

Seeds

Healthy as well as diseased seeds of *Arachis hypogea* infected with *Macrophomina phaseolina* were procured from the local market and stored at room temperature.

Bacterial and Fungal Strains

Strains of fluorescent pseudomonads were isolated from the rhizosphere of groundnut (G) and sunflower (S) by the serial dilution agar plate method. The strains were morphologically and biochemically characterized according to *Bergey's Manual of Determinative Bacteriology* [16] and were maintained by regular subculturing on succinate agar medium and tryptic soy agar medium at 4°C.

The fungal pathogen *M. phaseolina* was isolated from diseased seeds of groundnut by using the blotter technique and identified using standard mycological literature. The fungal culture was maintained by regular subculturing on Czapek-Dox Agar at 4°C.

Siderophore, Hydrocyanic Acid (HCN), and Indole Acetic Acid (IAA) Production

Siderophore production by the bacterial isolates was tested by the chrome azurol S (CAS) assay [28]. Production of HCN was tested by the method of Castric and Castric [7] and Bric *et al.* [5] respectively.

Phosphate Solubilization

Phosphate solubilization was performed by spot inoculation of organisms on Pikovskaya's medium [23]. The plates were incubated for 4–5 days at 28°C and formation of clear zone was observed.

Antagonistic Activity Against *M. phaseolina*

All strains of fluorescent pseudomonads were initially screened for their ability to inhibit *M. phaseolina* by using a dual culture technique [29]. Mycelial discs (5 mm diam.), obtained from a five days grown culture of *M. phaseolina*, were placed on four corners of tryptic soya agar plates. A loopfull of 24-h-old culture of fluorescent *Pseudomonas* strains PS1 and PS2 was inoculated in the center of the agar plates and incubated at 28±1°C for 5 days and inhibition of the fungal growth was measured.

Experimental Design for Field Study

The field experiments were conducted in the northern part of India during the rainy season, from July to October in the year 2001, on a sandy loam soil with 76% sand, 14% silt, and 10% clay. The pH 7.8 soil had organic carbon 0.59%, total N 0.06%, available P 22.45 kg/ha and available K 167 kg/ha. No crop had been cultivated in the field for the last 2–3 years. The total area of 14.85 m^2 was divided into 9 plots each of 1.1×1.5 m for one set of experimentation. A uniform plant population was maintained with an intra row spacing of 15 cm. The data obtained were from triplicate experimental trials.

To demonstrate the role of pseudomonads as natural biocontrol agents against the charcoal rot of groundnut, two best strains of pseudomonads (PS1 and PS2) isolated from groundnut were used for the studies (Table 1). The experiment was set in the field in three groups, and for the purpose nine blocks (three block/group) were marked for treatment studies using bacterized and non-bacterized seeds. Each block was given a different treatment as follows:

Group 1, treatment 1: soil inoculated with groundnut seeds bacterized with PS1 (pseudomonad strain 1); Treatment 2: soil inoculated with groundnut seeds bacterized with PS1 and the pathogen (P) *M. phaseolina*. In Group 2, the above set of three

Table 1. Production of siderophore, HCN, and IAA, phosphate solubilization, and antagonism against *M. phaseolina* by fluorescent pseudomonad strains isolated from the rhizosphere of groundnut (G) and sunflower (S).

Strains of fluorescent pseudomonads	Siderophore production[a]	HCN production[b]	IAA production[c]	Phosphate solubilization[d]	Antagonism against *M. phaseolina*[e]
PS1(G)	+++	+A	+	+++	+
PS2(G)	+++	+A	+	+++	+
PS3(G)	–	–	–	–	–
PS4(G)	–	–	+	–	–
PS5(G)	–	–	+	–	–
PS6(S)	+	–	+	+	–
PS7(S)	–	–	–	–	–
PS8(S)	+	–	+	+	–
PS9(S)	–	–	–	–	–
PS10(S)	–	–	–	–	–

[a]+++, large halos ≥20 mm wide surrounding colonies; +, small halos <5 mm; –, absence of halo formation.

[b]+A, strong HCN production, –, no HCN production.

