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Plant and Soil **229:** 25–34, 2001.
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Potential for improving pea production by co-inoculation with fluorescent *Pseudomonas* and *Rhizobium*

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Received 6 September 1999. Accepted in revised form 1 September 2000

Key words: antibiosis, disease suppression, inoculation, plant growth promoting rhizobacteria, *Rhizobium leguminosarum* bv. viceae, seed bacterization, siderophores

Abstract

Seed bacterization with five plant growth promoting fluorescent *Pseudomonas* strains isolated from Indian and Swedish soils and three *Rhizobium leguminosarum* biovar viceae strains isolated from Swedish soils were shown to promote plant growth in *Pisum sativum* L. cv. Capella. Co-inoculation of the fluorescent pseudomonads and *Rhizobium* improved plant growth in terms of shoot height, root length and dry weight. Both the fluorescent pseudomonads and *Rhizobium* were shown to exhibit a wide range of antifungal activity against pathogens specific to pea. Seed bacterization with plant growth promoting strains alone and together with a rhizobial isolate, R 361-27 reduced the number of infected peas grown in *Fusarium oxysporum* infested soils. We found that the introduced organisms were able to colonize the roots, which was confirmed using immunofluorescence staining and drug resistant mutant strains. In a synthetic culture medium, all the plant growth promoting fluorescent pseudomonads strains produced siderophores, which shown to express antifungal and antibacterial activity. Our results suggest the potential use of these bacteria to induce plant growth and disease suppression in sustainable agriculture production systems.

Introduction

The extensive use of chemical fertilizers and pesticides in agriculture is currently under debate due to environmental concern and fear for consumers' health. Use of naturally occurring rhizobacteria, which protect and promote plant growth by colonizing and multiplying in the rhizosphere/root cortex, could be an alternative method for plant protection. Many microorganisms, especially fluorescent *Pseudomonas* and bacilli enhance plant growth, yield and/or suppress disease in many crop plants (Dileep Kumar, 1999; Dileep Kumar and Dube, 1992; Duijff et al., 1993; Scher and Baker, 1982; Schippers, 1993; Weller, 1988).

In most of the cases, the plant growth promoting activity of the fluorescent *Pseudomonas* resides in siderophores, low molecular weight compounds (below 1500 daltons) with a high affinity for Fe^{3+} (KFe < 10^{10}) produced by these organisms. In addition, many fluorescent pseudomonads produce a number of antibiotics, which also have a role in plant growth promoting activity (Dowling and O'Gara, 1994; Thomashow and Macrodi, 1997).

At the same time, *Rhizobium* or *Bradyrhizobium* are also widely used in agriculture for crop improvement because of their ability to induce plants to fix atmospheric nitrogen. Logically, potentials for improving plant yields by combining these plant growth promoting organisms by co-inoculation with plant growth promoting rhizobacteria (PGPR) and rhizobia have also been a subject of several investigators (Chanway et al., 1989; Dashti et al., 1997, 1998;

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Grims and Mount, 1984; Li and Alexander, 1988; Parmar and Dadarwal, 1999; Podile, 1995; Polonenko et al., 1987).

Following this context, we therefore investigated the effects of co-inoculation with PGPR and rhizobia in an attempt to improve the plant biomass of peas and to suppress fusarium wilt caused by *Fusarium oxysporum*.

Materials and methods

Soil and plant material

Soil collected from a field near the Uppsala campus of Swedish University of Agricultural Sciences (sandy-loam with a pH of 7.2, total nitrogen 0.02%, organic matter 0.4%, no previous treatment with pesticides) was used for the green house studies. Pea, *Pisum sativum* L. cv. Capella, was used as the test plant in the experiments.

Micro-organisms

Beneficial rhizobacteria
Pseudomonas *strains:* Strain FPO 4 was obtained from Dr C. Dileep, Central Plantation Crops research Institute (ICAR), Kayamgulam, India. Strains RBT 13 and RRLJ 134 were taken from the culture collection of Dr Dileep Kumar from the Soil Microbiology Division, Regional Research Laboratory (CSIR), Jorhat, India, while strains S 97 and S 510 were obtained from Dr Anna Mårtensson, Department of Soil Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden (originally isolated by Dr S. Alstrom, Department of Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden). All the strains were identified as fluorescent pseudomonads and showed plant growth promoting activity and/or disease suppression in earlier studies with various crop plants.

