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ROLE OF FLUORESCENT *PSEUDOMONAS* ASSOCIATED WITH ROOT NODULES OF SOYBEAN IN SUPPRESSING THE ROOT ROTTING FUNGI AND ROOT KNOT NEMATODE OF SOYBEAN IN SOIL AMENDED WITH SEEDS OF *VERNONIA ANTIHELMENTHICA*

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ABSTRACT

Soilborne plant pathogens, root rotting fungi and root knot nematodes are major yield limiting factors in the production of food, fiber and ornamental crops. In this study, *Pseudomonas aeruginosa* (PGPR104) and *Bradyrhizobium japonicum* (NFB-49) isolated from the root nodule of cultivated soybean plant (*Glycine max* (Merr) and examined for their nematicidal and fungicidal activity. *In vitro* studies of cell free culture filtrates of PGPR-104 and NFB-49 showed nematicidal activity against *Meloidogyne javanica*. The strains PGPR-104 and NFB-49 showed more than 90% mortality. Antifungal activity of PGPR strains were tested against three common and destructive plant pathogenic fungi viz., *Macrophomina phaseolina, Rhizoctonia solani* and *Fusarium solani*. Both the bacterial strains, PGPR-104 and NFB-49 significantly inhibited the growth of pathogenic fungi. The plant protection potential of theses strains were evaluated in screen house on soybean in soil amended with *Vernonia antihelmenthica* seeds and compared with biocontrol isolate of *P. aeruginosa* and *B. japonicum*, fungicides topsin-M and nematicide carbofuran. Biocontrol agents (PGPR, rhizobia) and organic amendment (*V. antihelmenthica* seeds powder) significantly ($p<0.001$) control the infection caused by root rotting fungi *M. phaseolina*, *R. solani* and *F. solani* and root knot nematodes *M. javanica* on soybean. Endo-nodule PGPR, rhizobia and *Vernonia* soil amendment significantly improved soybean growth by producing taller plants and greater fresh shoot weight.

INTRODUCTION

Pathogenic microorganisms affecting plant health are major threat to food production worldwide (Compant *et al.,* 2005). Soilborne plant pathogens, root rotting fungi and root knot nematodes causing root and crown rots, wilt and damping off and root knot disease are major yield-limiting factors in the production of food, fiber and ornamental crops (Ehteshamul-Haque *et al.*, 2007ab). Conventional control measures such as the use of resistant cultivars and synthetic pesticides are unable to control most of the soilborne diseases (Weller *et al.*, 2002). Moreover, there is an increasing awareness that pesticides and fertilizers cause damage to the environment and effect human health (Perkins & Patterson, 1997). As a consequence, there is a trend toward finding ways to minimize the use of pesticides (Maas & Galletta, 1997). The use of biocontrol agents (Elad & Shtienberg, 1996) and alternative treatments (e.g., cultural practices, cover crops, organic amendments) may be an alternative in controlling plant diseases as they are not harmful to environment (Cutler & Hill, 1994).

Soybean (*Glycine max* (L.) Merrill.) is an important nitrogen-fixing leguminous crop cultivated for food and feed. Soybean oil, soymilk and soymeal are some of the important products of soybean. The world soybean production increased by 4.6% annually from 1961 to 2007 and reached average annual production of 217.6 million tons in 2005-07 and it is predicted to increase by 2.2% annually to 371.3 million tons by 2030 (Masuda & Goldsmith, 2009). Losses caused by soybean disease in the United States alone were estimated at $135-140 million in 1980 (Sinclair, 1982) Among the soybean diseases, *Fusarium* spp., *Rhizoctonia solani, Macrophomina phaseolina* and *Pythium* spp., are considered as major soybean seedling pathogens which contribute to stand reduction (Sinclair & Backman, 1989).

