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ROLE OF CRUSTACEAN CHITIN, FUNGICIDES AND FUNGAL ANTAGONIST ON THE EFFICACY OF *PSEUDOMONAS AERUGINOSA* IN PROTECTING CHILLI FROM ROOT ROT

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Abstract

Application of *Pseudomonas aeruginosa*, a plant growth promoting rhizobacterium alone or with crustacean chitin, fungicides (benlate/captan) or *Paecilomyces lilacinus* (a biocontrol agent) significantly suppressed *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium oxysporum* and *F. solani* attacking roots of chilli. *Paecilomyces lilacinus*, an egg parasite of root knot and cyst nematodes, also caused significant suppression of root rot pathogens. *Pseudomonas aeruginosa* was found to be less effective against *M. phaseolina*, but more effective against *F. solani*, than benlate and captan. *Pseudomonas aeruginosa* and *P. lilacinus* together on crustacean waste powder produced better plant growth than when used alone. The use of crustacean waste powder resulted in better plant growth than the use of pure chitin.

Introduction

In recent years, interest in the use of bacteria for biological control of plant pathogenic fungi has increased (Weller, 1988; Haas & Keel, 2003), especially the use of plant growth promoting bacteria (Raaijmakers & Weller, 1998; Weller, 1988; Weller *et al*., 2002). The root-colonizing bacteria that have a beneficial effect on plants are termed plant growth promoting rhizobacteria (PGPR) (Kloepper *et al*., 1980). PGPR have been reported to improve plant growth either through direct stimulation of the plant or by suppression of pathogens (Weller, 1988; Weller *et al*., 2002). Of the various rhizospheric bacteria, those belonging to the fluorescent *Pseudomonas* are aggressive colonizers of the rhizosphere of various crop plants and have broad spectrum antagonistic activity against plant pathogens (Izhar *et al*., 1995; Weller, 1988; Weller *et al*., 2002). Among the fluorescent *Pseudomonas*, *P. aeruginosa* is known to produce siderophores; pyoverdine; pyochelin (Buysens *et al*., 1996) and salicylic acid (De Meyer & Hofte, 1997). Pyoverdine and pyochelin are necessary for controlling *Pythium* damping-off in tomato (Buysens *et al*., 1996), while salicylic acid is reported to be essential for inducing systemic resistance (ISR) in plants by some strains of *P. aeruginosa* (De Meyer & Hofte, 1997; De Meyer *et al*., 1999). Application of *P. aeruginosa* successfully induced ISR in bean against *Botrytis cinerea* (De Meyer & Hofte, 1997) and in tobacco against tobacco mosaic virus (De Meyer *et al*., 1999).

With the growing interest in the introduction of plant growth promoting bacteria into the rhizosphere, it becomes of particular importance to characterize the effective bacteria under different conditions. The rhizobacteria are frequently found to be ineffective because of microbial competition or adverse environmental conditions (Lazarovits & Nowak, 1997) and combinations of more than one method provide more protection than either component alone (Ehteshamul-Haque *et al*., 1995; Izhar *et al*., 1995).

When mixed with soil chitin, the most common polysaccharide, stimulates the micro-organisms and this chemically stable compound is mineralized in a short period (Alexander, 1977). Soil amendment with chitin has also resulted in significant control of root knot nematode (Mian *et al*., 1982; Ehteshamul-Haque *et al*., 1997) and root-infecting fungi (Bade & Wick, 1988; Sultana *et al*., 2000) by changing the soil micro-flora resulting in an increase in microorganisms around the roots, antagonistic to root pathogens (Godoy *et al*., 1983). Pre-plant chitin amendment of soil significantly reduced disease incidence and severity of *Fusarium* yellows of celery caused by *Fusarium oxysporum* (Bell *et al.,* 1998). Most of the processing discards of shell fish are either not utilized or dumped as waste, thus producing an environmental problem (Shahidi *et al*., 1992) or adding cost for their disposal.

Seeds and roots are often treated with fungicides or biocontrol agents to protect the young seedlings from root diseases (Ramos & Ribeiro, 1993; Sharma & Nowak, 1998). Use of chemical pesticides is a routine practice to suppress soilborne and seedborne pathogens. Application of chemical pesticides with a compatible bacterium could be exploited for the control of root diseases, as the combination can provide benefits not possessed by either component alone (Jacobsen & Backman, 1993). The present report describes the effect of the addition of crustacean chitin, fungicides and a biocontrol agent *Paecilomyces lilacinus* on the efficacy of *Pseudomonas aeruginosa* in controlling root rot diseases of chilli.