[c]+, IAA positive; –, IAA negative.

[d]+++, ≥12 mm radial clear zone of phosphate solubilization; ++, ≤5 mm radial clear zone of phosphate solubilization, –, phosphate solubilization negative.

[e]+, antagonism against *M. phaseolina*; –, no antagonism against *M. phaseolina*.

treatments were repeated as treatments 4, 5, and 6 using seeds bacterized with the other strain of pseudomonad (PS2). Group 3 was the control, where germination of seeds (non-bacterized) were studied in the presence and absence of fungal pathogen (P) as follows: treatment 7: soil inoculated with groundnut seeds; treatment 8: soil inoculated with seeds and supplemented with chemical fertilizer; treatment 9: soil inoculated with seeds and pathogen. The method of bacterization is given below.

Chemical fertilizers were applied (g/ha): urea 100, potash 100, and super phosphate 200. The fertilizers were added after the germination of seeds and the field was irrigated once. Inoculum of *M. phaseolina* was prepared by growing fungus on Czapek-Dox broth at 28±1°C for 4–5 days. It was mixed in soil 12–15 days prior to sowing of seeds. Pure culture of *M. phaseolina* was vortexed and washed with sterilized distilled water (2–3 times). The inoculum consisted of mycelial fragments plus sclerotia. The inoculum was mixed with soil and left for a week for proper establishment. The density of pathogen measured after a week was 250 propagules/g oven-dried soil before applying to the field. The plants were watered with tap water whenever required and harvested after 105 days of sowing.

Seed germination (%) was recorded on day 15 after sowing and plant growth was monitored in terms of shoot height and root length, fresh shoot and root weight, dry shoot and root weight, weight of nodules per plant, number of pods and number of seeds per pod, and grain yield, up to 105 days at an interval of 15 days. Ten plants from each plot were randomly selected for measuring the early vegetative growth and pre and post flowering plant parameters. The data were statistically analyzed by using analysis of variance (ANOVA) for individual parameters on the basis of mean values to find out the significance at 1% and 5% levels.

Seed Bacterization

The method of Weller and Cook [34] was followed for seed bacterization. The seeds were surface-sterilized with 0.1% $HgCl_2$ [mercury (II) chloride] for 3–5 min and then washed and rinsed in sterilized distilled water for 3–4 times and dried overnight under a sterile air stream. Cells of fluorescent pseudomonads PS1 and PS2 were grown in succinate broth for 24 h at 28±1°C under shaking conditions and finally centrifuged at 7,000 ×*g* for 15 min at 4°C. The supernatant was discarded and the pellet was washed with sterilized distilled water and re-suspended to obtain a population density of 10^7 CFU/ml. This suspension was mixed with 1% carboxy methyl cellulose (CMC). Seeds were allowed to air-dry overnight under aseptic conditions after coating with a CMC slurry of bacterial culture. Care was taken to avoid clumping of seeds. Seeds coated with a slurry of CMC (without bacteria) served as control.

Development of Antibiotic-Resistant Marker

For the development of a resistant marker, *Pseudomonas* spp. were evaluated for antibiotic sensitivity. Antibiotic discs (5 mm diam.) of different concentrations were placed at four corners over the surface of the seeded culture of *Pseudomonas* in succinate agar medium. Plates were incubated at 28±1°C for 24 h and the inhibition zone was recorded. Resistance marker strains were developed by subjecting the culture successively to low concentration to high concentration of streptomycin. The seedlings of groundnut, raised from seeds bacterized with PS1 (Strep+) and PS2 (Strep+), were sampled for root colonization after one, two, and three months of sowing, and the bacterial population on the root was measured.

Root Colonization

Plants were carefully removed with a shovel, and soil particles adhering to the roots were gently removed. Care was taken not to remove soil particles tightly adhered to the roots. The roots were then cut into 1-cm-long segments and 1 g of root segments was dipped into 5 ml of sterilized distilled water and vortexed 4–5 times to release the rhizosphere bacteria into the water. Dilutions of the bacterial suspensions were pour-plated on succinate agar medium containing streptomycin (100 mg/l) for enumerating the introduced PSI (Strep+) and PS2 (Strep+) and poured on nutrient agar medium to evaluate the population of fluorescent pseudomonads and aerobic bacteria. After 24 h of incubation at 28±1°C, CFUs were counted.