Rhizobia are symbiotic soil bacteria characterized by their nodule formation ability in leguminous plants where they fix atmospheric nitrogen (Long, 2001). Legume seeds are treated with appropriate rhizobial strains prior to planting for improving nodulation and biological nitrogen fixation (Brockwell *et al*., 1995). Some of the *Rhizobium* strains are known to reduce disease severity caused by *Pythium ultimum* (Ozkoc & Deliveli, 2001), *Phytophthora clandestine* (Simpfendorfer *et al*., 1999), *Fusarium solani* (Estevez de Jensen *et al*., 2002), *F. oxysporum*, *Rhizoctonia bataticola* and *Pythium* sp., (Nautiyal, 1997) and *Macrophomina phaseolina*, *R. solani* and *Fusarium* spp., on both leguminous and non-leguminous plants under field condition (Ehteshamul-Haque & Ghaffar, 1993, 1995) in soil, naturally infested with these pathogens. Similarly root colonizing bacteria that have a beneficial effect on plants are termed as plant growth promoting rhizobacteria (PGPR) (Kloepper *et al*., 1999) have been reported to improve plant growth either through direct stimulation of the plant by producing growth regulators or by suppression of pathogens (Brown, 1972; Kloepper *et al*., 1999; Weller *et al*., 2002;

Raaijmakers *et al*., 2002). 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 (Ehteshamul-Haque, *et al.*, 2007ab; Siddiqui *et al*., 2000; Siddiqui & Ehteshamul-Haque, 2001).

Several nonchemical methods including organic amendments of soil is an effective method for controlling soilborne pathogens and diseases in various field crops (Chellemi, 2002; Zhou & Everts, 2004). A variety of mechanisms, including chemical, such as antimicrobial compounds during decomposition of organic matter in soil are thought to be caused diseases suppression (Ehteshamul-Haque *et al*., 1995; Mansoor *et al*., 2007; Mazzola, 2004). The present report describes the isolation and identification of fluorescent *Pseudomonas* and rhizobia associated with root nodules of soybean and their biocontrol potential against root rotting fungi and root knot nematodes affecting soybean.

MATERIALS AND METHODS

Plant material: Soybean root nodule was obtained from experimental field of Crop Disease Research Institute, Pakistan Agricultural Research Council located at Karachi University.

Isolation of bacterial strains from root nodules: Root system of the test leguminous plants were washed in running water, a well formed, healthy pinkish nodule on the root was carefully cut out with a portion of the root attach to the nodule. After surface sterilization of nodules for 5 minutes in 0.1% mercuric chloride in water and then with sterile water. After that nodule was then washed in 70% ethyl alcohol for 3 minutes followed by washing with sterile water. The nodule crushed with sterile glass rod in a small aliquot of sterile water and serial dilution of the suspension were then made. Each dilution (100µl) were then poured onto Petri dish containing S-1 medium supplemented with trimethoprim (Gould *et al*., 1985; Basham *et al*., 1993) for the isolation of fluorescent *Pseudomonas*. Bacterial colonies after three days of growth at 28°C fluoresced under UV light at 366nm were purified on Kings B agar medium (King *et al*., 1954). For the isolation of rhizobia, appropriate dilutions were poured (100µl) on the YEMA medium supplemented with 2.5ml of 1% Congo red/litre. After 5 days of incubation, distinct colonies (gummy white-creamy) of bacteria are picked up and transferred to agar slants (Subba Rao, 1977). Bacteria were identified according to Bergy's manual (Krieg & Holt, 1984).

***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 diameter, disc of test fungi like *Macrophomina phaseolina, Rhizoctonia solani* and *Fusarium solani* were inoculated. The dishes were incubated at 28ºC and zone of inhibition were recorded from 3-7days (depends upon the growth of test fungus).

Cell free culture filtrate of bacteria: Bacterial strains *Pseudomonas aeruginosa* were grown on KB Broth at 30ºC for 72 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. Whereas *Bradyrhizobium japonicum* was grown on YEM Broth at 30ºC for 72 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.

