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UTILIZATION OF ENDO-ROOT FLUORESCENT *PSEUDOMONAS* OF CHILLI FOR THE MANAGEMENT OF ROOT DISEASES OF CHILLI

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Abstract

Chili (*Capsicum annuum*) is an important vegetable and spice crop worldwide produced and consumed as fresh or processed. However production is increasingly constrained by chili plant diseases. The four diseases that lead to wilting in chili are *Phytophthora* root rot, *Verticillium* wilt, *Rhizoctonia* root rot, and *Fusarium* wilt. The association of *Fusarium* species with *Rhizoctonia solani* or root knot nematodes *(Meloidogyne* spp.) caused huge losses. In this study, seven strains of *Pseudomonas aeruginosa* were isolated from inner roots of healthy chili plants growing under field condition. *In vitro* test cell free culture filtrate of some strains showed nematicidal activity against *Meloidogyne javanica* root knot nematode by killing the 2nd stage juveniles and by retarding the egg hatching. In dual culture plate assay, one strain of *P. aeruginosa* inhibited the radial growth of all the four test root rotting fungi *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani* and *F. oxysporum* by producing the zone of inhibition. While other strains caused growth inhibition of at least 2 or 3 test fungi. Some bacterial strains also caused lysis of fungal hyphae. In screen house, application of some of these bacterial strains caused significant suppressive effect on root rotting fungi and root knot nematode infecting chili roots. Some *Pseudomonas aeruginosa* strains also showed positive impact on plant growth by increasing the plant height and fresh shoot weight and were found to produce indole-acetic acid at varying degree.

Introduction

Chili (*Capsicum annuum* L.) is an important vegetable and spice crop worldwide, produced and consumed as fresh or processed. The popularity and demand for chili are providing a boost to the chili industry, but production is increasingly constrained by chili plant diseases. The chili wilt has been found as the most frequently encountered disease problem (Skaggs *et al*., 2000). Several microorganisms may be involved in causing wilt diseases in chili. The four diseases that lead to wilting in chili are *Phytophthora* root rot, *Verticillium* wilt, *Rhizoctonia* root rot, and *Fusarium* wilt. *Rhizoctonia* root rot caused by *Rhizoctonia solani* generally affects seedlings, but *R. solani* can also infect mature plants and induce root rot, which leads to wilting and death of chili plants. To date, there are no commercially acceptable chili cultivars that are resistant to *R. solani* (Muhyi & Bosland, 1992). Fusarium wilt of chili first described by Leonian (1919), caused by *Fusarium annuum*, and at that time the disease was apparently present only in New Mexico. Species of *Fusarium* like *F.oxysporum* and *F. solani* are most common species of *Fusarium* found associated with diseased chili crop in Pakistan (Ehteshamul-Haque & Ghaffar, 1994; Ehteshamul-Haque, 2006; Qureshi *et al*., 2004). The association of *Fusarium* species with *R. solani* or root knot nematodes *(Meloidogyne* spp.) is causing huge losses

in Sindh, Pakistan (Ehteshamul-Haque, 2006). The root diseases of chili described above have a significant impact on chili production and present a formidable challenge for chili producers.

Endophytes have been defined as bacteria that are able to colonize living plant tissue without harming the plant or gaining benefit other than securing residency (Kado, 1992). Several studies have shown beneficial interaction between plants and some endophytic bacteria, such as plant growth promotion and biocontrol potential against plant pathogens (Hallmann, *et al*., 1995; Siddiqui & Ehteshamul-Haque, 2001). Little work carried out on the effect of endophytic fluorescent *Pseudomonas* on root rotting fungi and root knot nematodes attacking chili roots. The present report describes the effect of endo-root fluorescent *Pseudomonas* isolated from healthy chili roots on root rotting fungi and root knot nematode affecting chili roots.

Materials and Methods

Collection of root samples for the isolation of fluorescent *Pseudomonas*: For the isolation of fluorescent *Pseudomonas* root samples of healthy chili plants were collected from experimental fields of Karachi University and Hub, Balochistan and kept at 4°C until isolation was made within 24 hours.

Isolation of fluorescent *Pseudomonas*: For the isolation of endophytic bacteria, 1 g root from healthy plants was disinfested with 1% $Ca(OCl)_2$ for 3 minutes and rinsed twice with sterile water, submerged for 30 s in 15% H_2O_2 and rinsed twice again in sterile water. The roots were macerated in 10 ml of 0.1 M $MgSO_4$ solution with 0.02% Tween-20 and 100 µl aliquots from serial dilution were transferred on S1 medium supplemented with antibiotics trimethoprim (Gould *et al*., 1985). Dishes were incubated for three days at 28°C. Bacterial colonies fluoresced under UV light at 366 nm were purified on King's B agar medium (King *et al*., 1954) and identified according to Krieg & Holt (1984).

