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MANAGEMENT OF ROOT ROTTING FUNGI AND ROOT KNOT NEMATODE WITH ENDOPHYTIC FLUORESCENT *PSEUDOMONAS* ASSOCIATED WITH *SALVADORA* SPECIES

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Abstract

Root diseases caused by soilborne plant pathogens are responsible for the losses of billions of dollars in agricultural crops annually. The biological control of soilborne pathogens with microbial antagonists is gaining popularity in the crop protection system, due to the adverse effects of chemicals. In this study 40 isolates of fluorescent *Pseudomonas* were isolated from roots, shoots and leaves of *Salvadora persica* L. and *S. oleoides* Decne. Most of them showed strong suppressive effect on root rotting fungi *Macrophomina phaseolina, Rhizoctonia solani, Fusarium solani* and *F. oxysporum In vitro.* Significant nematicidal activity against root knot nematode (*Meloidogyne javanica*) was also observed by the cell-free culture filtrates of these isolates. Identification of six potential isolates was confirmed by using molecular biology tools. Out of which 3 were identified as *Pseudomonas aeruginosa,* one each as *P. putida* and *P. monteilii*, while one appeared as uncultured *Pseudomonas.* Experiments conducted on sunflower both in clay pots and field plots, test isolates of endophytic *Pseudomonas* were found effective in suppressing the root rot disease with improved plant growth as compared to untreated control. Efficacy of some isolates was found comparable with commercial fungicide carbendazim. Some isolates were also found effective in reducing the infestation of root knot nematode under field condition with improved flower weight.

Key words: Endophytic, Fluorescent *Pseudomonas, Salvadora,* Root rotting fungi, Root knot nematode, Sunflower.

Introduction

Crop yield losses caused by plant diseases can be reduced by the application of agrochemicals, but their affect on the environment is hazardous. The utilization of biological materials is an alternative and safe way to protect plants from phytopathogens (Pandya & Shelat, 2017; Urooj *et al.,* 2018). The control of plant pathogens by the application of plant associated bacteria have been demonstrated repeatedly (Afzal *et al.,* 2013; Costa *et al.,* 2013; Habiba *et al.,* 2016; 2017; Noreen *et al*., 2015). Besides, direct suppression of plant pathogens some bacteria also induced systemic resistance in plants against them (De Meyer & Hofte, 1997; De Meyer *et al.,* 1999; Rahman *et al*., 2016).

Bacteria isolated from surface sterilized tissues of plant have no negative impact on plant growth are called as endophytic bacteria (Schulz & Boyle, 2006). Nowadays they are gaining scientific and commercial interest because of their positive effect on plant growth, reduction of diseases and induction of systemic resistance against biotic and abiotic stresses (Afzal *et al.,* 2013; Boddey *et al*., 2003; Khan & Lee, 2013; Rahman *et al.,* 2016; 2017; Ryan *et al*., 2008). Among the bacterial antagonists, fluorescent *Pseudomonas* are gaining attention as biocontrol agents like rhizo bacteria (Afzal *et al.,* 2013; Habiba *et al*., 2016; 2017; Tariq *et al*., 2009; Shafique *et al*., 2015). Besides siderophore production, antifungal metabolites and siderophores produced by the plant growth promoting bacteria and induction of systemic resistance in plants against pathogens are considered as mechanisms involved in biocontrol of plant diseases (DeMeyer & Hofte 1997; DeMeyer *et al*., 1999; Ramamoorthy *et al*., 2001; Shafique *et al*., 2015; Siddiqui *et al.,* 2000; 2001).

In Pakistan, *Salvadora* plants, are known as miswak tree and its roots are used as a tooth brush (Chelli-Chentouf *et al*., 2012). They are able to tolerate a wide range of soil pH, salinity, water logging and drought (Korejo *et al*., 2010; 2014). It was hypothesized that plants from unique environments may harbor unique endophytes with biocontrol potential against a wide range of plant pathogens (Ehteshamul-Haque *et al*., 2013; Korejo *et al.,* 2014). In our previous study we have reported antibacterial and antifungal potential of cell free culture filtrates of endophytic fluorescent *Pseudomonas* associated with *Salvadora* spp. (Korejo *et al.,* 2017). In the present study we are reporting the suppressive effect of endophytic fluorescent *Pseudomonas* isolated from *Slvadora* species on root rot and root knot pathogens affecting sunflower.