Results and Discussion

Ten strains of fluorescent pseudomonads were isolated from groundnut and sunflower. All the isolates were Gram-negative and produced transparent smooth-margined, small colonies with diffusible greenish yellow pigments, which fluoresce under UV light on succinate agar medium and King's B medium. These fluorescent pseudomonads were screened for siderophore production and only PS1 (G) and PS2 (G) produced large orange halos around their colonies. The presence of siderophore in fluorescent *Pseudomonas* has been reported by several authors [2, 4]. The same strains, PS1 (G) and PS2 (G), were also better producers of HCN (Table 1).

Hydrocyanic acid production by fluorescent *Pseudomonas* isolated form potato and wheat rhizosphere was reported by Bakker and Schippers [2], and Haas *et al.* [14] analyzed the HCN production by strains of *P. fluorescens* that helped in suppression of *Thielaviopsis basicola* causing black root rot of tobacco. The production of IAA by some strains (Table 1) corroborates the earlier findings wherein the production of IAA and some other auxins has been reported, by the cultures of *P. cepacia* and *P. fluorescens* isolated from maize, bean, and tobacco rhizospheres [14]. Different *Pseudomonas* strains caused variable phosphate solubilization, PS1 (G) and PS2 (G) being the best (Table 1). Kloepper *et al.* [18] reported solubilization of minerals such as phosphorus as one of the most readily available for plant growth. The same strains PS1 (G) and PS2 (G) showed *in vitro* antagonism against *M. phaseolina* (Table 1). The maximum inhibition was caused by PS2 (G) (74%) as compared with that of PS1 (G) (71%) after 5 days (Table 2). As a result, these two strains were selected for further studies. Suppression of *Pythium*, *Rhizoctonia*, *Erwinia*, *Sclerotinia*, and *Fusarium* by pseudomonads of fluorescent group has been in practice for several years [8]. The mechanism of growth promotion and disease reduction is generally believed to be due to antagonistic interaction by siderophore-mediated competition, which results in the

Table 2. Antagonistic effects of PS1 and PS2 against *M. phaseolina* on TSM plates at 28±1°C.

Incubation (h)	Growth in control (mm)	Growth in interaction with PS1 (mm)	[a]Colony growth inhibition (%)	Growth in interaction with PS2 (mm)	[a]Colony growth inhibition (%)
48	25.4	15.7	38.1	14.9	41.3
72	42.6	17.7	58.4	16.4	61.5
96	69.7	23.9	65.7	20.6	70.4
120	85.3	24.6	71.1	22.1	74.0

[a]Colony growth inhibition (%)=C–T/C×100, where C=colony growth of *M. phaseolina* in control and T=colony growth of *M. phaseolina* in interaction.

exclusion of fungal pathogens in the rhizosphere by reduction in the availability of iron for spore germination and hyphal growth [21, 27].

Fluorescent pseudomonads have been shown to produce a wide variety of secondary metabolites such as fluorescent pigment, siderophore, antibiotics, enzymes, phytohormones associated with microbial antagonism, reducing phytopathogenic fungi, and deleterious rhizobacteria, with significant increase in plant growth and root development. However, most of these findings are limited to pot experiments [1, 10, 11, 15, 22, 25, 33]. The bacterial strains PS1 and PS2 tested in the present field trials produced a wide range of secondary metabolites including fluorescent pigment, siderophore, HCN, IAA, and solubilized phosphate, and showed potential efficacy not only in antagonizing fungal pathogen but also in inducing plant growth. In field trials, *P. aeruginosa* GRC_1 enhanced the growth of *Brassica compestris* [25] and *Arachis hypogea* [12]. Bacterization with PS1 and PS2 showed early and enhanced seed germination (Table 3), and *Pseudomonas* (PS1and PS2)-coated seeds sown in *M. phaseolina*-infested soil significantly increased seed germination by 8% and 14%, respectively, as compared with control (Table 3).