Materials and Methods

Crustacean waste and chitin extraction: Crustacean waste of shrimp, prawn and crab collected from Empress Market, Karachi was air dried, powdered in an electric blender and stored in polyethylene bags until used. Chitin was extracted from crustacean waste by the modified method of Bade & Wick (1988), where shells were demineralized in 5% HCl for 2 hours at room temperature (25-30^{o}C) and then deproteinized with 5% KOH solution for 2 hrs., at 100^{o}C. Chitin was separated on a coarse glass sintered funnel, washed with distilled water to neutral pH and then with acetone to remove the water followed by oven drying at 105^{o}C for 1 hr. The purified chitin was stored in polyethylene bags until used.

Pot experiment: Dry chitin powder from shrimp, prawn or crab was mixed in sandy loam soil, pH 8.0, at 0.1% w/w. Since the percentage of chitin varied in shrimp (16.1%), prawn (14.5%) and crab (19.5%), the crustacean waste powder was mixed with soil according to the percentage of chitin (0.62 g of shrimp, 0.68 g of prawn and 0.51 g of crab per 100 g soil to give 0.1% w/w). The soil was naturally infested with 3-7 sclerotia of *Macrophomina phaseolina* g^{-1} of soil as determined by wet sieving and dilution plating (Sheikh & Ghaffar 1975), 2-6% colonization of *Rhizoctonia solani* on sorghum seeds used as baits (Wilhelm, 1955) and 3000 cfu g^{-1} of soil of a mixed population of *Fusarium oxysporum* and *F. solani* as assessed by soil dilution (Nash & Snyder, 1962). Amended soil was transferred to small earthen pots each containing 1 Kg soil. Pots were kept at 50% W.H.C by watering daily (Keen & Raczkowiski, 1921). After three weeks, aqueous suspensions of *Pseudomonas aeruginosa* (3.8 x10^{8} cfu/mL) grown on KB agar and *Paecilomyces lilacinus* (1.1x10^{8} cfu/mL) grown on potato dextrose agar were drenched onto amended and non-amended soil in pots @ 50 ml per pot. Aqueous

suspension (100 ppm) of fungicides, benlate and captan were drenched @ 50 ml per pot, when used alone and at 25 ml per pot when used with bacterial or fungal antagonists. The application rate of both *P. aeruginosa* and *P. lilacinus* was also reduced to 25 ml per pot, when used together or with fungicides. Four seedlings of chilli (*Capsicum annuum* L.) of equal size, raised in steam sterilized soil were transplanted into each pot. Pots without amendment/antagonists or fungicides served as controls. Each treatment was replicated 4 times and pots were placed in a screen house in a randomized complete block design.

Determination of fungal infection and growth parameter: To assess the efficacy of *P. aeruginosa* in suppression of root disease, plants were uprooted after 6 weeks of growth. To determine the incidence of fungi, roots were washed with running tap water then surface disinfested with 1% $Ca(OCl)_2$ and 1 cm long root pieces from tap roots (5 from each plant) were plated onto potato dextrose agar plates amended into penicillin (100,000 units/litre) and streptomycin (0.2 g/litre). After incubation for 5days at 28^0 C, the incidence of root infecting fungi was recorded. Infection percentage for each pathogen was calculated using the formula:

$$\text{Infection \% of a pathogen} = \frac{\text{Number of plants infected by a pathogen}}{\text{Total number of plants}} \text{ X } 100$$

Plant growth parameters, such as plant height and fresh weight of shoot were also recorded. The experiment was conducted twice. Because the data from the two experiments were statistically similar, the data were combined for statistical analysis.

Statistical analysis: Data were analysed and subjected to analysis of variance and means were separated using Fisher's least significant difference (LSD) according to Gomez & Gomez (1984).

Results

Application of *Pseudomonas aeruginosa* and *Paecilomyces lilacinus* alone or with crustacean chitin or crustacean waste powder significantly ($p<0.05$) suppressed infection by *Macrophomina phaseolina*. Use of the fungicides benlate and captan used alone or with microbial antagonists *Paecilomyces lilacinus* and *Pseudomonas aeruginosa* also significantly ($p<0.05$) inhibited *Macrophomina phaseolina*. Complete control of *M. phaseolina* infection was achieved when *P. lilacinus* was used alone or with prawn chitin or prawn powder or where *P. aeruginosa* was used with prawn powder (Table 1).

Significant ($p<0.05$) control of *Fusarium solani* was achieved by the application of *P.aeruginosa,* benlate or captan alone and by the combined use of *P.aeruginosa* or *P. lilacinus* with fungicides or crustacean chitin or crustacean waste powder. The addition of prawn chitin resulted in complete control of *F. solani* infection. More than 50% reduction in *F. solani* infection was achieved with *P. aeruginosa* used with benlate, prawn or crab chitin or their waste powder. Moreover *P. lilacinus* caused more than 50% reduction in *F. solani* infection when used with benlate or captan or with prawn or crab chitin. Use of shrimp and crab chitin also produced more than 50% reduction in *F. solani* infection (Table 1).