***In vitro* test against root rotting fungi:** Dual culture plate method was used to determine the antifungal activity of bacterial strains (Drapeau *et al*., 1973). The bacterial strains/ isolates were streaked on one side of the Petri dishes containing Czapek's Dox agar pH 7.2. On the other side of Petri dishes, a 5 mm diam. disc of root rotting fungi like *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani* and *Fusarium oxysporum* were inoculated. The dishes were incubated at 28°C and zone of inhibition (if any) were recorded from 3-7 days (depends upon the growth of test fungus).

Nematicidal activity of cell free culture filtrates of bacteria: Bacterial strains were grown in KB Broth at 30°C for 48 hours in dark and centrifuged twice at 3000 rpm for 20 minutes. The pellets were discarded and culture filtrates were collected in beakers and few drops of chloroform was added to kill the bacterial cells if any. The bacterial filtrate was left open under laminar flow hood for 1 hour to evaporate the chloroform vapours before use. For the nematicidal activity, one ml of freshly hatched second stage juvenile suspension (25 juveniles) and 1 ml cell free culture filtrate of bacterial strains were transferred in glass cavity blocks and kept at 25 ± 5°C. There were three replicates of each treatment and juvenile mortality was recorded after 48 hours. The experiment was conducted twice.

Effect of PGPR on nematode's egg hatching: Method described by Ara *et al*., (1997) was used to determine the effect of cell free culture filtrates of PGPR on nematode's eggs hatching, where healthy egg masses of equal size were placed in glass cavity blocks and 2 ml of cell free culture filtrate was added. Juveniles hatched were recorded after 48 hours and compared with non-bacterized control. Egg masses were then transferred to cavity blocks containing sterile water and left for another 48 hours to confirm the anti hatching activity of culture filtrates.

Determination of indole acetic acid (IAA) produced by endo-root fluorescent *Pseudomonas*: Test bacteria were grown in phosphate glucose broth for 48 hours and production of indole acetic acid (IAA) was determined by Salkowski's reagent method (Patten & Glick, 2002).

Screen house experiment: Three weeks old chili (*Capsicum annuum)* seedlings of equal size, raised in steam sterilized soil in 35-cm diam. clay pots were used in this experiment. Endo-root *Pseudomonas aeruginosa* strains grown on King's B medium at room temperature (25-30^{o}C) for 3 days, were scrapped with the help of a sterilized bent spatula after adding 10 ml sterilized 0.5% gum arabic in each plate and pooled in a beaker. Roots of seedlings were dipped in each *P. aeruginosa* strain suspension viz., PGPR-110 (2.4 x 10^8 cfu/ml), PGPR-111 (1.8 x 10^8 cfu/ml), PGPR-112 (1.4 x 10^8 cfu/ml), PGPR-113 (1.0 x 10^8 cfu/ml) and PGPR-114 (1.6 x 10^8 cfu/ml) for 1 hour, then transplanted in 12 cm diam clay pots (each containing 1 kg soil) at 4 seedlings per pot. The soil had a natural infestation of 3-6 sclerotia/gm of soil of *Macrophomina phaseolina* as determined by wet sieving and dilution technique (Sheikh & Ghaffar, 1975), 5-10% colonization of *Rhizoctonia solani* on sorghum seeds used as baits (Wilhelm, 1955) and 3000 cfu/gm of soil of mixed population of *Fusarium solani* and *F. oxysporum* (Nash & Snyder, 1962). After one week of seedlings establishment, roots in each pot was inoculated with 2000 eggs/J_2 of *Meloidogyne javanica*. Seedlings treated with 0.5% gum arabic without bacteria served as control. Whereas Topsin M served as positive control against root rotting fungi and carbofuran served as +ve control against nematode. There were four replicates of each treatment and pots were randomized on a screen house bench and kept at 50% WHC (Keen & Raczkowiski, 1921).

To assess the efficacy of endo-root PGPR in suppression of root disease, plants were uprooted 6 weeks after nematode inoculation and roots were washed under tap water. Nematode infection was determined by counting the number of galls per root system (Taylor & Sasser, 1978). To determine nematode penetration and infection by root-infecting fungi, roots from each plant were cut into 1-cm long pieces and five pieces of tap roots from each plant were used for assessment of fungal infection. The remaining roots were mixed thoroughly, and 1-gram sub-sample was wrapped in muslin cloth and dipped in boiling 0.25% acid fuchsin stain for 3-5 minutes. Roots were left in the stain to cool, and then washed under tap water to remove excess stain. Roots were transferred to vials containing glycerol and water (1:1 v:v) with a few drops of lactic acid. Roots were macerated in an electric blender for 45 seconds and the resulting suspension was suspended in 50 ml water. Numbers of juveniles and females in five 5 ml sub samples were counted with the aid of dissecting microscope and numbers of nematode/g root were calculated (Siddiqui & Ehteshamul-Haque, 2001). To determine the incidence of fungal infection, 1-cm long root pieces from tap roots (five pieces from each plant) were surface

