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Effect of *Pseudomonas fluorescens* on Barley Root Rots

A. Foroutan
Agricultural and Natural Resources Research Center of Mazandaran, Sary, Iran

Abstract: Twelve *Psuedomonas fluorescens* strains were selected for disease control in greenhouse and field trial. They were screened on the basis of their *in vitro* antagonistic activity towards *Cochliobolus sativus* (*Cs* D-18) and *Fusarium graminearum* (*Fg* B-4) the inducers of barley root rots. The selected strains were identified as Biovar1 *Psuedomonas fluorescens*. *P. fluorescens* D-3, D-5 and D-8, were the most effective for disease controlling under field conditions, in which significantly ($p \leq 0.05$) reduce disease severity and increase yield.

Key words: Barley, bio control, *Cochliobolus*, *Fusarium*, fungicide, root rot

INTRODUCTION

Barley root rots caused by several species of fungi such as *Gaeumannomyces graminis* var. *tritici, Cochliobolus sativus, Fusarium graminearum, F. culmorum, Rhizoctonia cerealis, Pythium* sp. (Paulitz *et al*., 2002; Smiley *et al*., 2005; Wiese, 1987), is an important root rot disease in Mazandaran province Iran.

Among the described causal agents, *C. sativus* and *F. graminearum* are the common pathogen of barley root rot, in which yield losses estimated up to 30% (Foroutan *et al*., 2001).

Recently there have been some reports that, in the areas where barley is the only option for plantation and planted continuously, excessive and repeated use of fungicides is being common. As a result it may spoil the soil, ground water and pollute even the atmosphere. These problems have renewed public interest in exploring alternative or supplementary non-polluting sources of pesticides i.e., the biopesticides.

Since the 1980s rhizobacteria and other microorganisms have been investigated as possible replacements for chemicals used to control a broad range of plant diseases. Isolates from the genus *Pseudomonas* have been tested due to their widespread distribution in soil, ability to colonize the rhizospheres of host plants and ability to produce a range of compounds antagonistic to a number of serious plant pathogens (Anjaiah *et al*., 1998; Maurhofer *et al*., 1991; Rodriguez and Pfender, 1997; Mathivana *et al*., 2004; Ross *et al*., 2000). Weller and Cook (1983) reported that 10-25% of the yield increases by the application of root-colonizing bacteria antagonistic to take-all pathogen as a seed treatment at planting time. Raaijmakers and Weller (2001) could suppressed take-all disease by means of seeds coating with the strains Q 8r1-96, Q2-87 and 1M1-96. They also figured out a relationship between take-all suppression and the rhizosphere population density of DAPG-producing *Pseudomonas* sp.

In Iran, suppression of wheat Take-all by bacterial and fungal isolates was reported by Sedaghatfar *et al*. (2002) and Foroutan *et al*. (2001) in greenhouse studies.

Not much study has been conducted on the use of biological agents in control of barley root rot diseases in Iran. Therefore this was the first study in the area, with the aim of finding the species of *Pseudomonas* existing on barley rhizosphere to replace with chemical control.

MATERIALS AND METHODS

Barley (*Hordeum vulgare*) seeds of Valfajr facultative cultivar, which is the commercial in the province, *Cochliobolus sativus* D-18 and *Fusarium graminearum* B-4 (the most aggressive strains of the pathogens among the other strains), obtained from Agricultural and Natural Resources Research Center of Mazandaran.

For isolation of barley rhizobacteria various samples were collected from different parts of barley fields in Baykola (Neka), Dashtnaz (Sari), Kolet (Neka), Gharakheil (Ghaemshar), Firouzkandeh (Sari), Jouibar, Semeskandeh (Sary) and Zirvan (Behshar) of Mazandaran province in Iran during 2002-2003. The samples were taken from the root zone of one month old barley seedlings. Barley plants were dug out and gently shaken off and then the soil closely adhering the roots were collected. Rhizosphere bacteria were isolated using nutrient agar (NA) and King B Medium (Weller and Cook, 1983). One gram of roots and associated soils was suspended in 5 mL of sterile distilled water and was vigorously shaken for 15-20 min.