Table 1. Effect of a fungal antagonist (*Paecilomyces lilacinus*), fungicides and crustacean chitin on the efficacy of *Pseudomonas aeruginosa* for the control of root rot diseases of chilli caused by *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani* and *F. oxysporum*.

Treatments	Infection %			
	M.phaseolina	***R.solani***	***F.solani***	***F.oxysporum***
Control	37	12	75	18
P. aeruginosa	31	00	43	12
P. lilacinus	00	00	81	12
Benlate	18	00	62	00
Captan	25	00	62	00
P. aeruginosa + *P. lilacinus*	18	00	56	00
P. aeruginosa + Benlate	18	00	37	00
P. aeruginosa + Captan	25	00	43	00
P. lilacinus + Benlate	06	06	31	00
P. lilacinus + Captan	31	00	25	00
Shrimp chitin	06	00	25	18
Prawn chitin	31	00	00	00
Crab chitin	06	00	31	25
Shrimp powder	25	00	50	56
Prawn powder	18	00	56	00
Crab powder	25	00	81	06
Shrimp chitin + *P. aeruginosa*	06	00	68	25
Prawn chitin + *P. aeruginosa*	12	00	25	12
Crab chitin + *P. aeruginosa*	25	25	31	00
Shrimp powder + *P. aeruginosa*	18	06	62	00
Prawn powder + *P. aeruginosa*	00	31	37	31
Crab powder + *P. aeruginosa*	25	00	18	00
Shrimp chitin + *P. lilacinus*	25	00	62	06
Prawn chitin + *P. lilacinus*	00	00	25	06
Crab chitin + *P. lilacinus*	06	00	31	00
Shrimp powder + *P. lilacinus*	12	00	56	06
Prawn powder + *P. lilacinus*	00	12	68	00
Crab powder + *P. lilacinus*	25	25	68	25

$LSD_{0.05}$, Treatments = 6.5[1], Pathogen = 2.8[2]

[1] Means values of treatments in columns showing differences of LSD values are significantly different at $p<0.05$

[2] Means values of pathogens in rows showing differences of LSD values are significantly different at $p<0.05$

Only 12% of the control plants were found to be infected with *Rhizoctonia solani*. *Pseudomonas aeruginosa* alone or with benlate, captan or with *P. lilacinus* completely inhibited *R. solani.* Crustacean chitin or crustacean waste powder also showed complete suppression of *R. solani* infection. Captan and benlate alone or with *P. aeruginosa* or *P. lilacinus* completely prevented *Fusarium oxysporum* infection. Complete suppression of *F. oxysporum* was achieved when *P. aeruginosa* was used with *P. lilacinus*, crab chitin or shrimp or crab waste powder. Complete reduction in *F. oxysporum* infection was also found when prawn chitin or prawn powder was used alone or *P. lilacinus* was used with prawn powder or crab chitin (Table 1).

Table 2. Effect of a fungal antagonist (*Paecilomyces lilacinus*), fungicides, crustacean chitin and *Pseudomonas aeruginosa* on growth of chilli seedlings.

Treatments	Plant height (cm)	Fresh shoot weight (g)
Control	7.6	0.51
P. aeruginosa	9.6	0.57
P. lilacinus	8.6	0.48
Benlate	6.9	0.48
Captan	7.7	0.41
P. aeruginosa + *Pi lilacinus*	9.8	0.72
P. aeruginosa + Benlate	7.8	0.61
P. aeruginosa + Captan	7.6	0.58
P. lilacinus + Benlate	8.1	0.65
P. lilacinus + Captan	7.9	0.58
Shrimp chitin	8.4	0.45
Prawn chitin	7.2	0.42
Crab chitin	7.9	0.44
Shrimp powder	11.0	0.73
Prawn powder	12.4	1.21
Crab powder	12.2	0.94
Shrimp chitin + *P. aeruginosa*	9.1	0.54
Prawn chitin + *P. aeruginosa*	7.6	0.49
Crab chitin + *P. aeruginosa*	10.0	0.49
Shrimp powder + *P. aeruginosa*	12.2	1.13
Prawn powder + *P. aeruginosa*	11.8	1.31
Crab powder + *P. aeruginosa*	10.5	0.71
Shrimp chitin + *P. lilacinus*	7.5	0.41
Prawn chitin + *P. lilacinus*	7.3	0.46
Crab chitin + *P. lilacinus*	10.2	0.62
Shrimp powder + *P. lilacinus*	12.2	1.01
Prawn powder + *P. lilacinus*	12.8	1.45
Crab powder + *P. lilacinus*	12.0	0.86
$LSD_{0.05}$	1.90[1]	0.14[1]

[1] Means values of treatments in columns showing differences of LSD values are significantly different at $p<0.05$

Greater plant height resulted when *P. lilacinus* was used with prawn waste powder followed by *P. aeruginosa* used with shrimp powder or prawn powder used alone (Table 2). Maximum fresh shoot weight resulted when *P. lilacinus* was used with prawn powder followed by *P. aeruginosa* used with prawn powder. Crustacean waste powder alone or with microbial antagonists produced better plant growth than pure chitin (Table 2).

