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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 3 (3): 368-371, 2000

Biocontrol of Flax Seedling Blight with Mixtures of Pseudomonas Spp.

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Abstract: Antagonism between flax rhizobacteria and *R. solan,* the causal of flax seedling blight, was studied *in vitro. Pseudomonas flurorescens, Pseudomonas cepacia* and *Pseudomonas* sp. were the most effective antagonists. Linear growth of *R. solani* was inversely proportional to the concentration of the culture filtrates of *Pseudomonas* strains. Individual strains as well as their mixtures significantly increased the percentage of surviving seedlings in greenhouse and field trials; however, the mixtures were much more effective than the individual strains. Moreover, the mixtures were more effective in increasing seed yield and straw yield in field trials.

Key words: Biocontrol, flax seedling blight, *Pseudomonas* Spp.

Introduction

Flax is susceptible to infection with several fungal diseases such as seedling blight caused mainly by *R. solani* Kuhn; however, *Pythium* spp. and *Fusarium* spp. may also be involved in the disease although their role is less important (Nyvall, 1981). Some rhizosphere microorganisms can enhance plant growth by inhibiting the pathogenic activities of both major and minor soil-borne pathogens. inhibition mechanisms include production of antibiotics. The production of antibiotics in the rhizosphere has been suggested to be the mechanism for biological control of plant diseases by Pseudomonad (Kapulnik, 1991). Many *Pseudomonas* strains are known to suppress fungal growth *in vitro* by the production of one or more antifungal antibiotics, which have been identified as siderophores (Kloepper *et al*., 1980a, b; Pietr and Kempa 1989; Hebbar *et al*., 1992; Cassinelli *et al*., 1993; Buysens *et al*., 1996), pyrrolnitrin (Dahiya *et al*., 1988; Homma and Suzui, 1989; Kraus and Loper, 1992), pyoluteorin (Kraus and Loper, 1992; Maurhofer *et al*., 1992), other antibiotic metabolites included pyocanin, phenazine carboxamide (Dahiya *et al*., 1988), Pseudan compounds Homma and Suzui, 1989) and 2, 4-diacetylphloroglucinol (Maurhofer *et al*., 1992; Shanahan *et al*., 1992).

The culture filtrates of antagonistic bacteria were shown to inhibit the plant pathogens *in vitro* and *in vivo* (Singh and Deverall, 1984). The inhibitory action of the filtrates was related to the antagonistic capacity of the microorganisms and to the chemical nature of antibiotic substances they produce (Sedra and Maslouhy, 1995). The combinations of antagonistic microorganisms would be more effective in controlling soil borne pathogens than single microorganisms. The rationale is that multiple antagonists would offer more verstality in mechanisms of action against pathogens and also increase the probability of antagonists interacting with pathogens over a large range of microclimates. This should also broaden the spectrum of disease control in the field when two or more diseases are operating in tandem (Fukui *et al*., 1994). In general, certain combinations of strains enhanced yield, whereas other mixtures and strains used individually did not (Pierson and Weller, 1994). Combined treatment with two strains of *P. fluorescens* reduced disease incidence more than single treatment (Yeom and Park, 1995).

In this study, we investigated the possibilities of suppressing the incidence of seedling blight pathogens of flax by bacterization with combination of *Pseudomonas* spp. and their culture filtrates.

Materials and Methods

Bacteria isolation and their antagonistic test: Pseudomonads strains were isolated from rhizosphere of the Egyptian double-purpose flax (*Linum usitatissimum* L.) variety Giza 8. One gram of soil sample was inoculated in 100 ml of the King's Medium B (KMB) and incubated at 20°C for 24 h. Bacterial growth were isolated and purified by streaking plates. These isolates were maintained on KMB slants at 4°C (Seeley, 1989). *In vitro* tests for antagonism of all pure isolates of bacteria toward *Rhizoctonia solani* were made using plate assay. A bacterial culture was streaked over the surface of Potato-Dexterose-Agar (PDA) at the periphery of the plate and mycelial discs (4 mm diameter) of *R. solani* were placed at opposite side of plate periphery. The assay plates were incubated at 28°C and observations were made up to 7 days on the inhibition of mycelial growth. The experiment included the inoculated plates in addition to plates inoculated only with *R. solani* as control. Three replicates from each treatment were used.

