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MANAGING THE ROOT DISEASES OF OKRA WITH ENDO-ROOT PLANT GROWTH PROMOTING *PSEUDOMONAS* AND *TRICHODERMA VIRIDE* ASSOCIATED WITH HEALTHY OKRA ROOTS

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Abstract

Okra [*Abelmoschus esculentus* (L.) *Moench*] is an important vegetable crop is grown world wide including Pakistan. However, diseases are the limiting factor in okra production. In Pakistan okra crop is attacked by various soilborne plant pathogenic fungi like *Macrophomina phaseolina, Rhizoctonia solani, Fusarium* spp., and root knot nematodes *Meloidogyne* spp. Considerable evidence has been accumulated in recent years to support and identify the benefits associated with the use of endophytic bacteria and fungi in crop protection. In this study some strains of endophytic fluorescent *Pseudomonas* isolated from roots of healthy okra plants were identified as *Pseudomonas aeruginosa.* The *P. aeruginosa* isolates showed significant activity against root rotting fungi and root knot nematode *in vitro* by producing zone of inhibition against test fungi and killing 2nd stage juveniles of root knot nematode at varying degrees. In screen house experiment, application of some potential strains of *P. aeruginosa* alone or with *Trichoderma viride*, an endophytic fungus and biocontrol agent showed significant biocontrol activity against *M. phaseolina, R. solani, F. solani, F. oxysporum* and *Meloidogyne javanica*, the root knot nematode infecting okra roots. Application of most of the *P. aeruginosa* isolates alone or with *T. viride* showed positive impact on plant growth by improving plant height, fresh shoot weight and root length. Endophytes colonize an ecological niche similar to that of phytopathogens and biological control with endophytes offers an effective strategy for pest management.

Introduction

Okra [*Abelmoschus esculentus* (L.) Moench] is an important vegetable crop is grown world wide including Pakistan (Athar & Bokhari, 2006). Okra is a warm, rainy season crop and requires high temperature. However, diseases are the limiting factor in okra production. In Pakistan okra crop is attack by various soilborne plant pathogenic fungi like *Macrophomina phaseolina, Rhizoctonia solani, Fusarium* spp., and *Meloidogyne* spp., the root knot nematodes (Ehteshamul-Haque *et al.,* 1996; Parveen *et al.*, 1994; Sultana *et al.,* 2005). Most soilborne pathogens are difficult to control by conventional strategies such as the use of resistant cultivars and synthetic fungicides (Weller *et al.,* 2002). Endophytic bacteria or fungi are those bacteria or fungi that live in plant tissues without doing substantive harm or gaining benefit other than residency (Kobayashi & Palumbo, 2000). Considerable evidence has been accumulated in recent years to support and identify the benefits associated with the use of endophytic bacteria in crop protection (Siddiqui & Ehteshamul-Haque, 2001; Hallmann *et al.*, 1995; 1997, 1998; Tariq *et al*., 2009). Besides, endophytes also promote plant growth by a number of mechanisms. These include phosphate solubilization activity (Altomare *et al.*, 1999), indole acetic acid production (Tariq *et al.*, 2009) and production of a siderophore (Leong, 1986). Moreover, a number of other beneficial effects on plant growth have been attributed to endophytes including osmotic adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals and alteration of nitrogen accumulation and metabolism (Compant *et al.*, 2005). Among the endophytic plant growth promoting bacteria, species of *Pseudomonas* have has been shown to improve plant growth and it is known to synthesize growth-stimulating plant hormones (Ehteshamul-Haque *et al.*, 2007; Tariq *et al.*, 2009). Plant hormones produced by *Pseudomonas* include auxins and cytokinins, as well as volatile signals such as ethylene 2, 3-butanediol and acetonin (Persello-Cartieaux *et al*., 2003), which implicated in stimulation of root growth. Similarly *Trichoderma* spp., are free living fungi that are common in soil and root ecosystem are well known for their biocontrol potential (Ehteshamul-Haque & Ghaffar, 1992; Qureshi *et al.,* 2012; 1990; Woo *et al.*, 2006).