During the initial stages of growth, both PS1 and PS2 showed similar effects although, the latter strain was relatively better at later stages. Application of fertilizer and bacteria enhanced nodular weight per plant in comparison with bacterial application alone, but no significant increase in vegetative growth parameters was recorded. Maximum number of pods as well as number of seeds per pod was recorded in plants treated with PS2 as compared with PS1 after 105 days of germination (Table 3). Similarly, the use of PS1 and PS2 enhanced grain yield by 65.7% and 77.1%, respectively, whereas in the case of pathogen alone, there was a 57% decrease in yield (Table 4). Such plant growth stimulation by fluorescent pseudomonads has also been reported by a number of workers [3, 35]. The number of plants showing disease symptoms of *M. phaseolina* indicated distinct decrease in the number of diseased plants as a result of seed bacterization, as the application of PS1 and PS2, respectively, resulted in 68.96% and 70.41% decrease in disease symptoms. The statistical analysis showed that disease suppression was significant at 0.01 levels (Table 5).

The population density of normal resident bacteria and antibiotic-resistant marker strains PS1 and PS2 was estimated, in rhizosphere of peanut roots, and a successive increase in population density of PS1 and PS2 and normal resident bacteria was recorded (Table 6). As a result of root colonization, the population of fluorescent *Pseudomonas*

Table 3. Effect of seed bacterization of fluorescent pseudomonads (PS1 and PS2) on seed germination and vegetative growth of groundnut, 105 days after germination.

Treatment	Seed germination (%)	Root length[a] (cm)	Shoot height[a] (cm)	Fresh root weight[a] (g)	Fresh shoot weight[a] (g)	Dry root weight[a] (g)	Dry shoot weight[a] (g)	Nodule weight/plant[a] (g)	Number of pods[a]	Number of seeds/pod[a]
PS1	73.75	29.37**	97.08**	5.62527**	199.7**	1.354**	46.9**	1.619**	27.4**	4.3**
PS1+F	82.5[#]	24.55*	93.58**	4.9818**	190.1[ns]	1.1884*	36*	1.86**	25.4**	4.7**
P+PS1	73.75	28.54**	104**	5.4917**	183[ns]	1.0786[ns]	48.5**	0.978**	19.3[ns]	3.6**
PS2	91.25	30.22**	97.11**	5.3635**	205.5**	1.4008**	48.9**	1.93**	28.5**	4.8**
PS2+F	86.25[#]	28.37**	96.79**	5.0249**	197.3**	1.1933*	47.3**	1.75**	28.1**	4.6**
P+PS2	77.50	28.29**	104.2**	5.6112**	188[ns]	1.1847[ns]	48.5**	1.024**	23.8**	3.9**
Control	65.00	21.85	78.56	3.7675	183.6	0.9878	30.4	0.607	18.1	3.1
Control+F	71.25[#]	27.53**	88.28**	5.2459**	183.9[ns]	1.0829[ns]	40.1**	0.972**	21.6*	3.4[ns]
Pathogen	43.75	10.68[ns]	45[ns]	3.5648[ns]	98.3[ns]	0.6653[ns]	29[ns]	0.17[ns]	5.2[ns]	1.5[ns]

Values are the mean of ten replicates.

[a], Significant at 0.01 level of analysis of variance (ANOVA). **, Significant at 0.01 level of LSD as compared with control; *, Significant at 0.05 level of LSD as compared with control; [ns], Not significant at 0.05 level of LSD as compared with control.

P, plant pathogen *M. phaseolina*, PS1 and PS2, pseudononad strains.

Table 4. Effect of seed bacterization of fluorescent pseudomonads on grain yield of groundnut.

Treatment	Yield/plot (kg)	% Increase over control
PS1	2.9*	65.71
PS1+F	2.8*	60.00
P+PS1	1.95^{ns}	11.42
PS2	3.1**	77.14
PS2+F	2.95*	68.57
P+PS2	2.1^{ns}	20.00
Control	1.75	–
Control+F	2.3^{ns}	31.42
Pathogen	0.75^{ns}	–57.1

Values are the mean of three replicates.