Rhizobial strains: Strains R 304, R 313 and R 361-27 were used for the study. All strains were earlier identified as *Rhizobium leguminosarum* biovar viceae and were taken from the culture collection at the Department of Soil Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Fungal pathogens

Aphanomyces euteiches, *Phoma medicaginis* var. *pinodella* (obtained from Plant Pathology Division of Swedish University of Agricultural Sciences, Uppsala), *Fusarium oxysporum* (isolated from wilted *Pisum sativum* L. cv. Capella) and *Rhizopus oryzae*, and *Pythium* sp. (obtained from Prof. Winkelmann, Microbiology and Biotechnology, University of Tübingen, Germany), were used in this study.

In vitro *antibiosis against fungal pathogens of pea*

In vitro antagonism of the bacterial isolates against *Aphanomyces euteiches*, *Fusarium oxysporum* and *Phoma medicaginis* var. *pinodella* was investigated using potato dextrose agar (PDA), King's medium B (KB) and Yeast-mannitol agar (YMA) (Li and Alexander, 1988), as described by Dileep Kumar and Dube (1992). For this, a loopful of the test rhizobacterial suspension was streaked along one side of Petri plates containing one of the media mentioned above. Mycelial discs of the test fungi (6 mm discs), cut from actively growing culture, were placed at the opposite side to the bacterial streak and the zone of inhibition was noted after 7 days of growth at 28±2 °C.

To verify whether the inhibition was due to the presence of siderophores, the above test was repeated using *Phoma medicaginis* var. *pinodella* on KB and YMA media amended with 50 μm of $FeCl_3$ per lit, recording growth inhibition as above.

Seed bacterization

Seed bacterization was carried out by a method of Dileep Kumar and Dube (1992) with some modifications as follows. Pea seeds (*Pisum sativum* L. cv. Capella) were surface sterilized by placing seeds in a 2.5% sodium hypochlorite for 5 min followed by rinsing in 1:29 mixture of hydrogen peroxide: distilled water for 30 min and dried under a sterile condition. *Pseudomonas* and rhizobial strains grown in KB and YMA medium, respectively, were harvested with a sterile glass rod and suspended in 20 ml of 1% sterile carboxymethylcellulose (CMC) suspension. Five grams of surface sterilized seeds were steeped in this bacterial suspension for 1 h and dried overnight (at 20 °C) in sterile Petri dishes. The treated seeds were examined for colony forming units (CFU) on KB and YMA and were adjusted to give 1.2×10^7 CFU/seed for individual treatments and $1.0 \times$

10^4CFU/seed for each organism in combination (co-inoculation). Seeds treated with only 1% CMC served as the control. The treated and control seeds were sown into plastic pots (five seeds per pots, six pots per treatment) containing, respectively, sterile (autoclaved two times at $1 \cdot 10^5$Pa for 2 h and 120°C at an interval of 24 h and left at room temperature for 3 weeks prior to use) and non-sterile soil and grown under greenhouse conditions with a day time temperature of 18°C and a night time temperature of 15°C. Natural light was supplemented by Silvania incandescent and cool-white lamps, 400 W, 400–700 nm, with a 16–18 light dark cycle at 22–18°C and 83% relative humidity. The growth in terms of shoot height, root length and dry weight were noted after 28 days of growth. The percentage of germination was noted after 7 days and nodulation was noted after 28 days of growth.

Root colonization

Preparation

Root colonization by R 361-27 and S 510 were studied through immunofluroscence techniques according to Van Vuurde and Van der Wolf (1995). For this, bacterized pea seeds, prepared as before, were raised under greenhouse conditions as above in sterilized plant compartments consisting of glass tubes (30 mm in diameter, 170 mm length), containing 20 g of oven baked clay balls (diameter 3 mm, Leca^{TM}, Svensk Leca, AB Uppsala Cementgjuteri, Hällbygatan 27, SE-752 28 Uppsala, Sweden) together with 14 ml 0.02 m sodium phosphate buffer, pH 7.0, for 12 days. The first lateral root was then used for immunofluorescence observations by carefully removing adhering soil particles. The second and third roots were used for colonization studies. The selected root pieces, choosen for immunofluorescence studies, were 'finger printed' by placing them on KB and YMA medium (with 1% agar and pour plated as a very thin layer) followed by incubation at 22°C for 2 days. The agar bacterial growth medium was then dried at 50°C with a hot air drier. The dried medium was then cut into small pieces which were, respectively, transferred into a single well of a 24 wells, polystyrene, flat-bottom microplate.