Materials and Methods

Collection of plant samples for the isolation of fluorescent *Pseudomonas:* Plant samples (root, shoot and leaves) from healthy *Salvadora persica* L. and *S. oleoides* Decne were collected from Karachi University campus, Malir from Karachi division and Maklee from Thatta district. Isolation of endophytic *Pseudomonas* was made within 24 hrs.

Isolation of fluorescent *Pseudomonas* from plant samples, genomic extraction and PCR amplifications: Endophytic fluorescent *Pseudomonas* were isolated from surface sterilized roots, shoot and leaves of *Salvadora* spp., as described earlier (Afzal *et al.,* 2013; Ji *et al.,* 2014). Briefly plant parts were dipped in bleach (1%) for 3 min. and washed with sterile water, then with 70% alcohol, then chopped into blender with 100 mL of water and dilution was made. One hundred µL from 1: 10^{-4} dilution was transferred onto S-1 medium plates (Gould *et al*., 1985; Bashan *et al*., 1993). Fluorescent colonies of bacteria grown after 3 days at 28ºC were purified (King *et al*., 1954). Bacteria were initially identified on the basis of biochemical tests (Garrity *et al*., 2005). PCR amplification, restriction pattern and sequence analysis of molecular marker genes including ribosomal "16S rDNA" and the sigma 70 factor subunit of DNA polymerase "*rpoD"* were used to confirm the identification of selected isolates (Anzai *et al.,* 2000; Spilker *et al.,* 2004; Mulet *et al*., 2009). Genomic extraction and PCR amplification has already been reported (Noreen *et al.,* 2015).

The PCR products were purified by a PCR clean-up kit (QIAprep® Qiagen, Germany) and either subjected to restriction pattern analysis and/or sequenced using ABI Prism 377 in the DNA Sequencing facility at the Centralized Science Laboratory (CSL) of the University of Karachi. Sequence searches (nBLAST), multiple sequence alignment (CLUSTAL W), and distance matrix analysis were performed using an online NCBI server (www.ncbi.nlm.nih.gov).

Restriction fragment length polymorphism (RFLP) and BOX PCR analysis: RFLP analysis was used to establish preliminary heterogeneity between the *Pseudomonas* isolates. The restriction enzyme digest was prepared in a 20µL total reaction volume using the restriction enzyme *Alu*I (Promega, USA) utilizing the virtual cutting site (Restriction Mapper V3, available online) in the 16S rDNA and rpoD genes. The components were mixed gently by pipetting before and after adding the enzyme. The reaction tubes were then incubated in a PCR machine (Master cycler *ProS*, Eppendorf Germany) at an optimum temperature of 37°C for 4 h as suggested by the manufacturer. The reaction mix was added with 6x loading dye (Fermentas, USA) and subjected to 2% agarose gel and visualized on a UV trans-illuminator after staining with ethidium bromide. BOX PCR analysis was also performed to establish intra-strain variations using the BOX-A1R 5'-CTACGGCAAGGCGACGCTGACG-3' primer ideally as described by Marques *et al.,* (2008).

Nematicidal activity: Endophytic fluorescent *Pseudomonas* were grown in KB broth for 48 hrs. and cell free culture filtrate was obtained as described earlier (Afzal *et al.,* 2013; Siddiqui *et al.,* 2001). Nematicidal effect of cell free culture filtrate was determined by placing 1 mL aqueous suspension of freshly hatched juveniles of *Meloidogyne javanica* (2nd stage 20 juveniles) along with one mL culture filtrate in glass cavity blocks. Experiment was conducted at room temperature (25± 5°C) with three replicates and juveniles mortality was recorded after 24 and 48 hrs. The experiment was repeated twice.

Antifungal activity: Antifungal activity of endophytic fluorescent *Pseudomonas* was determined against root rotting fungi (*Macrophomina phaseolina* (Tassi) Goid, *Rhizoctonia solani* Kuhn, *Fusarium solani* (Mart.) Appel & Wollenw., and *F. oxysporum* Schlecht. emend. Snyder & Hansen) using dual culture plate assay on Czapek's Dox agar (Afzal *et al.,* 2013; Ji *et al.,* 2014). The experiment was repeated twice with four replicates.