The antagonistic effects of the bacterial isolates were determined by observing the free inhibition zone of *R. solani* after 7 days when the surface of the control plate was covered by the mycelium of *R. solani.*

Identification of antagonistic bacteria: The identification of the highly antagonistic isolates toward to *R. solani* were carried out by standard bacteriological tests (Holt *et al.,* 1994).

***In vitro* antibiosis among bacterial strains:** *In vitro* antibiosis was determined for three strains by streaking various combination on KMB and PDA. The first bacterial strain was streaked onto agar media and incubated at 28°C for 48 h; then the cells were scraped off the surface of the media. The remaining cells were killed with chloroform vapor. The second bacterial strain then was inoculated onto culture media by streaking vertically to the streak of the first strain and incubated at 28°C. Growth of the second strain near the streak of the first strain was examined over a 72 h period. The tests were done twice (Fukui *et al*., 1994).

Effect of culture filtrates of antagonistic bacteria on growth of *R. solani*: Cultures filtrates were obtained by growing antagonistic bacteria in Erlenmeyer flask (250 ml), each containing 50 ml of liquid PDA medium for 10 days at 28°C. The cultures were filtered through bacterial filter to separate bacterial growth.

Adequate volumes of filtrates were added to conical flasks containing 47.5, 45 and 37.5 ml warm sterilized PDA medium to produce concentrations of 5, 10 and 25 percent of the medium before solidification. The media were then poured in five petri dishes (replicates) for each treatment.

Fungal isolation and preparation of Irioculurn: *R. solani* Kuhn, isolated from roots of flax seedling blight was used throughout this study. Isolation, purification and identification of this fungus were

carried out at Plant Pathology Lab. Sakha Agric. Res. Station. ARC. The inoculum was prepared by growing *R. solani* in 500 ml bottles containing barley grain medium (100 g of barley grains + 50 ml water), then incubated at 20°C for 20 days. The inoculum was mixed throughout with the soil at a rate of 0.1 g/kg of soil weight.

Preparation of inocula from the antagonistic microorganisms: Inocula of antagonistic strains were obtained by growing organisms in Erlenmeyer conical flasks (250 ml) containing 50 ml of liquid medium for 10 days at 28°C (nutrient broth). The mixed culture of *Pseudomonas* spp. was prepared by adding 1.5 ml cell suspension of each strain.

Bacterial seed treatment: For single-strain inoculation, 1.5 ml of a bacterial suspension or filterate and 15 g of flax seeds were mixed in small plastic bag. For mixture of strains inoculation or their filterate, 1.5 ml of the mixture was applied to 15 g of seeds.

Experimental conditions: Pseudomonads strains suspensions or filtrates and their mixtures were tested against *R. solani.* Greenhouse study was conducted by using clay pots of 20 cm in diameter. In the field experiment, treatments were sown in 1.5 × 3 m plots, seed rate was 64.3 gm/plot which are equivalent to 60 kg/feddan. The soil used in both experiments was a natural clay soil (pH 7.4, clay 63.2 percent, E.C. 1.3 mmhos/cm). The design of layout of both trails was a randomized complete block design with four replications, greenhouse and field experiments were carried out during 1997/1998 and 1998/1999 growing seasons at Sakha Agric. Res. Station, ARC.

Variables of the tested plants: In greenhouse test, 15 flax seeds treated with bacterial suspensions or filtrates (individually or in mixtures) were planted one week after soil infestation. Percentage of surviving seedlings, were recorded 40 dafrom sowing in both greenhouse and field experiments. Seed yield and straw yield were recorded at the end growth seasons in the field trials.

Statistical analysis: Percentage data of greenhouse and field experiments were transformed into arc sine angles before carrying out analysis of variance (ANOVA) to produce approximately constant variance. ANOVA was performed by the software (A Microcomputer Program for the Design, Management and Analysis of Agronomic Research Experiments MSTATC, Michigan State Univ., USA).

Results and Discussion

Data presented in Table 1 showed the antagonism between isolates of *Pseudomonas* spp. and *R. solani.* Isolates 4, 7 and 13 were the most antagonistic isolates because they inhibited *R. solani* by over growth. Isolates 2, 8 and 15 were moderately antagonistic, the fungal mycelium appeared flaccid and collapsed at the line of contact with any of these isolates. Isolates 3 and 10 were inhibited by the fungal growth. The other isolates showed no inhibition and were not affected by the fungal growth.