Immunofluorescence colony staining

The antisera used in the following were prepared as described by Somasegaran and Hoben (1994). IgG was purified and conjugated with fluorescein isothiocynate (FITC) as described by Van Vuurde and Van der Wolf (1995) and stored frozen (−20°C) prior to use. Two ml of FITC conjugated antiserum were then added to the root segments placed in the microplate wells (1:100, 1:200 and 1:800 dilution of the antisera were used). The plates were incubated overnight at 22°C. Nonbound conjugate were then removed by washing with phosphate bufffered saline (0.85% NaCl, 0.05 m sodium phosphate buffer pH 7.2). The agar plates were then examined under a microscope for the presence of the introduced bacteria shown as fluorescent colonies.

For FPO4, RBT 13 and RRLJ 134, drug resistant mutant strains (resistant to $100 \text{ mg} \cdot \text{l}^{-1}$ of streptomycin sulphate) were developed as previously reported (Dileep Kumar and Dube, 1992) and used to monitor and confirm the root colonization from the second and third roots. For this, the roots raised from the bacterized plants were cut into 1 cm long pieces and 1 g (wet weight) of these pooled root pieces was added to 5 ml of sterile distilled water and shaken for 3–4 min to wash rhizoplane bacteria into the water. Serial dilutions of the bacterial suspensions were made and pour plated on KB medium containing $100 \text{ mg} \cdot \text{l}^{-1}$ of streptomycin sulphate and nutrient agar for introduced and total aerobic bacteria counts, respectively. The number of CFU/g root (from average of three readings) was estimated after 48 h of growth.

Disease suppression studies

Seed bacterization (as described above) were carried out with FPO4, RBT13, RRLJ 134, S 97, S 510, R 361-27 and combinations (co-inoculation) of the first five organisms with R 361-27 were examined for their ability to suppress wilt disease caused by *Fusarium oxysporum* on peas grown under greenhouse conditions as above. For this, bacterized seeds were sown into pots infested with fungal conidia (mixed with 25 ml of fungal conidial suspension containing $1.0 \cdot 10^7 \text{conidia} \cdot \text{ml}^{-1}$) and raised in greenhouse for 28 days. At harvest, the number of plants showing disease symptoms such as wilting of plants, drooping of leaves and root discoloration was determined. Plants raised from non bacterized seeds grown in soil without fungal conidia were used as the control and disease severity was recorded with the non bacterized seeds into soil infested with fungi. Again, 30 plants were used.

Extraction and purification of siderophores

For extraction and purification of siderophores, the method of Bezbaruah et al. (1996) was used. Bacterial inoculum (0.5 ml) obtained from an 18-h-old

culture of all fluorescent pseudomonads were introduced into succinate medium (100 ml in 250 ml flasks) and incubated for 48 h in a rotary shaker at 120 rpm. The grown medium from all the cultures were pooled after 48 h and 200 mg · l^{-1} $FeCl_3$ was added. After stirring for 20 min, the suspension were centrifuged at 12 000 rpm for 20 min. The supernatant was then passed through a 1.5 cm wide and 20 cm long amberlite XAD-2 (ICN) column. The adsorbed column was washed three times with distilled water and then eluted with methanol. The eluted methanolic fraction was concentrated and then passed twice through a cm-Sephadex C-25 (Pharmacia) column (diameter 1.5 cm, length 20 cm) to enhance the purity and eluted with methanol. This methanolic elute was dried and used for further studies.

In vitro *antibiosis of the extracted siderophore against fungal pathogens*

The extracted crude siderophores from four strains (FPO4, RRLJ 134, S 97 and S 510) were tested against the following fungal pathogens to observe their antifungal nature: *Aphanomyces euteiches*, *Fusarium oxysporum*, *Phoma medicaginis* var. *pinodella*, *Pythium* sp., *Rhizopus oryzae*.

For the bioassay, 200 μl (per litre medium) of the spore/conidial suspension (1.0×10^7 ml^{-1}) from different fungi were incorporated to cooled autoclaved PDA medium and pour plated. Filter discs impregnated with 20 μl of siderophores from various PGPR organisms were placed over the medium and the zone of inhibition was noted after 2 days for *Rhizopus oryzae*, while the same was recorded after 7 days for the other test fungi. Three replicates were used in the experiment.