***In vitro* juvenile mortality test:** One ml of freshly hatched second stage juvenile suspension (20-25 juveniles) and 1 ml cell free culture filtrate of bacterial strains were transferred in glass cavity blocks and kept at 26 ± 5ºC. There were three replicates of each treatment and juvenile mortality was recorded after 24 and 48 hours.

Juvenile's mortality test of water extract of *Vernonia Antihelmenthica* seeds: Water extract of *Vernonia* seeds was obtained by soaking the seeds in distilled water, homogenized and filtered. The filtrate was used for nematicidal activity against juveniles of *Meloidogyne javanica.* A concentration of 0.01, 0.1, 1.0 and 10 mg/ml were prepared in cavity block. Twenty hand picked juveniles were transferred in each cavity block and juveniles mortality was recorded after 48 hours.

Screen house experiment: The experiment was carried out to examine efficacy of rhiozobia and *Pseudomonas* in controlling root rotting fungi and root knot nematode on soybean in soil amended with a botanical toxicant *Vernonia antihelmenthica* seeds. Dry seeds powder of *Vernonia* was mixed in sandy loam soil, pH 8.0 at 0.5 and 0.1% w/w and transferred into 16 cm diameter clay pots, 1kg in each. 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 determined by soil dilution (Nash & Snyder, 1962). The pots were watered daily to allow for the decomposition of the organic substrate.

After 2 weeks decomposition of botanical toxicant, six seed of soybean were sown per pot. *Bradyrhizobium japonicum* (NFB-49) (1.64 x 10^8 cfu/ml) and *P. aeruginosa* (PGPR-104) (1.08 x 10^8 cfu/ml) were drench in each pots at 25ml per pot. *Pseudomonas aeruginosa* (PGPR-11) that showed significant results in our previous studies were also applied at 1.95 x 10^8 cfu/ml to compare the results. Pots without biocontrol agent or soil amendment served as control. Topsin-M at 200 ppm (100 ml/ pot) served as positive control against root rotting fungi and carbofuran (0.1g/Kg) served as positive control against root knot nematode. Four replicates of experiment were randomized in complete block design and adjusted at 50% W.H.C (Keen & Raczkowski, 1921). After germination four seedlings were kept in each pots and excess were removed. Each pots were then inoculated with 500 eggs/J_2 of root knot nematode.

Efficacy of botanical toxicant or biocontrol agents were determined against soil-borne pathogen of soybean, plants were uprooted after 6 weeks of nematode inoculation. For the incidence of root infecting fungi, one cm long root pieces from tap root, 5 from each plant after surface sterilization with 1% Ca (OCL) $_2$ for 3 minutes were transferred onto PDA plates, containing penicillin (100,000 units/liter) and streptomycin (0.2 g/liter). Plates were incubated for 5 days at 28ºC and root infecting fungi *M. phaseolina, R. solani* and *F. solani* grown were recorded.

For determination of nematode infection, number of knots per root system were counted. Nematode's penetration in roots were also examined, where remaining roots (left after using fungal infection) from each plant were cut into one cm long pieces and mixed thoroughly. One-gram sub-sample after washing in running tap water, was wrapped in muslin cloth and dipped for 3-5 minutes in boiled 0.25% acid fuchsine stain. Roots were left in the stain till to cool then washed under tap water to remove excess stain. Roots were transferred in vials containing 1:1 glycerol and water with few drops of lactic acid. Roots were macerated in an electric blender for 45 seconds and macerate suspended in 100 ml water. Number of J_2, J_3, J_4 and female in 5 samples of 5 ml each were counted with the acid of low power microscope (6X) and number of nematodes/gram root was calculated (Siddiqui *et al.*, 2000). Data on plant growth and numbers of nodules per plant were also recorded.

RESULTS

Isolation of fluorescent *Pseudomonas* and rhizobia from root nodules: One potential strain of *Psudomonas aeruginosa* (PGPR-104) and one strain of *Bradyrhizobium japonicum* (NFB-49) were isolated and identified.