Numerous microorganisms with biocontrol activity are discovered each year but their actual use in agriculture is negligible due to their inconsistent performance. To combine the disease-suppressive activity of two (or more) beneficial microbes in a biocontrol preparation is assumed to overcome this problem (Meyer & Roberts, 2002). The current report describes the impact of endo-root fluorescent *Pseudomonas* isolated from healthy okra plants alone or with endophytic *Trichoderma viride* on root rotting fungi and root knot nematode affecting okra roots.

Materials and Methods

Isolation of endophytic fluorescent *Pseudomonas* and *Trichodrma viride*: For the isolation of endophytic fluorescent *Pseudomonas* roots samples of healthy okra plants were collected from Karachi University Campus and farmer's fields in Malir, Karachi. Samples were brought to the laboratory and kept at 4°C until isolation was made within 24 hours. One gram roots were washed with running water then with 70% alcohol for 2-3 minutes finally with distilled water for about 1 minute and chopped into small pieces in a blender with 50 ml of water so as to give the dilution of 1:50. A dilution of root suspension upto $1:10^4$ was transferred (0.1 ml/ Petri plate) onto Petri plates containing S-1 medium, supplemented with 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. Bacteria were identified with reference to Bergey's Mannual (Krieg & Holt, 1984). Most of the fluorescent *Pseudomonas* were identified as *P.*

aeruginosa, whereas *Trichoderma viride* was isolated from surface sterilized roots of okra. After washing with tap water, roots were cut in to small pieces (0.5 cm) and surface sterilized with 70% alcohol for 3 minutes and then washed with sterile water. The root pieces were then sterilized with 1% Ca $(OCl)_2$ for 3 minutes and after washing with sterile water transferred onto potato dextrose agar plates supplemented with penicillin (100000 units/litre) and streptomycin (0.2 g/litre). Fungal colonies of *Trichoderma* emerged from root pieces after incubation at 28°C for 5 days were identified with reference to Rafai (1969).

***In vitro* test against root infecting 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 test fungi *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani* and *F. oxysporum* were inoculated. The dishes were incubated at 28°C and zone of inhibition (if any) were recorded from 3-7 days (depending on the growth of test fungus).

Cell free culture filtrates of bacteria and their nematicidal activity: Bacterial strains were grown in King's B 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 a beaker for use. One ml aqueous suspension of freshly hatched second stage juvenile of *Meloidogyne javanica* (25-40 juveniles) and 1 ml cell free culture filtrate of bacterial strains were transferred in glass cavity blocks and kept at 26±5°C. One ml centrifuged broth with one ml distilled water served as control. There were three replicates of each treatment and juvenile mortality was recorded after 48 hours. The experiment was repeated twice.

Screen house experiment: Six isolates of *P. aeruginosa* viz., PAE-13, PAE-15, PAE-16, PAE-18, PAE-21 and PAE-23 which caused growth inhibition of all the four test root rotting fungi *In vitro* and showed more than 50% nematicidal activity were selected for screen house study. The soil used in this experiment was obtained from experimental field of the Department of Botany. The sandy loam soil (pH 8.05) was naturally infested with 3-7 sclerotia of *M. phaseolina* g^{-1} of soil as determined by wet sieving and dilution plating(Sheikh & Ghaffar 1975), 2-6% colonization of *R. solani* on sorghum seeds used as baits (Wilhelm, 1955) and 3000 cfu g^{-1} of soil of a mixed population of *F. oxysporum* and *F. solani* as determined by soil dilution technique (Nash & Snyder, 1962). The soil was transferred in 12 cm diam., clay pots at 1 Kg per pot. A 25ml cell suspension of five-day-old culture of *P. aeruginosa* isolates viz., PAE-13, (1.3 x 10^8 cfu/ml), PAE-15 (1.5 x 10^8 cfu/ml), PAE-16 (3.2 x 10^8 cfu/ml), PAE-18 (1.4 x 10^8 cfu/ml), PAE-21 (7.1 x 10^8 cfu/ml), PAE-23 (1.6 x 10^8 cfu/ml) and *T. viride* (2.1 x 10^7 cfu/ml) were drench in each pots. Topsin-M served as positive control against root rotting fungi, while carbofuran (0.5 g/ pot) served as positive control against root knot nematode. In an other set pots were also applied mixed application of *Pseudomonas* and *T. viride* for comparison. Seeds of okra [*Abelmoschus esculentus* (L.) Moench.], purchased from local seed store were sown in each pot (6 seeds per pot). After germination (one week) four seedlings were kept in each pots and excess were removed. Each pots were inoculated with *M. javanica* eggs/juveniles at 2000/pot. There were four replicates of each treatments and the pots were randomized on a screen house bench in block design and kept at 50%W.H.C. (Keen & Raczkowiski, 1921) with daily watering.