Different concentrations of the extracted crude siderophores from three organisms (RRLJ 134, S 97 and S 510) were also tested for their effect on growth of *Phoma medicaginis* var. *pinodella*. Four concentrations (25, 50, 75 and 100 mg · l^{-1}) of the crude siderophore in ferric and deferric form were amended to potato dextrose broth (PDB) inoculated with 20 μl of spore (approximately 1.0×10^7 ml^{-1}) suspension of the fungus and allowed grow and dry mycelial weight were recorded after 7 days of growth. PDB without siderophore served as the control. Three replicates were used for the experiment.

Table 1. In vitro antibiosis of plant growth promoting fluorescent pseudomonads against pathogenic fungi to pea, *n*=3

Pathogenic fungi	Media	Zone of inhibition by the respective isolate (in cm)					
		Control	FPO4	RBT 13	RRLJ134	S 97	S 510
Aphanomyces euteiches	KB[a]	0.0	3.0[b]	2.0[b]	3.0[b]	NI[a,b]	2.0[b]
	PDA[a]	0.0	2.0[b]	2.0[b]	2.0[b]	NI	1.5[b]
Phoma medicaginis var. *pinodella*	KB	0.0	1.5[b]	1.0[b]	2.0[b]	0.5[b]	1.0[b]
	PDA	0.0	1.5[b]	1.0[b]	1.5[b]	0.2	1.0[b]
Fusarium oxysporum	KB	0.0	1.5[b]	1.5[b]	1.0[b]	0.1	0.5[b]
	PDA	0.0	1.5[b]	1.5[b]	1.0[b]	0.2	0.5[b]

[a] KB = King's medium B, PDA = Potato dextrose agar, NI = no inhibition.
[b] = Values are statistically significant over the control ($P<0.05$).

Statistical analyses

The statistical analyses of the germination, shoot height, root length, fresh and dry weights were done applying SAS (SAS Institute Inc. USA). Disease suppression and *in vitro* antibiosis data were analyzed with Sigma Plot (Jandel Corporation, USA) at $P<0.05$ level. All experiments were conducted twice.

Results

In vitro *antibiosis of the organisms against fungal pathogens*

All the fluorescent *Pseudomonas* strains studied, except S 97 against *Aphanomyces euteiches,* were shown to be antagonistic against all test fungal pathogens (Table 1). However, no single organism was dominant against all the pathogenic fungi. FPO4 showed the maximum inhibition in most cases, followed by RRLJ 134. Statistical analysis of the zones of inhibition produced showed them to be statistically significant ($P<0.05$) in all cases except S 97. S 97 had the least effect and only induced a growth suppression against *Phoma medicaginis* var. *pinodella* on KB media. Amongst the rhizobial strains tested, R 361-27 shown a clear antibiosis against all the tested fungi (Table 2). This was followed by R 304 and R 313. R 313 had no effect on the growth of *Aphanomyces euteiches*. The inhibition zones noted were significant for all the organisms ($P<0.05$).

Addition of iron into the medium generally reduced *in vitro* antibiosis (from 0 to 100%) of *Pseudomonas* strains against *Phoma medicaginis* var.

Table 2. *In vitro* antibiosis of *Rhizobium leguminosarum* bv. viceae against pathogenic fungi to pea, n=3

Pathogenic fungi	Media	Zone of inhibition by the respective bacterial isolate (in cm)			
		Control	R 304	R 313	R 361-27
Aphanomyces	YMA[a]	0.0	NI[a]	NI	0.5[b]
euteiches	PDA[a]	0.0	0.5[b]	NI	0.6[b]
Phoma	YMA	0.0	0.2	0.3[b]	0.3[b]
medicaginis var. *pinodella*	PDA	0.0	0.5[b]	0.3[b]	0.5[b]
Fusarium	YMA	0.0	0.5[b]	0.3[b]	0.8[b]
oxysporum	PDA	0.0	1.0[b]	1.5[b]	1.0[b]

[a]PDA = Potato dextrose agar, YMA = Yeast-mannitol agar, NI = no inhibition.
[b]Values are statistically significant over the control (P<0.05).

Table 3. Effect of $FeCl_3$ (50 $\mu g \cdot l^{-1}$) on antibiosis of *Phoma medicaginis* var. *pinodella* with plant growth promoting *Pseudomonas* strains on King's media B, n=3

Treatments	Zone of inhibition by the respective bacterial isolate (in cm)				
	FPO4	RBT 13	RRLJ 134	S 97	S 510
Control (without iron)	1.5	1.0	2.0	0.5	1.0
With iron	0.9[a]	1.0	1.5	0.0[a]	0.0[a]

[a]Values are statistically significant over the control (P<0.05).

pinodella (Table 3). The amendment had no effect on RBT 13 antagonism, while a complete elimination of the inhibition was noted for S 97 and S 510 and a partial decrease of inhibition occurred for FPO4. However, statistically the data was found significant only for FPO4, S 97 and S 510.