The bacterial isolates no's. 4, 7 and 13, which showed the highest antagonistic effect against *R. solani,* were identified as *P. fluorescens, P. cepacia* and *Pseudomonas* sp., respectively, according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). These Pseudomonads were belonging to the, Group 4: gram-negative aerobic/microaerophilic rods and cocci, subgroup 4A in the Genus Pseudomonas.

Table 2 showed that the linear growth of *R. solani* was inversely proportional to the concentration of culture filtrates of the tested bacterial strains. In general, filtrate of f-4 was the most efficient in suppressing the fungal growth, while filtrate of sp-13 was the least efficient. Filtrate of c-7 showed intermediate efficiency. The antagonistic activity of *Pseudomonas* cultures (Table 1) or filtrates (Table 2) was due to the production of wide spectrum antifungal secondary metabolites such as pyrrolnitrin, phenazine and pyocyanine (Rosales *et al*., 1995).

Patterns of inhibition among the tested strains on KB and PDA are

Table 1: Antagonism between some bacterial isolates *(Pseudomonas* spp.) selected from the *rhizosphere* of flax seedlings and *R. solani*

Isolate No.	Inhibition zone
1	-
2	+
3	±
4	+ +
5	-
6	-
7	+ +
8	+
9	-
10	±
11	-
12	-
13	+ +
14	-
15	+

+ + Inhibition of pathogen *(R. solemn)* by overgrowth
\+ Inhibition of pathogen on contact with the potential antagonist
\- No inhibition
± Inhibition of the potential antagonist by the pathogen

Table 2: Effect of different concentrations of culture filtrates of *Pseudomonas* spp. on the linear growth (cm) of *R. solani*

Organisms	Conc of filtrate (%)	Growth diameter after 5 days	Reduction in fungal growth (%)
Control	0	8.50	0.0
P. fluorescens f-4	5	7.04	17.2
	10	4.40	48.2
	25	2.30	72.9
P. cepacia c-7	5	7.22	15.1
	10	4.72	44.5
	25	2.46	71.1
Pseudomonas sp. sp-13	5	7.64	10.0
	10	2.94	41.9
	25	2.68	68.5
LSD (P = 0.05)		0.34	
(P = 0.01)		0.46	

Table 3: *In vitro* antibiosis between *Pseudomonas* strains on agar media

Strains inoculated	Strains inoculated 1st on King's medium B		
Second	f-4	c-7	sp-13
f-4	+	+	+
c-7	+	+	+
sp-13	-	-	+
	Strains inoculated first on potato-dextrose agar		
	f-4	c-7	sp-13
f-4	-	-	-
c-7	+	-	-
sp-13	±	-	-

Growth of strain inoculated second:
+ = Normal growth, equivalent to the growth in single inoculation
± = Reduced growth, compared to the growth in single inoculation
- = No growth after 24 hr, but slight growth occasionally observed after 72 h.

shown in Table 3. On KB strain sp-13 was inhibited by the strain f-4 and c-7, whereas strains f-4 and c-7 were not inhibited by any strain. Strong inhibition was observed among the tested strains on PDA, the strains were also self inhibitory on this medium. The results of the present study suggest that the application of *Pseudomonas* strains in mixtures would be a promising approach for increasing efficiency of biocontrol of flax seedling blight. ANOVA of Table 4 indicated that treatments and application methods were highly significant sources of variation in seedling survival, while their interaction was not significant. Cultures and filtrates of *Pseudomonas* spp., individually or in combinations, significantly increased percentage of surviving seedlings compared to the untreated control. *Pseudomonas* cultures were more effective in increasing seedling survival than *Pseudomonas* filtrates. Mixtures of *Pseudomonas* strains were more effective in reducing disease incidence than individual strains. The mixture of the three strains gave the maximum percentage of surviving seedlings under greenhouse and field conditions (Table 5 and 6). The individual strains as well as their mixtures significantly increased seed yield and straw yield, however, the mixtures were