Serial dilution was done by transferring 1 mL suspension to 9 mL sterile distilled water. A 0.1 mL

Table 1: Areas and code of bacterial antagonist isolates

Area	Code	Number of isolates
Baykola (Neka)	B	18
Dashtnaz (Sari)	D	18
Kolet (Neka)	K	16
Gharakheil (Ghaemshar)	G	15
Firouzkandeh (Sari)	F	24
Jouibar	J	15
Semeskandeh (Sari)	S	28
Zirvan (Behshar)	Z	16

suspension of 10^7 to 10^9 dilutions were spread on solidified either nutrient agar or King B Media. The plates then were kept for incubation at 25 to 28°C for 24 h. The individual bacteria colonies were picked on NA slants and after incubation at 28°C for 24 h, the slants were kept at 4°C for short period maintenance. All bacterial isolates were purified, coded and preserved. The code were made based on locations in which the samples had been collected such as: B (Baykola), D (Dashtnaz), K (Kolet), G (Gharakheil), F (Firouzkandeh), J (Jouibar), S (Semeskandeh) and Z (Zirvan) as showed in Table 1.

The bacteria were screened on the basis of their *in vitro* antagonistic activity towards *Cs* D-18 and *Fg* F-4 (Weller and Cook, 1983). Twelve superior bacteria were selected to disease control in greenhouse and field conditions.

Identification of antagonist bacteria was done on the bases of the ability of isolates to produce fluorescent by plating bacteria on King's medium B, biochemical and physiological tests such as; Gram, hyper sensitive reaction in tobacco, oxidizes, nitrate reduction and fluorescence production (King *et al*., 1954; Krieg and Holt, 1984; Holt *et al*., 1994; Schaad *et al*., 2001).

Disease control

In greenhouse: Valfajr barley seeds were bacterised with 12 superior bacterial isolates. The seeds were soaked for 4 h in pre-grown selected bacterial isolates on nutrient broth yeast extract agar (NBYA) separately, at the rate of 10^9 colony forming unit cfu mL^{-1}, approximately OD 0.2-0.3 at A620 n (Weller, 1988). Nutrient broth yeast extract agar was prepared based on 2, 2, 0.5, 2.5 and 1.5 g of nutrient broth, yeast extract, K_2HPO_4, $K_2H_2PO_4$, glucose and agar respectively per liter distilled water (Baudoin *et al*., 1988).

The seeds also were coated with Rovral-Ts (2 g kg^{-1}) as selected fungicide. This fungicide was screened following *in vitro* inhibition assay, among the other candidate fungicides i.e., propiconazole, Benomyl. Fungicide was used in greenhouse and field evaluation studies, to compare the efficacy of bio-control versus chemical control against the disease.

The coated seeds were air dried for 5 h in a laminar flow cabinet, then were planted on pre-inoculated potted soil with *Cs* D-18 and *Fg* B-4 separately. There were three replication pods for each treatment, based on a randomized completed design (Balasubramaniyan and Palaniappan, 2004), with 29 treatments. The treatments were include: 1-13. Seed coated with antagonists and fungicide and planted in inoculated soil with *Cs* D-18, 14. Control 1 (non treated seeds were planted in inoculated plots with *Cs* D-18), 15-27. Seed coated with antagonists and fungicide and planted in inoculated soil with *Fg* B-4, 28. Control 2 (non treated seeds were planted in inoculated plots with pathogens *Fg* B-4), 29. Control 3 (non treated seeds were planted in non inoculated plots with the pathogens).

Effect of antagonists then was done after disease progress on inoculated control plants with the pathogens (control 1 and 2). Disease rating was done on the bases of 0-4 scale, where, 0 = no disease and 4 = death of plants.

In field: The experiment was carried out at the Dashtnaz Agricultural Research Station of Mazandaran. The site was cultivated with an offset disk plough to 15 cm depth and again to a 10 cm depth on Oct.11, 2004. The soil was sterilized with methyl bromide at the rate of 40 g m^{-2} on Oct.12, 2004 to removal any pre-contamination of the trial soil. Cress plant (*Lepidium sativum*) seeds were grown as bioassay test to insurance the removal of methyl bromide hazard effects on Nov. 11. 2004.