Iron amendment into YMA media resulted in a total elimination (100%) of the inhibition against three test fungal pathogen by all the rhizobial strains (P<0.05).

Seed bacterization studies

Shoot growth

Seed bacterization improved shoot growth (Table 4). Inoculation with FPO4 and RBT 13 reduced shoot growth in both sterile and non-sterile soil. S 510 + R 361-27 showed the best enhancement in both sterile and non-sterile soil. Co-inoculation with S 510 + R 361-27 improved the growth under both experimental conditions. The statistical analysis revealed that the data were significant (P<0.05) for 20 and 18 treat-

Table 4. Effect of seed bacterization on shoot height and root length (in cm) of peas after 28 days of growth, n=30

Treatments	Shoot height		Root length	
	Sterile	Non sterile	Sterile	Non sterile
Control	18.1	18.2	21.3	20.2
FPO4	16.7	17.9	23.2	19.8
RBT 13	17.5	18.1	21.5	21.8
RRLJ 134	21.2[a]	20.5[a]	25.0[a]	19.8
S 97	21.7[a]	20.9[a]	24.3[a]	21.5
S 510	21.5[a]	21.2[a]	25.3[a]	20.5
R 304	20.8[a]	20.6[a]	24.0[a]	22.0
R 313	20.8[a]	19.5[a]	23.8[a]	22.5[a]
R 361-27	18.5	19.2	25.0[a]	23.2[a]
FPO4 + R 304	21.4[ab]	21.2[ab]	24.3[a]	25.0[abc]
FPO4 + R 313	20.8[ab]	20.9[abc]	27.2[abc]	23.7[ab]
FPO4 + R 361-27	21.1[abc]	22.0[abc]	26.0[ab]	23.7[ab]
RBT 13 + R 304	20.7[ab]	20.3[ab]	25.7[ab]	23.7[a]
RBT 13 + R 313	20.4[ab]	19.3[b]	25.7[ab]	25.7[abc]
RBT 13 + R 361-27	19.9[abc]	19.2[b]	25.7[ab]	24.3[b]
RRLJ 134 + R 304	22.0[ac]	22.5[abc]	26.0[a]	23.3[ab]
RRLJ 134 + R 313	21.2[a]	21.8[abc]	23.7[a]	23.8[ab]
RRLJ 134 + R 361-27	21.9[ac]	21.6[abc]	24.2[a]	24.3[ab]
S 97 + R 304	21.4[a]	20.9[a]	26.0[a]	23.3[a]
S 97 + R 313	21.3[a]	21.6[ac]	26.0[a]	24.0[a]
S 97 + R 361-27	22.1[ac]	21.6[ac]	25.7[a]	23.5[a]
S 510 + R 304	22.2[ac]	21.2[a]	24.8[a]	24.3[ab]
S 510 + R 313	21.3[a]	21.1[ac]	25.2[a]	24.0[ab]
S 510 + R 361-27	22.4[ac]	22.6[abc]	26.8[ab]	24.3[ab]

[a]Values are statistically significant over the control (P<0.05).
[b]Values are statistically significant over the respective PGPR strain alone (P<0.05).
[c]Values are statistically significant over the respective *Rhizobium* strain alone (P<0.05).

Table 5. Effect of seed bacterization on dry weights (g) of peas after 28 days of growth, *n*=30

Treatments	Dry weight	
	Sterile	Non sterile
Control	0.40	0.50
FPO4	0.34	0.48
RBT 13	0.35	0.51
RRLJ 134	0.46	0.54
S 97	0.49	0.54
S 510	0.56	0.60[a]
R 304	0.53	0.55
R 313	0.53	0.55
R 361-27	0.47	0.55
FPO4 + R 304	0.56	0.64[ab]
FPO4 + R 313	0.64	0.60[ab]
FPO4 + R 361-27	0.65	0.72[abc]
RBT 13 + R 304	0.51	0.64[abc]
RBT 13 + R 313	0.48	0.57
RBT 13 + R 361-27	0.38	0.52
RRLJ 134 + R 304	0.55	0.61[abc]
RRLJ 134 + R 313	0.54	0.58[abc]
RRLJ 134 + R 361-27	0.57	0.57[abc]
S 97 + R 304	0.56	0.53[b]
S 97 + R 313	0.54	0.58[a]
S 97 + R 361-27	0.57	0.50[b]
S 510 + R 304	0.56	0.53[bc]
S 510 + R 313	0.58	0.55
S 510 + R 361-27	1.76[abc]	0.61[abc]

[a] Values are statistically significant over the control ($P<0.05$).
[b] Values are statistically significant over the respective PGPR strain alone ($P<0.05$).
[c] Values are statistically significant over the respective *Rhizobium* strain alone ($P<0.05$).

ments in sterile and non-sterile soil, respectively, over their controls.