Table 4: Analysis of variance of the effect of Pseudomonads cultures and filtrates on susceptibility flax to *R. solani* seedlings under greenhouse conditions

Source of variation[a]	D.F	M.S.	F
Replications	3	35.84	
Treatments (T)	8	741.59	47.94**
Application methods (M)	2	909.48	58.79**
T × M	16	12.24	0.79
Error	78	15.47	

[a]Replications are random while treatment (T) and application method are fixed ** Indicate $p < 0.01$

Table 5: Effect of Pseudomonads cultures and filtrates, individually and in combinations on susceptibility of flax seedlings to *R. solani* under greenhouse conditions

Treatments	Application method			Mean
	Culture	Filtrate	None[d]	
P. fluorescens f-4	51.68[a] (46.00[b])	46.65[a] (43.06)[b]	60.00[a] (50.80)[b]	52.78[a] (46.62[b])
P. cepacia-7	46.68 (43.09)	40.00 (39.20)	56.65 (48.83)	47.78 (43.71)
Pseudomonas sp. Sp-13	35.00 (36.20)	36.65 (37.24)	53.33 (46.92)	41.66 (40.12)
f-4 + c-7	71.68 (57.96)	58.33 (49.80)	76.65 (61.17)	68.89 (56.31)
f-4 + sp-13	58.33 (49.83)	53.33 (46.92)	70.00 (56.83)	60.55 (51.19)
c-7 + sp-13	50.00 (45.00)	46.65 (43.06)	65.00 (53.80)	53.88 (47.29)
f-4 + c-7 + sp-13	73.35 (59.10)	63.30 (52.89)	80.00 (83.60)	72.22 (58.53)
Nutrient broth	31.65 (34.07)	31.65 (34.21)	46.65 (43.06)	36.65 (37.11)
Control[c]	30.03 (33.14)	31.68 (34.17)	46.65 (43.06)	36.12 (36.79)
Mean	49.82 (44.93)	45.36 (42.28)	61.66 (52.01)	
L.S.D. for treatments (T)	= 3.20 (p = 0.05) or 4.24 (p = 0.01)			
for application methods	= 1.84 (p = 0.05) or 2.45 (p = 0.01)			

[a]Percentage of surviving seedings [b]Arc sine-transformed data [c]Natural soil infested with *R. Solani*
[d]Natural soil non infested with *R. solani* and non treated with culture of filtrate

Table 6: Effect of *Pseudomonas* spp. individually and in combinations on susceptibility of flax to seedling blight and on yield under field conditions

Treatments	Seedling survival (%)				Seed yield/fed (kg)		Straw yield/ded (t)	
	1997/98		1998/99		1997/98	1998/99	1997-98	1998/99
P. fluorescens f-4	64.1[a]	(53.20)[b]	61.48[a]	(51.68)[b]	402.60	374.51	1.893	1.772
P. cepacia c-7	62.33	(52.14)	60.70	(51.18)	402.78	373.89	1.841	1.761
Pseudomonas sp-13	60.08	(50.82)	58.38	(49.82)	399.71	368.34	1.865	1.760
f-4 + c-7	67.38	(55.20)	68.68	(56.01)	411.24	385.23	1.940	1.835
f-4 + sp-13	65.40	(54.01)	68.55	(55.92)	405.91	381.14	1.971	1.780
c-7 + sp-13	64.23	(53.29)	63.15	(52.63)	403.35	377.28	1.925	1.777
f-4 + c-7 + sp-13	70.68	(57.26)	70.13	(56.91)	418.03	392.86	1.973	1.888
Nutrient broth	50.58	(45.33)	49.38	(44.64)	392.85	358.20	1.811	1.718
Control	50.85	(45.49)	49.28	(44.58)	389.36	355.84	1.818	1.714
LSD (p = 0.05)		2.38		2.42	13.00	8.21	0.077	0.035
(p = 0.01)		3.24		3.30	17.67	11.16	0.105	0.048

[a]Percentage of surviving seedlings [b]Arc sine-transformed data

more efficient in increasing seed yield and straw yield.

These results are in agreement with those previously reported by other workers (Wolk and Sarkar, 1993; Fukui *et al*., 1994; Pierson and Weller, 1994; Sedra and Maslouhy, 1995; Yeom and Park 1995).

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