***In vivo* experiments:** The biocontrol potential of some isolates of endophytic fluorescent *Pseudomonas* against soilborne plant pathogens was evaluated in a screen-house and also under field conditions in 2013 and repeated in 2014 using sunflower (*Helianthus annuus*) as a test plant.

Screen-house experiments: The experiment was conducted in clay pots, where 1Kg naturally infested soil was transferred in each pot and seeds of sunflower were sown at 6 seed per pot. Infestation of root rotting fungi in soil (3-7 sclerotia g^{-1} of soil of *M. phaseolina*, 3-10% colonization of *R. solani* on sorghum seeds and mixed population of *F. oxysporum* and *F. solani* 3000 cfu g^{-1} of soil) was found, as determined by the methods described by the Sheikh & Ghaffar (1975), Wilhelm (1955) and Nash & Snyder (1962) respectively. Cell suspension (from 5 d old cultures) of fluorescent *Pseudomonas* EFPS-8, EFPS-19, EFPS-22, EFPS-20 and EFPS-36 (10^8cfu/mL each) was applied in each pot soil and 6 seeds of sunflower were sown. Four seedlings were kept in each pot after germination, while 25 mL of carbendazim (200 ppm) was applied in positive control, whereas plants not received any treatment were kept as control. Pots were kept in randomized block design with four replicates. Plant growth promoting potential of fluorescent *Pseudomonas* and suppressive effect on root pathogens were recorded after six weeks as described by Habiba *et al.,* (2016).

The experiment was repeated under similar condition in 2014 to confirm the results.

Field plot experiments: The experiment was conducted in 2x2 m plots with four replicates in a randomized block design. The field was found to be infested with *M. phaseolina* at 5-16 sclerotia/g of soil. It was infested by *R. solani* (5-12 % colonization on sorghum seeds) and a mixed population of *F. oxysporum* and *F. solani* (2500cfu/gm of soil). A cell suspension of each of five isolates of fluorescent *Pseudomonas* EFPS-8, EFPS-19, EFPS-22, EFPS-20 and EFPS-36 ((10^8cfu/mL each) was applied as a drench in planting rows at 100 mL/m and sunflower seeds were sown (at 30 seeds per row). Each row, after germination of seeds was inoculated with eggs/juveniles (at 2000/2 m) of row of *Meloidogyne javanica*. Carbofuran at 1g/m and carbendazim (200 ppm) at 200 mL/m were kept as standard against nematode and fungal infection. Observations was recorded after 40 and 75 days of growth, 4 plants from each replicate were uprooted and data on plant growth and fungal infection was taken. Number of galls per root system were counted for the estimation of nematode disease.

The experiment was repeated in the same field in 2014 in similar condition to confirm the results.

Data analysis

Three way ANOVA was used to analyze the data on fungal infection and means were separated using least significant difference (LSD) at (p=0.05). Whereas for other parameters one way ANOVA was used (Gomez & Gomez, 1984).

Results

Isolation and identification of endophytic fluorescent *Pseudomonas:* From roots, shoots and stems of *S.persica* and *S. oleoidesa* forty isolates of endophytic fluorescent *Pseudomonas* were isolated and identified (Table 1).

Identification of selected *Pseudomonas* using molecular biology tools: Selected *Pseudomonas* isolates F1 to F6 were subjected to PCR based amplification of the 16S rDNA gene indicated that all isolates belonged to the same genus, the *Pseudomonas.* Fig. 1a). The genus specific primers of 16S rDNA not only provided identical PCR products but also their restriction maps (with the exception of F6) whereas the species specific *rpoD* gene provided an excellent differential fragmentation pattern within the *Pseudomonas* isolates (Fig. 1b). These results were further confirmed by BOX PCR analysis showing the intra-strain variation (Fig. 1c). In order to complement RFLP and BOX results, standard 16S rDNA gene sequencing was performed followed by sequence searches by nBLAST, multiple alignment by CLUSTAL W (Anzai *et al*., 2000), and distance matrix analysis was performed using an online NCBI server. Based on extensive bioinformatics analysis, *Pseudomonas* isolates F2, F3 and F5 were identified as *Pseudomonas aeruginosa* whereas F1 was identified as *Pseudomonas monteilii* and F6 as *Pseudomonas putida* (Fig. 1d). Isolate F5 gave the highest sequence similarity with uncultured *Pseudomonas* species.