To determine the efficacy of *P. aeruginosa* and *T. viride* on the root pathogens and plant growth, plants were uprooted after six weeks of nematode inoculation and data on plant height, fresh shoot weight, root length, root weight were recorded. Nematode infection was recorded by counting the numbers of galls per root system. To examined the incidence of root infecting fungi, roots were washed in running tap water, five one cm long root pieces from tap roots, surface disinfected with 1% Ca $(OCl)_2$ were placed onto Potato Dextrose Agar plates supplemented with penicillin (100000 units/ litre) and streptomycin (0.2 g/litre). The dishes were incubated for 5 days and incidence of fungi grown were recoded. The experiment was conducted twice. Data were subjected to analysis of variance (ANOVA) and means were separated using the least significant difference (LSD) according to Gomez & Gomez (1984).

Results

***In vitro* antifungal activity of endophyic *Pseudomonas*:** Out of 14 isolate of *P. aeruginosa* tested, seven viz., PAE-11, PAE-13, PAE-15, PAE-16, PAE-18, PAE-21 and PAE-24 showed growth inhibition of all the four test root infecting fungi viz., *M. phaseolina*, *F. solani*, *F. oxysporum* and *R. solani* by producing the zone of inhibition (Table 1). Other isolates also inhibited the radial growth of atleast two or three test pathogens.

***In vitro* juvenile's mortality of root knot nematode:** Cultural filtrates of *Pseudomonas* isolates showed significant nematicidal effects by killing the second stage juveniles at varying degree. Culture filtrate of PAE-11, PAE-12, PAE-13, PAE-14, PAE-15, PAE-16, PAE-17, PAE-18, PAE-19, PAE-20, PAE-21, PAE-22, PAE-23 and PAE-24 showed 63.5%, 86.9, 68.2, 73.9, 65.5, 72.4, 77.3, 65.8, 58.5, 77.3, 52.8, 88.4, 79.8, 60% juveniles mortality (Table 2).

Screen house experiment: *Pseudomonas aeruginosa* strains of PAE-13, PAE-15, PAE-23 and topsin-m significantly ($p<0.05$) reduced *M. phaseolina*, *F. solani*, *F. oxysporum* and *R. solani* infection on okra roots (Table 3). Application of PAE-18, PAE-23 alone or mixed application of *T. viride* with PAE-15 or PAE-18 caused complete reduction of *M. phasolina* infection on okra roots. Plants treated with *P. aeruginosa* isolates PAE-15, PAE-18 and PAE-21 showed no infection of *R. solani*. Where *P.aeruginosa* isolates PAE-13 and PAE-16 used alone or where PAE-15, PAE-16, PAE-21 and PAE-23 isolates used with *T. viride* showed complete suppression of *F. solani* infection (Table 3). Topsin-m, *P. aerugionsa* isolate PAE-13 and PAE-16 caused complete suppression of *F. oxysporum* (Table 3).