Root emergence

The plantlets raised from bacterized seeds in sterile soil had longer roots than those did in non-sterile roots (Table 4). Co-inoculation with FPO4 + R 313 and RBT 13 + R 313 improved root lengths best under both sterile and non-sterile conditions. In sterile soil, the data were statistically significant ($P<0.05$) for all treatments except for FPO4 and RBT 13 while they were not significant in six treatments of non-sterile soil over their respective controls (Table 4).

Plant production

An enhanced dry weight was noted for most of the inoculation treatments (Table 5). However, statistically the data was found significant only for co-inoculation with S 510 + R 361-27 in sterile soil. The enhancement in dry weight was more pronounced in non-sterile soil where nine treatments were found significant at the 0.05 level over the control. Only one combined treatment was found statistically significant in sterile soil over their respective individual treatments, whereas seven of the combination treatments had significant enhancement ($P<0.05$) in sterile soils.

Germination

The percentage of germination varied between 50 and 90% in different treatments (data not shown). However, in most of the cases, see below for what was noted for the control (70 and 80%, respectively, in sterile and non-sterile soil). The data were not statistically significant in all the cases.

Root colonization and nodulation

The positive nodulation recorded for the *Rhizobium* strains and their combinations (co-inoculation) clearly indicates that the rhizobia were able to colonize and induce nodulation of the roots (data not shown). Later, the immunofluorescence study with S 510, R 361-27 and their combination (i.e. S510 + R 361-27) confirmed the root colonization of both strains on plants (data not shown).

Figure 1 indicates that the introduced drug mutant strains of FPO4, RBT 13 and RRLJ 134 were able to colonize and multiply along the roots of pea plants independently whether rhizobia was present or not.

Disease suppression

Bacterization reduced the number of plants with wilt symptoms (Table 6). FPO4 showed the best result ($P<0.05$) and was able to protect 60% of the plants from disease. Co-inoculation with FPO4+R 361-27, S 510, S 510+361-27, RRLJ 134, RRLJ 134+R 361-27 also showed significant reduction. Co-inoculation with RBT13+R 361-27 was not different from the wilt causing fungus alone.

Extraction and purification of siderophores

We were able to isolate pyoverdine type siderophores from all the PGPR strains tested, which had a characteristic peak in or around 405 nm. The yield was around 250 – 300 mg $\cdot$ l^{-1} (crude weight) in all the test strains.