disinfested with 1% $Ca(OCl)_2$ and plated onto potato dextrose agar amended with penicillin (100,000 units/litre) and streptomycin (0.2 g/litre). After incubation for 5 days at 28^{o}C, colonies of *Macrophomina phaseolina*, *Rhizoctonia solani* and species of *Fusarium* were recorded. Data on plant height and fresh shoot weight were also recorded. The experiment was conducted twice. Date were analyzed and subjected to analysis of variance (ANOVA) followed by least significant difference according to Gomez & Gomez (1984).

Results

***In vitro* antifungal activity of endo-root fluorescent *Pseudomonas*:** *Pseudomonas aeruginosa* strains isolated from healthy roots of chili plants were tested for antifungal activity against four highly pathogenic root rotting fungi *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani* and *F. oxysporum*, isolated from diseased chili roots. *Pseudomonas aeruginosa* strain PGPR-110 inhibited all the four tested fungi by producing zone of inhibition. PGPR-110 also caused lysis of mycelium of *F. solani* and *F. oxysporum* (Table 1). Where as PGPR-113, PGPR-114, PGPR-124 and PGPR-125 inhibited the radial growth of *M. phaseolina*, *F. solani* and *F. oxysporum* producing zone of inhibition. PGPR-113 and PGPR-114 did not produced zone of inhibition against *F. solani*, but caused lysis of mycelium (Table 1).

***In vitro* nematicidal activity of endo-root fluorescent *Pseudomonas*:** Culture filtrates of rhizobacterial strains showed significant nematicidal activity by killing second stage larvae of *Meloidogyne javanica* at varying degrees (Table 2). Maximum killing was observed by PGPR-110 followed by PGPR-113 and PGPR-114.

Effect of endo-root fluorescent *Pseudomonas* on hatching of nematode's eggs: Cell free culture filtrate of PGPR caused retardation of nematode's egg hatching. After 48 hours, no egg was found hatched exposed to PGPR-110. Whereas PGPR-114 exposed egg masses showed 2 hatched larvae per egg mass compared to 60 larvae per egg mass in untreated control (Table 2).

Production of indole acetic acid by endo-root fluorescent *Pseudomonas*: The PGPR strains were found to produce indole acetic acid at varying degrees (Table 3).

Screen house experiment: Application of some endo-root *P. aeruginosa* strains caused suppression of root rotting fungi on chili roots. PGPR-110 was found highly effective against *F. solani*, *F. oxysporum* and *M. phaseolina* (Table 4). Other strains were found effective against at least one pathogenic fungi.

Bare-root dip treatment of chili seedlings with endo-root *P. aeruginosa* also caused a suppressive effect on nematode infection by reducing the gall formation on roots and nematode's penetration in roots (Table 4). PGPR-110 caused maximum reduction in gall formation on roots, whereas PGPR-113 treated plants showed minimum nematode's penetration in roots (Table 4).

Endo-root *P. aeruginosa* also showed positive impact on plant growth. Greater plant height was produced by PGPR-110 followed by PGPR111 (Table 5). Maximum fresh shoot weight was produced by *PGPR*-111 followed by PGPR-112.

Table 1. *In vitro* growth inhibition of root rotting fungi *Macrophomina phaseolina, Rhizoctonia solani, Fusarium solani* and *F. oxysporum* by the strains of *Pseudomonas aeruginosa*.

PGPR strains	Zone of Inhibition (mm)			
	M. phaseolina	*R. solani*	*F. solani*	*F. oxysporum*
PGPR-110	20	19.6*	10.6	19.3*
PGPR-111	0	22	0	26.6
PGPR-112	0	16.3*	0	24.6
PGPR-113	22.6	28.6*	0*	15.0
PGPR-114	19.3	20.6	0*	12.0
PGPR-124	20.6	24.3	0	19.4
PGPR-125	16.3	19.3*	0	15.4

0= No inhibition, * = Fungal mycelium lysed

Table 2. *In vitro* nematicidal activity of cell free culture filtrates of fluorescent *Pseudomona, P. aeruginosa* on *Meloidogyne javanica* after 48 hours.

P. aeruginosa strains	Juveniles mortality %	No. of juveniles hatched/ egg mass
Control (Distilled water)	0.0	82
Control (KB broth)	12.0	60
PGPR-110	68.0	0.0
PGPR-111	28.0	5.0
PGPR-112	16.0	10
PGPR-113	56.0	35
PGPR-114	52.0	2.0
PGPR-124	44.0	5.0
PGPR-125	48.0	3.0

Table 3. Production of Indole acetic acid (IAA) by endo-root *Pseudomonas aeruginosa* in phosphate-glucose broth.