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

P. aeruginosa strains	*M. phaseolina*	*R. solani*	*F. solani*	*F. oxysporum*
	Zone of inhibition (mm)			
PAE-11	19	29	24.3	30
PAE-12	34	0	0	23
PAE-13	34	3	28.6	25
PAE-14	0	27	32	24
PAE-15	9	31	25	22
PAE-16	32	28	22	24
PAE-17	28*	25	28	3
PAE-18	23	25	7	2
PAE-19	38	24	3	33*
PAE-20	18	0	29*	24
PAE-21	31	29	8	29
PAE-22	28	26*	22	18
PAE-23	28	25	28	23
PAE-24	0	3	33	15

0= No inhibition, * = Fungal mycelium lysed

Table 2. *In vitro* nematicidal activity of cell free culture filtrates of *Pseudomonas aeruginosa* (PAE) on *Meloidogyne javanica* after 48 hours.

P. aeruginosa strains	Juveniles mortality %
Control (KB broth)	03.3
PAE-11	63.3
PAE-12	86.6
PAE-13	68.3
PAE-14	73.3
PAE-15	65
PAE-16	71.6
PAE-17	76.6
PAE-18	65
PAE-19	58.3
PAE-20	76.6
PAE-21	51.6
PAE-22	88
PAE-23	80
PAE-24	60

Application of most of the *P. aeruginosa* isolates alone or with *T. viride* showed positive impact on plant growth by improving plant height, fresh shoot weight and root length. Greater plant height was achieved with mixed application of PAE-21 and *T. viride*, followed by PAE-16 used alone (Table 4). Plants treated with PAE-16 or PAE-23 with *T.viride* showed maximum fresh shoot weight (Table 4).

Application of endophytic *P. aeruginosa* alone or with *T. viride* also showed adverse effect on nematode infection on okra roots. Plants treated with *P. aeruginosa* isolates alone or with *T. viride* showed less number of nematode's galls on roots as compared to untreated control (Table 4).

Discussion

Endophytic microbial communities, both bacteria and fungi are known to affect root health. In the present study some strains of endophytic fluorescent *Pseudomonas* isolated from roots of healthy okra plants were identified as *P. aeruginosa* which showed significant activity against root rotting fungi and root knot nematode both *In vitro* and *In vivo.* Several studies have shown that the interaction between plants and some endophytic bacteria was associated with beneficial effects such as plant growth promotion and biocontrol potential against plant pathogens (Hallmann *et al.,* 1995). The root colonizing bacteria that have a beneficial effect on plants are termed as plant growth promoting rhizobacteria have been reported to improve plant growth either through direct stimulation of the plant by producing growth regulators or by suppression of pathogens (Weller *et al.,* 2002; Raaijmakers *et al.,* 2002; Inam-ul-Haq *et al.,* 2012). Of the various rhizospheric bacteria, the bacteria belonging to the fluorescent *Pseudomonas* which colonize roots of a wide range of crop plants are reported to be antagonistic to soil-borne plant pathogens (Siddiqui & Ehteshamul-Haque, 2001). The production of certain antibiotics (Raaijmakers *et al.,* 2002) and siderophores (De Meyer & Hofte, 1997) by *P. aeruginosa* has been regarded as one of the mechanism involved in antagonism. Raajimakers & Weller, (1998) reported role of 2, 4-diacetylphloroglucinol an antifungal metabolite from species of fluorescent *Pseudomonas* in plant root disease suppression.

Table 3. Effect of endophytic *Pseudomonas aeruginosa* (PAE) and *Trichoderma viride* on the infection of *Macrophomina phaseolina, Rhizoctonia solani, Fusarium oxysporum, Fusarium solani* and *Pythium* sp., on okra roots.