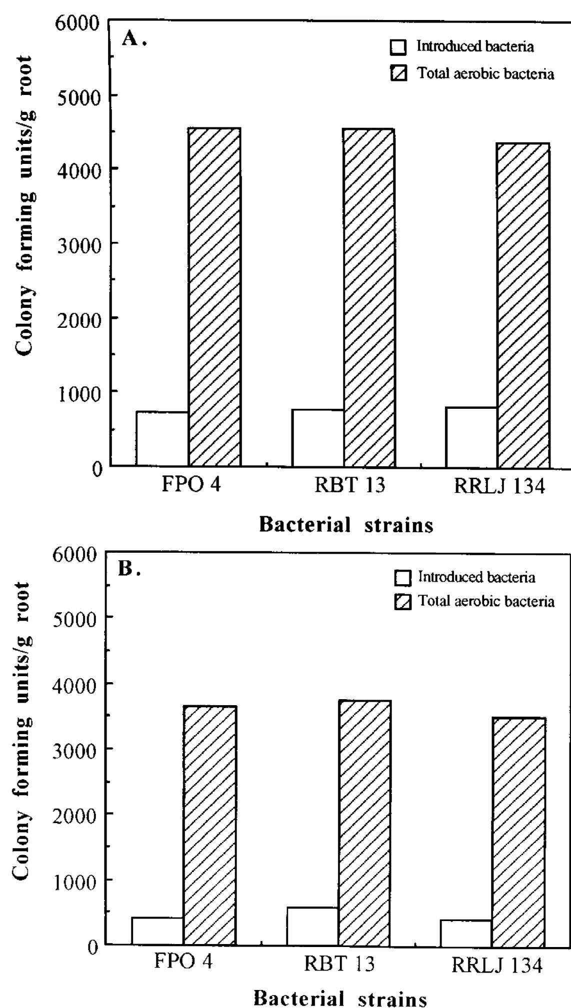

Figure 1. Colonization of plant growth promoting rhizobacterial strains FPO 4, RBT 13 and RRLJ 134 on pea root rhizoplane in relation to total aerobic rhizoplane colonizing bacteria and (A) in presence of *Rhizobium leguminosarum* bv. viceae 361-27 and (B) in absence of 361-27. Data from 30 pooled individual plants.

Table 6. Effect of seed bacterization on development of disease syndrome of *Fusarium oxysporum* on pea plants, n = 30. Values given within the parentheses show the percentage decrease over the fungus alone

Treatments	Number of plants showing symptoms
Control	0.0[a] (100)
FPO 4	12.0[a] (53.0)
RBT 13	25.0 (3.9)
RRLJ 134	20.0[a] (23.1)
S 97	22.0 (15.4)
S 510	19.0[a] (26.9)
R 361-27	26.0 (0.0)
FPO 4 + R 361-27	13.0[a] (30.8)
RBT 13 + R 361-27	26.0 (0.0)
RRLJ 134 + R 361-27	18.0[a] (30.8)
S 97 + R 361-27	25.0 (3.9)
S 510 + R 361-27	19.0[a] (26.9)
Fungus alone	26.0

[a] Values are statistically significant from the fungus alone (P <0.05).

Antifungal properties of the extracted siderophore

Siderophores extracted from FPO4, RRLJ 134, S 97 and S 510 were tested for their antifungal activity against *Aphanomyces euteiches*, *Fusarium oxysporum*, *Phoma medicaginis* var. *pinodella*, *Pythium* sp. and *Rhizopus oryzae* on PDA (Table 7). All the PGPR strains except S 97 against *P. medicaginis* var. *pinodella* produced statistically significant (P <0.05) inhibition zones.

Effect of iron on antifungal activity

Incorporation of different concentrations of siderophore extract from RRLJ 134, S-97 and S 510 in most cases statistically decreased dry weights of the *Phoma medicaginis* mycelium (Table 8). The decrease of mycelial weight was proportional to the increase in

Table 7. Antifungal activity of the extracted crude siderophore of four plant growths promoting pseudomonads against fungal pathogens on potato dextrose agar medium, $n = 3$

Organisms	Zone of inhibition by the siderophore (in cm)				
	Aphanomyces euteiches	*Fusarium* sp.	*Phoma medicaginis* var. *pinodella*	*Pythium* sp.	*Rhizopus oryzae*
Control	0.0	0.0	0.0	0.0	0.0
FPO 4	1.0^a	4.0^a	4.0^a	1.2^a	2.0^a
RRLJ 134	1.0^a	4.0^a	4.0^a	1.4^a	0.8^a
S 97	0.4^a	3.0^a	0.2	0.4^a	2.0^a
S 510	1.0^a	4.0^a	4.0^a	1.2^a	0.8^a

[a] Values are statistically significant from the control ($P<0.05$).

Table 8. Effect of extracted siderophore (ferric and deferric form) from three plant growths promoting pseudomonads on the growth of *Phoma medicaginis* var. *pinodella* after 7 days of growth, (dry weight of the mycelium in mg)

Bacterial strain	Form of siderophore	Concentration of the crude siderophore				
		0 (control)	25	50	75	100
RRLJ 134	Deferric	17.67	10.67^a	8.67^a	7.00^{ab}	7.00^{ab}
	Ferric	17.67	9.67^a	9.00^a	9.33^a	9.00^a
S 97	Deferric	17.67	16.33	13.67^{ab}	14.00^a	12.67^{ab}
	Ferric	17.67	17.33	16.00^a	16.00^a	17.67
S 510	Deferric	17.67	16.00^{ab}	15.33^a	12.33^{ab}	6.33^{ab}
	Ferric	17.67	16.67	16.33	15.67	17.00

[a] Values are statistically significant over the control ($P<0.05$).
[b] Values are statistically significant compared to their respective ferric forms ($P<0.05$).

concentration of the siderophore extract. The increase in mycelial weight in the respective ferric form confirmed that the siderophore had a role in fungal pathogen inhibition. However, the reduction was very similar for both ferric and deferric forms of RRLJ 134.