***In vitro* antifungal studies:** Both the *Pseudomonas aeruginosa* and *Bradyrhizobium japonicum* inhibited the radial growth of root infecting fungi *Macrophomina phaseolina, Fusarium solani* and *Rhizoctonia solani*. The PGPR-104 produced 15mm zone of inhibition against *Macrophomina phaseolina*, while NFB-49 produced 5mm inhibition zone against *M. phaseolina*. PGPR-104 and NFB-49 also inhibited the growth of *R. solani* and *F. solani* and lysed the fungal hyphae, although did not showed zone of inhibition (Table 1).

Table 1. *In vitro* growth inhibition of *Macrophomina phaseolina, Fusarium solani* and *Rhizoctonia solani* by *Pseudomonas aeruginosa* (PGPR-104) and *Bradyrhizobium japonicum* (NFB-49).

S.No.	Treatments	Zone of inhibition (mm) for different Fungi		
		M. phaseolina	*F. solani*	*R. solani*
1.	*B. japonicum* (NFB-49)	5mm	*	*
2.	*P. aeruginosa* (PGPR-104)	15mm	*	*

* = No zone was formed but fungal hyphae lysed

Juvenile's mortality test of bacterial strains: Culture filtrate of the *Pseudomonas* and rhizobial strains showed nematicidal effect by killing the second stage juveniles. PGPR-104 and NFB-49 showed 98% and 96% juveniles mortality respectively (Table 2).

Table 2. Effect of cell free culture filtrate of *Pseudomonas aeruginosa* (PGPR-104) and *Bradyrhizobium japonicum* (NFB-49) on juvenile mortality of *Meloidogyne incognita* (RKN) after 48 hour.

S.No.	Treatments	Juvenile mortality %	
		24 hour	48 hour
1.	Control	04	08
2.	*B. japonicum* (NFB-49)	88	96
3.	*P. aeruginosa* (PGPR-104)	94	98

***In vitro* juvenile's mortality test of water extract of *Vernonia antihelmenthica* seeds:** Water extract of *Vernonia* seed showed nematicidal effect by killing the second stage juveniles and showed 28, 84, 72 and 54% juveniles mortality at 10, 1.0, 0.1 and 0.01 mg/ml respectively (Table 3).

Table 3. Effect of different concentrations of water extract of *Vernonia* seeds on juvenile mortality of *Meloidogyne javanica* (RKN) after 48 hour.

S.No.	Treatment	Juvenile mortality %	
		24 hour	48 hour
1.	Control	08	12
2.	Water extract of *Vernonia* at 10 mg/ml	78	98
3.	Water extract of *Vernonia* at 1 mg/ml	40	84
4.	Water extract of *Vernonia* at 0.1 mg/ml	24	72
5.	Water extract of *Vernonia* at 0.1 mg/ml	12	54

Screen house experiment: Application of *Pseudomonas aeruginosa* (PGPR-104) significantly reduced infection of *R. solani, F. solani* and *F. oxysporum*, while *Bradyrhizobium japonicum* (NFB-49) suppressed *R. solani* and *F. oxysporum* (Table 4). Whereas PGPR-11 significantly reduced infection of *M. phaseolina, R. solani, F. solani* and *F. oxysporum. Vernonia* soil amendment alone was found effective against *R. solani*, *F. solani* and *F.oxysporum*. All the test bacterial strains were found effective against *M. phaseolina* when used with *V*ernonia (Table 4). All the bacterial strains and *Vernonia* caused a suppressive effect on nematode infection by reducing the gall formation on roots or nematode's penetration in roots (Table 5). Mixed application of PGPR and *Vernonia* showed greater prevention in nematode's penetration in roots.

Bacterial strains and *Vernonia* application caused positive impact on plant growth by producing taller plants and greater fresh shoot weight (Table 6).

Table 4. Effect of *Pseudomonas aeruginosa, Bradyrhizobium japonicum* alone or with *Vernonia,* botanical toxicants on root infecting fungi of soybean in soil artificially infested with nematodes.