An experimental design was carried out as randomized complete block (Balasubramaniyan and Palaniappan, 2004), with 29 treatments and in 3 replication, the method was the same as described above. Plots size was 0.9 × 6 m. The trial was received 60 kg h^{-1} diammonium phosphates, 50 kg h^{-1} P_2O_5 and 100 kg h^{-1} urea. The plots were pre inoculated either with 2 weeks old colonized oat (*Avena fatua*) seeds with strains *Cochliobolus sativus* D-18, *Fusarium graminearum* B-4 separately, or sterile oat (*Avena fatua*) seeds as control 3.

The trial was sown with bacterised and fungicide coated seeds of Valfajr barley and at the rate of 60 kg ha^{-1} on Nov. 15. 2004. The seeds were sown in four rows (6 m long and 30 cm of row spacing). The trial was kept weeds free by hand weeding in several times as required.

Population dynamic of antagonists: Samples of barley plants were collected after 1 and 2 months of planting dates. Five to six randomly selected plants were dug out

from each replicated at each time. Root samples were prepared for isolation of the bacteria, to determine the population size of antagonists. One gram of roots and associated soils was suspended in 5 mL of sterile distilled water and was vigorously shaken for 15-20 min. Serial dilution and other measurements were done as described above. The numbers of antagonist bacteria colonies were enumerated after 48 h.

Disease evaluation: Disease evaluation, was done after disease progress on inoculated plants with the pathogens (control 1 and 2), on the bases of 0-4 scale, (the same method as described in greenhouse method), in the end of April 2005. Two rows of the each plot were harvested by hand and grain yields were obtained using a small thresher and analyzed on May 24, 2005.

RESULTS

A total of 32 barley rhizobacteria out of 220 isolated from the rhizosphere of different barley fields in Mazandaran province were showed to have inhibition zone toward pathogens on PDA medium. The antagonism of the bacteria was tested following dual culture technique. Twelve out of 32 had maximum inhibition against *Cs* D-18 and *Fg* B-4. The selected strains were identified as Biovar 1 *Psuedomonas fluorescens*. They could significantly ($p \leq 0.05$) affect the mycelial growth of the pathogens.

The most effective bacteria include; *P. fluorescens* B6 *P. fluorescens* B11, *P. fluorescens* D3, *P. fluorescens* D5, *P. fluorescens* D8, *P. fluorescens* F12 *P. fluorescens* F13, *P. fluorescens* J 22, *P. fluorescens* K 4, *P. fluorescens* N 6, *P. fluorescens* S 5 and *P. fluorescens* S 10.

Population dynamic of antagonists and effects of antagonists on infection degree and yield were summarized at Table 2.

Effect of bacterial isolates on disease severity and yield induced by *Cs* and *Fg* in greenhouse and field are showed at Table 3.

Table 2: Population rates of antagonists on barley roots

	Population densities (cfu g^{-1} root) monthly	
Treatments	1	2
P. fluorescens B6	ND*	ND*-1x10^2
P. fluorescens B11	ND*	ND*-1x10^2
P. fluorescens D3	ND*	2x10^5-2x10^6
P. fluorescens D5	ND*	2x10^5-2x10^7
P. fluorescens D8	ND*	2x10^5-2x10^7
P. fluorescens F12	ND*	1x10^3-1x10^4
P. fluorescens F13	ND*	1x10^3-1x10^4
P. fluorescens J 22	ND*	1x10^3-1x10^4
P. fluorescens K 4	ND*	1x10^3-1x10^4
P. fluorescens N 6	ND*	1x10^3-1x10^4
P. fluorescens S 5	ND*	1x10^3-1x10^4
P. fluorescens S 10	ND*	1x10^3-1x10^4
Rovral-Ts	ND*	1x10^2-1x10^3
Control (non-infected)	ND*	1x10^3-1x10^5
Control (infected)	ND*	ND*

*non detected

Table 3: Effect of bacterial isolates on disease severity and yield induced by *Cs* and *Fg* in greenhouse and field