Discussion

All the fluorescent pesudomonads and rhizobial strains were highly inhibitory to the investigated plant pathogens. The reduction of inhibition zones in the presence of iron implicates that siderophores may be involved in the mechanism of antagonism. The strain RBT 13 had no change in the inhibition zones in the presence of iron, while S 97 and S 510 completely lost their inhibition capacity. This indicates that something other than siderophores may be involved in the mechanism of antagonism of RBT 13, whereas the inhibition of S 97 and S 510 appeared to be siderophore mediated. A later study by us confirmed that RBT 13 was able to produce a phenazine type of antibiotic in KB medium (data not shown). Fungal pathogen control through siderophore and antibiotic producing *Pseudomonas* has been reported by many workers (Dowling and O'Gara, 1994; Weller, 1988).

Seed bacterization with both fluorescent *Pseudomonas* strains and *Rhizobium* and their combinations (co-inoculations) brought distinct crop enhancement in most cases. These were positively correlated with each other with respect to shoot height, root length and dry weights, but were negatively correlated with the germination. Co-inoculation resulted in the best plant development, indicating that these types of combinations can be considered suitable for further testing, in for example, national field experiment programmes. Successful establishments of these type of combinations have also been reported by others workers (Chanway et al., 1989; Dashti et al., 1997, 1998; Grims and Mount, 1984; Li and Alexander, 1998; Parmar and Dadarwal, 1999; Polonenko et al., 1987).

Dashti et al. (1997) reported that the application of PGPR (*Serratia liquefaciens* 2-68 or *Serratia proteamaclans* 1-102) along with *Bradyrhizobium japonicum* increased the protein and dry matter yield in soybean. Another study by Dashti et al. (1998) on soybean also confirmed an accelerated nodulation and increased nitrogen fixation by nitrogen fixing bacteria in the presence of plant growth promoting rhizobacteria. Chanway et al. (1989) also reported that plant growth promoting *Pseudomonas* strains in combination with rhizobial strains enhanced the growth and nitrogen fixation of western Canadian lentils (*Lens esculenta* Moench) and pea (*Pisum sativum* L) cultivars in field and laboratory conditions. Parmar and Dadarwal (1999) also observed that co-inoculation of *Pseudomonas* and *Bacillus* sp. with *Rhizobium* strains enhanced the nodule weight, root length, shoot biomass and total plant nitrogen in chickpea, when grown in sterilized jars or under pot culture conditions. They noted an enhanced level of flavonoids like compounds by these nodule enhancing rhizobacteria, which may trigger the plant to be more susceptible towards rhizobial infections and thus improving plant productivity.

Our observed reduction of the number of plants becoming infected with *Fusarium oxysporum* indicates that the seed bacterization brought a distinct decrease in the severity of the disease. Thus, our observations indicate that the exploitation of these types of bacterial combinations may be tried in other vascular wilt diseases as these are systemic and very difficult to

control through chemicals.*P. fluorescens* and *P. putida* have been reported as successful biocontrol agents of *Fusarium* spp. (Dileep Kumar, 1999; Duijff et al., 1993; Elad and Baker, 1985a, b; Lemenceau et al., 1992; Scher and Baker, 1980, 1982; Sneh et al., 1984; Van Peer et al., 1990).

Root colonization studies confirmed the colonization of introduced organisms along the roots of the plants. Recently, Betelho et al. (1998) reported that a rifampicin resistant mutant strain derived from a broad spectrum antifungal, plant growth promoting strain of fluorescent *Pseudomonas* had colonized the endorhizosphere of maize.

Extracted crude siderophores had a strong antifungal and anti bacterial activity. This indicates that the growth promoting activity and disease suppression ability of these organisms may reside in their siderophores as reported in some other cases. However, an increase in the concentration of the deferric form of the siderophore of RRLJ 134 did not bring about a drastic enhancement of the mycelial weight of test fungi indicating that the crude siderophore extract may have a lesser role in the inhibition of this test fungus.

In conclusion, our results show a potential use of combinations of PGPR and rhizobia in improving plant growth and/or suppressing diseases in pea plants.

Acknowledgements

Financial support from the Department of Biotechnology, Government of India, New Delhi, India for awarding one of the authors (DK) a DBT Overseas Associateship (long-term) and Swedish Council for Agriculture and Forestry Research is gratefully acknowledged.

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