Discussion

Chitin, a major component of discarded shellfish is reported from Pakistan to contain 16.1% chitin in shrimp, 19.5% in crab and 14.5% in prawn (Sultana *et al.*, 2000). There are reports that chitin and chitosan amendments to soil effectively reduced soilborne diseases (Benhamou & Theriault, 1992; Bell *et al.*, 1998). Addition of small quantities of

chitin to soil resulted in a marked suppression of the severity of root rot of bean caused by *Fusarium solani* f.sp. *phaseoli* and vascular wilt of radish caused by *F.oxysporum* f.sp. *conglutinans* (Mitchell & Alexander, 1961). Bell *et al.,* (2000) reported a reduction in the abundance of the plant parasitic nematodes *Heterodera trifolii* in white clover and *Paratylenchus* sp., in ryegrass roots by the application of chitin to soil. In the present study, use of crustacean chitin at 0.1% or crustacean waste powder (having 0.1% chitin) alone or with *Pseudomonas aeruginosa* or *Paecilomyces lilacinus* was efficacious in the control of root-infecting fungi attacking chilli roots. Rodriguez-Kabana *et al*., (1984) reported that the addition of chitin (1% or more) to soil control root knot nematode. Others reported that the use of chitin alone or with organic materials reduced fungal infection (Bade & Wick, 1988; Sultana *et al*., 2000) and root knot nematode establishment (Ehteshamul-Haque *et al*., 1997). Similarly Manjula & Podile (2001) reported that a chitin-supplemented formulation improved biocontrol activity and plant growth promoting potential of *Bacillus subtilis*.

Soil amendment with chitin stimulates microorganisms (Mitchell & Alexander, 1962) and produces toxins (Mankau & Das, 1969) creating an environment adverse to plant pathogens. In soil, approximately 90-99% of the chitinoclastic population are actinomycetes which digest the chitin (Alexander, 1977). This digested chitin is then utilized by soil bacteria as a nitrogen source resulting in an increase in their population in amended soil. These bacteria, present in the rhizosphere, inhibit root-infecting fungi by producing antibiotics (Raaijmakers & Weller, 1998; Weller *et al*., 2002; Haas & Keel, 2003) and enhanced plant growth by producing phytohormones such as auxin derivatives and gibberellin-like substances (Brown, 1972). It is interesting to note that in our study crustacean waste powder showed more positive effect on plant growth than their pure chitin used alone or with microbial antagonists. Presumably other nitrogenous materials present in the waste may provide an additional food base for saprophytic and introduced bacteria, that have a positive effect on plant growth. *Pseudomonas aeruginosa* was found to be less effective against *Macrophomina phaseolina* but more effective against *Fusarium solani* than benlate and captan. Presumably *F. solani* is more sensitive to the antibiotics produced by *P. aeruginosa* (Buysens *et al*., 1996; De Meyer & Hofte, 1997; De Meyer *et al*., 1999), while *M. phaseolina* is less affected by the antibiotics of *P. aeruginosa* due to its hard sclerotial structure. In this study *Pseudomonas aeruginosa* used with *Paecilomyces lilacinus* resulted in greater fresh shoot weight than either used alone. Similarly *P. aeruginosa* used with *P. lilacinus* caused greater suppression of root knot disease in tomato than either component alone (Siddiqui *et al*., 2000), and the use bradyrhizobia with *P. lilacinus* resulted in greater plant growth and root nodulation in mungbean (Ehteshamul-Haque *et al*., 1995).

The delivery of microbial antagonists with urban and agricultural wastes as mulches was found very effective in suppressing root pathogens of avocado and citrus (Casale *et al.,* 1995). Similarly the addition of some composts to soil increased the population of PGPR in the tomato rhizosphere resulting in antagonism towards *Fusarium oxysporum* f.sp. *radicis-lycopersici*, *Pyrenocheta lycopersici*, *Pythium ultimum* and *Rhizoctonia solani* (Alvarez *et al.,* 1995). The results of the present study suggest that biocontrol bacteria may be applied with chitin or chitin-containing materials like crustacean waste, or carrier materials supplemented with chitin-containing substances for better protection of plant roots from attack by soilborne pathogens. The application of chitin and chitosan with chitin-degrading microbes to soil, provided effective protection against wilt disease

(Toyoda *et al.*, 1996). Furthermore application of chitosan may enhance the vitality of plant cells and the plant's ability to degrade the walls of fungi upon entry (Benhamou & Theriault, 1992).

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