S.No.	Treatments[1]	Infection %			
		M. phaseolina[2]	*R. solani*[2]	*F. solani*[2]	*F. oxysporum*[2]
1.	Control	62.5	56.2	50	68.7
2.	Topsin-M	50	18.7	25	0
3.	Carbofuran	56.2	25	56.2	0
4.	*B. japonicum* (NFB-49)	62.5	31.2	68.7	12.5
5.	*P. aeruginosa* (PGPR-11)	25	31.2	25	43.7
6.	*P. aeruginosa* (PGPR-104)	56.2	0	37.5	0
7.	*Vernonia* (at 0.5% w/w)	56.2	0	31.2	25
8.	NFB-49 + *Vernonia* (at 0.5%)	37.5	12.5	75	12.5
9.	PGPR-11+ *Vernonia* (at 0.5%)	43.7	12.5	62.5	0
10.	PGPR-104+ *Vernonia* (at 0.5%)	31.2	37.5	18.7	62.5
11.	*Vernonia* (at 0.1% w/w)	56.2	0	31.2	0
12.	NFB-49 + *Vernonia* (at 0.1%)	25	18.7	18.7	18.7
13.	PGPR-11+ *Vernonia* (at 0.1%)	50	62.5	31.2	0
14.	PGPR-104 + *Vernonia* (at 0.1%)	0	0	75	0

$LSD_{0.05}$ = Treatments[1] = 23.18, Pathogen[2] = 12.39

[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 *Pseudomonas aeruginosa, Bradyrhizobium japonicum* alone or with *Vernonia,* a botanical toxicants on root knot disease and root nodule of soybean.

S.No.	Treatments[1]	Number of knots per root system	Number of J_2 female per plant	Number of nodules per plant
1.	Control	10	60	0.18
2.	Topsin-M	2	20	0.12
3.	Carbofuran	3	15	0.05
4.	*B. japonicum* (NFB-49)	4	30	0.31
5.	*P. aeruginosa* (PGPR-11)	3	60	0.12
6.	*P. aeruginosa* (PGPR-104)	3	40	0.12
7.	*Vernonia* (at 0.5% w/w)	2	2	0.37
8.	NFB-49 + *Vernonia* (at 0.5% w/w)	3	20	0
9.	PGPR-11+ *Vernonia* (at 0.5% w/w)	1	20	0.86
10.	PGPR-104 + *Vernonia* (at 0.5% w/w/)	2	35	0.56
11.	*Vernonia* (at 0.1% w/w/)	1	2	0.37
12.	NFB-49 + *Vernonia* (at 0.1% w/w)	1	20	0
13.	PGPR-11 + *Vernonia* (at 0.1% w/w)	2	3	0
14.	PGPR-104 + *Vernonia* (at 0.1% w/w)	1	2	0
	$LSD_{0.05}$	**1**	**33**	**ns**

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

ns = Non-significant

Table 6. Effect of *Pseudomonas aeruginosa, Bradyrhizobium japonicum* alone or with *Vernonia,* a botanical toxicants on the growth of soybean.

S.No.	Treatments[1]	Number of fruits per plant	Plant length (cm)		Fresh plant weight (g)	
			Shoot	Root	Shoot	Root
1.	Control	3	27	25	4	2
2.	Topsin-M	2	25	22	3	1
3.	Carbofuran	3	34	27	3.5	2
4.	*B. japonicum* (NFB-49)	4	36	35	4	3
5.	*P. aeruginosa* (PGPR-11)	3	40	37	4	2
6.	*P. aeruginosa* (PGPR-104)	3	44	32	4	2
7.	*Vernonia* (at 0.5% w/w)	4	46	39	4	1
8.	(NFB-49 + *Vernonia* (at 0.5% w/w)	3	41	34	5	1.5
9.	PGPR-11 + *Vernonia* (at 0.5% w/w)	3	40	31	3.5	3
10.	PGPR-104 + *Vernonia* (at 0.5% w/w)	3	30	35	3	4
11.	*Vernonia* (at 0.1% w/w)	4	44	39	4	2
12.	NFB-49 + *Vernonia* (at 0.1% w/w)	4	37	35	4.5	2
13.	PGPR-11+ *Vernonia* (at 0.1% w/w)	4	43	39	4.5	2
14.	PGPR-104 + *Vernonia* (at 0.1% w/w)	4	38	42	4	2
	$LSD_{0.05}$	**1**	**8**	**11**	**1.9**	**1.6**