	Disease severity (DS) and yield (Y)							
	Cs				*Fg*			
	Green house		Field		Green house		Field	
Treatments	DS*	Y**	DS***	Y***	DS*	Y**	DS***	Y***
P. fluorescens B6	1.320^b	35^b	2.11^b	2.378^c	1.292^b	35.2^b	2.23^b	2.380^c
B. subtilis B11	1.287^b	35^b	2.16^b	2.334^c	1.291^b	35^b	2.90^b	2.299^c
P. fluorescens D3	1.316^b	35.21^b	2.18^b	2.915^b	1.320^b	36^b	2.22^b	2.900^b
P. fluorescens D5	1.312^b	35.22^b	1.55^c	2.918^b	1.298^b	36.14^b	1.64^c	2.922^b
B. subtilis D8	1.318^b	35.11^b	1.34^c	2.920^b	1.322^b	35^b	1.42^c	2.916^b
P. fluorescens F12	1.312^b	35.4^b	2.13^b	2.352^c	1.322^b	35^b	2.18^b	2.296^c
B. subtilis F13	1.321^b	35.10^b	2.19^b	2.338^c	1.318^b	35.23^b	2.22^b	2.341^c
P. fluorescens J 22	1.316^b	35^b	2.19^b	2.324^c	1.324^b	35.12^b	2.26^b	2.333^c
P. fluorescens K 4	1.317^b	35^b	2.18^b	2.324^c	1.323^b	35.12^b	2.25^b	2.331^c
B. subtilis N 6	1.318^b	35^b	2.13^b	2.232^c	1.327^b	35^b	2.22^b	2.197^c
P. fluorescens S 5	1.322^b	35.11^b	2.13^b	2.371^c	1.299^b	35^b	2.18^b	2.355^c
P. fluorescens S 10	1.319^b	35.14^b	2.11^c	2.344^c	1.311^b	36^b	2.21^c	2.293^c
Rovral-Ts	1.314^b	35.13^b	1.59^b	2.865^b	1.294^b	35^b	1.62^b	2.858^b
Control (noninfected)	0.007^c	37.12^a	0.007^d	3.14^a	0.007^c	37^a	0.007^d	3.08^a
Control (infected)	3.114^a	28.22^c	2.66^a	2.214^d	3.110^a	26^c	2.44^a	2.198^d
CV%	4.44	3.32	16.23	14.18	5.26	9.51	11.36	15.22

*Mean of 5 plants /pod **1000 grain weight ***Mean of 5 plants /plot. Mean followed by different letter differ significantly at $p \leq 0.05$

DISCUSSION

Pseudomonas strains have been considered to have an attribute to biological control of some soil borne diseases (Capper and Campbell, 1986; Duffy and Weller, 1994).

In vitro experiments revealed that 12 out of 32 isolates showed antifungal activity and consistently induced reproducible zone of fungal inhibition on PDA medium. These isolates were selected for the antagonistic against the root rots diseases in greenhouse and in field trials. All 12 isolates could reduce of disease severity and increased the grain yield in greenhouse trials. But only 3 isolates (*P. fluorescens*.D-3, *P. fluorescens* D-5 and *P. fluorescens* D-8) were the most effective in controlling the disease under field conditions, but other selected bacteria did not have this ability. Because all factors in which were stabled in green house, could not be controlled in the field trial. Also population size of these three isolates showed their ability in colonizing of root system (Table 2). The 3 isolates as described above are the native and were obtained from the same field in which the study was done. So these strains could successfully adapt to the rhizosphere in that field as reported by Duffy and Weller (1994), Raaijmaker and Weller (2001) and Weller and Cook (1983). Ross *et al*. (2000) expressed that many biological control agents are found to be active only in certain soil types.

Effects of factors such as soil texture, organic matter, pH, water and oxygen availability and competition for nutrients with indigenous micro flora on dampen the biological activity of introduced inocula, also have been described by Capper and Higgins (1993) and Johnsson *et al*. (1998).

As conclusion 3 isolates (*P. fluorescens*. D-3, *P. fluorescens* D-5 and *P. fluorescens* D-8) in which were the most effective in controlling of two barley root rot diseases under field conditions, can be used in the integration with other disease management systems to afford greater levels of protection and sustain crop yield.

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