	IAA (µg/ml)	
	24 hours	48 hours
Control	0.0	0.0
PGPR-110	0.2	0.5
PGPR-111	0.1	0.8
PGPR-112	0.2	0.1
PGPR-113	0.4	0.1
PGPR-114	0.1	0.2
PGPR-124	0.5	0.8
PGPR-125	0.0	0.3

Discussion

In the present study, some strains of endo-root fluorescent *Pseudomonas*, *P. aeruginosa* showed significant activity against root rotting fungi and root knot nematode both *in vitro* and *In vivo*. Of the various rhizospheric bacteria, the bacteria belonging to fluorescent *Pseudomonas*, which colonize roots of a wide range of crop plants, are reported to be antagonistic to soilborne plant pathogens (Izhar *et al*., 1995; Ehteshamul-Haque *et al*., 2007; Siddiqui *et al*., 2000; Siddiqui & Ehteshamul-Haque, 2001). The production of certain antibiotics (Leavy *et al*., 1992) and siderophores (De Meyer & Hofte, 1997; Buysens *et al*., 1996) by *P. aeruginosa* has been regarded as one of the mechanism involved in antagonism.

Table 4. Effect of endo-root *Pseudomonas aeruginosa* on the infection of root rotting fungi *Macrophomina phaseolina, Rhizoctonia solani, Fusarium solani* and *F. oxysporum* infecting chili roots.

Treatments	Infection (%)			
	M. phaseolina	***R. solani***	***F. solani***	***F. oxysporum***
Control	31.2	31.2	75	62.5
Topsin-M	12.5	12.5	12.5	18.7
Carbofuran	18.7	25	43.7	37.5
PGPR-110	6.2	18.7	18.7	25
PGPR-111	12.5	18.7	56.2	37.5
PGPR-112	0.0	37.5	68.7	68.7
PGPR-113	31.2	18.7	62.5	31.2
PGPR-114	18.7	12.5	37.5	43.7
$LSD_{0.05}$	Treatments = 21.1[1]		Pathogens = 17.2[2]	

[1]Mean values in column showing differences greater than LSD values are significantly different at $p<0.05$.
[2]Mean values in rows showing differences greater than LSD values are significantly different at $p<0.05$.

Table 5. Effect of endo-root *Pseudomonas aeruginosa* on the infection of root knot nematode and growth of chili plants.

Treatments	Galls/ root system	No. of Juveniles/ females/ g root	Plant height (cm)	fresh shoot weight (g)
Control	24.9	71.2	12.2	0.81
Topsin-M	8.6	51.2	18.2	1.31
Carbofuran	7.5	22.5	14.9	1.12
PGPR-110	5.8	32.5	19.5	1.59
PGPR-111	17.2	29.6	18.6	1.93
PGPR-112	11.3	45.2	13.2	1.87
PGPR-113	8.8	28.4	14.4	0.93
PGPR-114	14.3	33.5	14.6	0.96
$LSD_{0.05}$	4.1[1]	41.2[1]	3.2[1]	0.68[1]

[1]Mean values in column showing differences greater than LSD values a re significantly different at $p< 0.05$.

In this study endo-root *Pseudomonas aeruginosa* was found active against root rotting fungi and root knot nematode infecting chili roots which is in agreement with our previous study (Siddiqui & Ehteshamul-Haque, 2001). *Pseudomonas aeruginosa* has been reported to suppress root knot infection on chili, watermelon, guar and pumpkin (Parveen *et al*., 1998), on okra (Ara *et al*., 1997) and on tomato (Siddiqui & Ehteshamul-Haque, 2001). There are reports that plant growth promoting endophytic rhizobacteria are involved with host plants in mutual interaction (Pandey *et al*., 2005). They promote plant growth directly or indirectly, via production of phytohormones, biocontrol of host plant diseases or improvement of plant nutritional status (Glick, 1995). Endophytic bacteria isolated from cotton, caused significant reduction in root galling by *M. incognita* (Hallman *et al*., 1998).

Endo-parasitic nematodes like *Meloidogyne* spp., form specialized feeding cells in the plant tissue and remain embedded in the tissue, whereas parasitic fungi also proliferate inside the host tissues and absorbs the nutrients. Due to protection by surrounding plant tissue, they are difficult to control by soil and rhizosphere microorganisms. Therefore endophytic microorganisms colonizing plant root tissue may be better able to manage endo-parasitic nematode and fungi due to fact that both occupy the same ecological niche and are close contact (Hallman *et al*., 1997). The use of endophytes for the control of root rotting fungi and parasitic nematodes is a relatively

new approach. Use of endo-root PGPR as biocontrol agent for control of soilborne diseases of plant is interesting because they provide protection to plant roots from both ecto-parasitic and endo-parasitic pathogens.

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