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

DISCUSSION

In the present study *Pseudomonas aeruginosa* (PGPR-104), and *Bradyrhizobium japonisum* (NFB-49) isolated from the inside of the root nodule of soybean showed antifungal activity against common plant pathogenic fungi like *Macrophomina phaseolina, Rhizoctonia solani* and *Fusarium solani in vitro.* Research on the mechanisms by which PGPR mediate their protective activity in plants and enhance nodule formation with production of plant hormones, the co-inoculation benefits has been reported (Chebotar *et al*., 2001). Legume-rhizobia symbioses actively fix nitrogen and are critical to agricultural crop production (Vance, 1997; Pepper, 2000). Enhancement of legume nitrogen fixation by co-inoculation of rhizobia with some plant growth promoting bacteria (PGPB) is a way to improve nitrogen availability in sustainable agriculture production systems. Most of the PGPB strains tested by co-inoculation with *Rhizobium* or *Bradyrhizobium* species are general rhizobacteria (Bullied *et al*., 2002; Subba Rao, 1979; Srinivasan *et al*., 1996; Zhang *et al*., 1996). In the present study, endophytic *Pseudomonas aeruginosa* isolated from root nodule of soybean showed significant antifugal and nematicidal activity both *In vitro* and *In vivo*. There are reports that fluorescent *Pseudomonas* associated with plant roots improve plant growth both indirectly and directly. Production of toxic metabolites, antibiotics, HCN or siderophores have adverse effect on plant pathogens and indirectly improve plant growth. Whereas production of growth regulators and enhanced uptake of nutrients directly caused a beneficial effect on plant growth (Kloepper, 1993; Glick, 1995). Promotion of nodule formation and nitrogen fixation in legumes by rhizobacteria has been reported due to the production of flavonides like compounds both by the bacteria and also by the host plant due to bacterial stimulation (Parmar & Dadarwal, 1999). In this study, endo-nodule *P. aeruginosa* significantly improved plant growth by producing taller plants and better root growth. Fluorescent *Pseudomonas* are also known to produce indole-3-acetic acid which stimulate root growth and consequently increased nutrient uptake (Barbieri & Galli, 1993; Srinivasan *et al*., 1996).

In the present studies *B. japonicum* significantly suppressed root-rotting fungi and root knot nematodes and improved the biomass of the plants, which is in agreement with our previous studies (Ehteshamul-Haque & Ghaffar, 1993; 1995ab, Ehteshamul-Haque *et al.*, 2007). In this study soil amendment with a botanical toxicant *Vernonia antihelmenthica* seeds alone or with PGPR or rhizobia showed adverse affect on root rotting fungi and root knot nematode affecting soybean roots. There are reports that botanical toxicants applied in soil produced many nematicidal and larvicidal compounds (Alam *et al*., 1978, 1979) and root dipping with these chemicals significantly inhibited gall formation on tomato and eggplant by root knot nematode (Siddiqui, 1986).

Soils of many potential soybean fields in many countries are characterized by low levels of biological nitrogen fixation (BNF) activities and often cannot support high soybean yields without addition of inorganic N fertilizers or external application of soybean rhizobia. Moreover, soybean production is not economically possible without the effective control measures against parasitic nematodes and soilborne diseases. The selection of candidate to be used as biocontrol agent against soil-borne plant pathogens having characteristics qualities like nitrogen fixation and plant growth promoting potential holds tremendous potential to overcome such a huge losses in economical term and may increase yield substantially as well.

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