Treatments	*M. phaseolina*	*R. solani*	*F. solani*	*F. oxysporum*	*Pythium* sp.
	Infection %				
Control	25	18.7	25	50	18.7
Topsin-M	12.5	6.2	12.5	0	0
Carbofuran	18.7	12.5	12.5	25	6.2
P. aeruginosa (PAE-13)	6.2	6.2	0	0	6.2
P. aeruginosa (PAE-15)	12.5	0	6.2	12.5	0
P. aeruginosa (PAE-16)	25	12.5	0	0	0
P. aeruginosa (PAE-18)	0	0	18.7	18.7	12.5
P. aeruginosa (PAE-21)	18.7	0	12.5	12.5	6.2
P. aeruginosa (PAE-23)	0	6.2	6.2	18.7	0
T. viride (TV)	12.5	12.5	12.5	31.2	6.2
P. aeruginosa (PAE-13) + TV	12.5	0	18.7	25	12.5
P. aeruginosa (PAE-15) + TV	0	0	0	0	0
P. aeruginosa (PAE-16) + TV	18.7	0	0	25	6.2
P. aeruginosa (PAE-18) + TV	0	0	6.2	25	0
P. aeruginosa (PAE-21) + TV	18.7	0	0	6.2	6.2
P. aeruginosa (PAE-23) + TV	25	6.2	0	12.5	0

$LSD_{0.05}$ = Treatments= 9.9[1], Pathogens= 5.9[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 4. Effect of endophytic *Pseudomonas aeruginosa* and *Trichoderma viride* on the growth of okra and infection of *Meloidogyne javanica.*

Treatments	Shoot length (cm)	Fresh shoot weight (g)	Root length (cm)	Fresh root weight (g)	No. of knots/ root system
Control	8.3	0.8	4.7	0.08	14
Topsin-M	9.47	0.9	3.8	0.15	2
Carbofuran	9.12	0.9	6.2	0.21	1
P. aeruginosa (PAE-13)	8.72	0.5	4.2	0.14	6
P. aeruginosa (PAE-15)	11.0	1.4	7.0	0.25	4
P. aeruginosa (PAE-16)	14	1.6	8.2	0.35	3
P. aeruginosa (PAE-18)	11.2	1.3	7.4	0.27	2
P. aeruginosa (PAE-21)	12	1.5	7.2	0.2	0
P. aeruginosa (PAE-23)	11.7	1.4	7.7	0.23	2
T. viride (TV)	11.51	1.3	7.9	0.24	8
P. aeruginosa (PAE-13) + TV	12.5	1.1	7.3	0.17	1
P. aeruginosa (PAE-15) + TV	12.9	1.3	8.4	0.2	0
P. aeruginosa (PAE-16) + TV	12.7	1.1	9.3	0.29	2
P. aeruginosa (PAE-18) + TV	12.6	1.3	6.6	0.22	1
P. aeruginosa (PAE-21) + TV	15	1.2	8.0	0.28	2
P. aeruginosa (PAE-23) + TV	13.3	1.6	9.7	0.33	0
$LSD_{0.05}$	3.4[1]	0.5[1]	2.7[1]	0.18[1]	0.27[1]

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

In this study, application of endophytic *Pseudomonas* alone or with *T. viride,* an endophytic biocontrol agent not only suppressed the infection of root rotting fungi and root knot nematode but also improved plant growth. *Trichoderma* spp., are free living fungi that are common in soil and root ecosystem and for many yeas they are known to produce a wide range of antibiotic substance and they also parasitize other fungi (Denis & Webster 1971; Woo *et al.,* 2006). Recent discoveries show that they are opportunistic, avirulent plant symbionts and induce systemic resistance in plants like rhizobacteria (Harman *et al*., 2004a). In this study, some isolates of *P. aeruginosa* with *T. viride* produced taller plants and better root growth than either used alone. However, their mixed application did not show a clear advantage against root pathogens over their separate use. Endophytes colonize an ecological niche similar to that of phytopathogens, which makes them suitable as biocontrol agents (Berg *et al.*, 2005). The plant growth-promoting endophytes are now being used in the developing areas of forest regeneration and phytoremediation of contaminated soils (Harman *et al.*, 2004b). Biological control with endophytes offers an effective strategy for pest management.

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