*, Significant at 0.05 level of LSD; **, Significant at 0.01 level of LSD; ^{ns}, Not significant.

P, plant pathogen *M. phaseolina*, PS1 and PS2, pseudononad strains.

Table 5. Effect of seed bacterization on the development of disease symptoms (number of plants showing disease symptoms[a]).

Days	P+PS1	P+PS2	Pathogen
30	7.8** (61.0)	6.6** (67.0)	20.0
60	11.0** (62.83)	10.0** (66.22)	29.6
90	12.0** (68.91)	12.6** (67.36)	38.6
105	15.0** (68.96)	14.3** (70.41)	48.33

[a]The data represent the mean value of three replicates for each.

**Significant at 0.01 level of LSD. Values in parenthesis represent percentage decrease in disease symptoms over *M. phaseolina* alone.

P, plant pathogen *M. phaseolina*, PS1 and PS2, pseudononad strains.

strains on the rhizosphere increased in the first 15 days after sowing but thereafter recorded constant increment up to 60 days. Root colonization with fluorescent *Pseudomonas* was aggressive. The continued presence for 105 days in soil showed that it had reached homeostasis after undergoing exchange with indigenous microflora and was not affected by the active and passive processes restricting the soil community. This indicates that the application of *P. aeruginosa* PS1 and PS2 did not result in any perturbations of the indigenous population of microflora. These findings were similar to root colonization of *P. aeruginosa* GRC1 in Indian mustard (2005) and peanut (2006). The results suggested that PS1 and PS2 were good root colonizers and not affected by *M. phaseolina*. The findings of root colonization by pseudomonad strains also support the biological control and plant growth enhancement abilities. The same view, that different fluorescent pseudomonads have differential ability to colonize a particular root niche, has been supported earlier by several authors and has been reviewed extensively [18, 19, 30, 33].

As a result of their phosphate solubilizing activity and capability to produce IAA and HCN, the fluorescent *Pseudomonas* strains, which are autochthonous in nature, proved better in comparison with allochthonous strains as far as their effect on groundnut is concerned. Further studies on the strains, standard performance, cost, storage, and methods of application of these bioproducts would be worked out for their commercial production.

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Table 6. Root colonization of groundnut by fluorescent pseudomonads (FP) and indigenous aerobic soil bacteria (AB).

FP	Population of bacteria in the rhizosphere (CFU/g root segments)*									
	15 days		30 days		60 days		90 days		105 days	
	FP[a]	AB[b]	FP[a]	AB[b]	FP[a]	AB[b]	FP[a]	AB[b]	FP[a]	AB[b]
PS1	6.6×10^{5}*	7.4×10^{6}*	5.9×10^{5}*	6.4×10^{6}*	8.4×10^{5}*	7.1×10^{6}*	5.8×10^{5}*	6.9×10^{6}*	7.7×10^{5}*	9.1×10^{6}**
P+PS1	5.4×10^{5}*	6.1×10^{6}*	3.4×10^{5}*	7.1×10^{6}*	5.9×10^{5}*	6.1×10^{6}*	4.2×10^{5}*	5.7×10^{6}*	3.4×10^{5}*	7.6×10^{6}*
PS2	8.6×10^{5}*	7.7×10^{6}*	7.5×10^{5}*	7.1×10^{6}*	9.6×10^{5}*	6.7×10^{6}*	6.7×10^{5}*	7.1×10^{6}*	6.9×10^{5}*	7.5×10^{6}*
P+PS2	6.9×10^{5}*	7.4×10^{6}*	4.9×10^{5}*	5.8×10^{6}*	7.1×10^{5}*	9.6×10^{6}**	5.1×10^{5}*	6.7×10^{6}*	4.1×10^{5}*	6.1×10^{6}*

The data represent the mean value of three replicates for each.

[a]Total population of fluorescent pseudomonads (FP).

[b]Total population of indigenous aerobic soil bacteria (AB).

*, Significant at 0.05 level of LSD. **. Significant at 0.01 level of LSD.

P, plant pathogen *M. phaseolina*, PS1 and PS2, pseudononadsstrains.

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