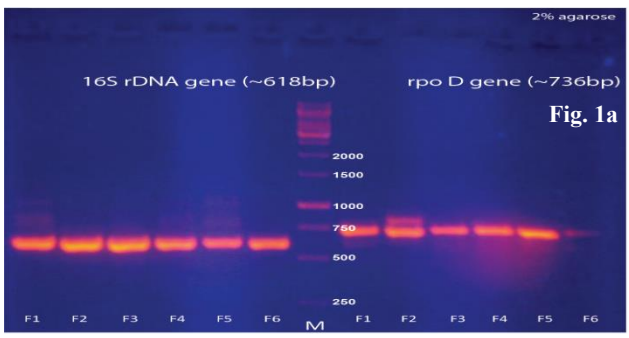
Table 1. Effect of cell free culture filtrate of endophytic fluorescent *Pseudomonas* (EFPS) isolated from different parts of *Salvadora* species on juvenile mortality of *Meloidogyne javanica,* the root knot nematode *In vitro.*

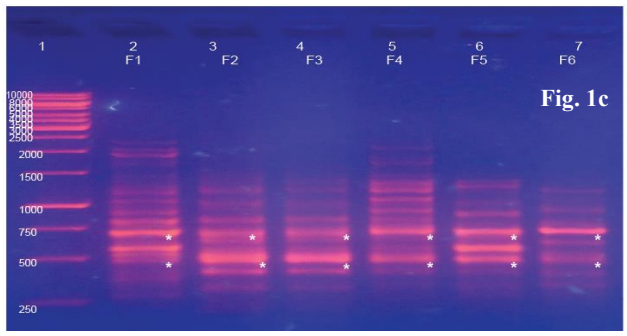
Culture #	Plant source	Name of bacteria	After 24 hours	After 48 hours	Locality
			Juvenile mortality %		
Control	KB broth		21.6	26.6	KU
EFPS-1	*S. oleoides*; stem	*Pseudomonas* sp.	41.6	91.6	”
EFPS-2	*S. oleoides*; leaf	*Pseudomonas* sp.	81.6	91.6	”
EFPS-3	*S. oleoides*; root	*Pseudomonas* sp.	43.3	65	”
EFPS-4	*S. oleoides*; stem	*Pseudomonas* sp.	81.6	91.6	”
EFPS-5	*S. oleoides*; leaf	*Pseudomonas* asp.	63.3	90	”
EFPS-6	*S. oleoides*; root	*Pseudomonas* sp.	31.6	78.3	”
EFPS-7	*S. oleoides*; stem	*Pseudomonas* sp.	81.6	88.3	”
EFPS-8	*S. oleoides*; leaf	*Pseudomonas aeruginosa*[2]	81.6	88.3	”
EFPS-9	*S. oleoides*; root	*Pseudomonas* sp.	78.3	88.3	”
EFPS-10	*S. oleoides*; stem	*Pseudomonas* sp.	71.6	78.3	Malir
EFPS-11	*S. oleoides*; leaf	*Pseudomonas* sp.	68.3	88.3	”
EFPS-12	*S. oleoides*; root	*Pseudomonas* sp.	40	81.6	”
EFPS-13	*S. oleoides*; stem	*Pseudomonas* sp.	73.3	88.3	”
EFPS-14	*S. oleoides*; leaf	*Pseudomonas* sp.	53.3	86.6	”
EFPS-15	*S. oleoides*; root	*Pseudomonas* sp.	76.6	81.6	”
EFPS-16	*S. oleoides*; stem	*Pseudomonas* sp.	60	68.3	Makl
EFPS-17	*S. oleoides*; leaf	*Pseudomonas* sp.	71.6	88.3	”
EFPS-18	*S. oleoides*; root	*Pseudomonas* sp.	35	70	”
EFPS-19	*S. persica*; stem	*Pseudomonas monteilii*[2]	71.6	88.3	KU
EFPS-20	*S. persica*; leaf	*Pseudomonas aeruginosa*[2]	75	88.3	”
EFPS-21	*S. persica*; root	*Pseudomonas aeruginosa*[2]	73.3	96.6	”
EFPS-22	*S. persica*; stem	*Pseudomonas* sp.[2]	56.6	93.3	”
EFPS-23	*S. persica*; root	*Pseudomonas* sp.	64	66	“
EFPS-24	*S. persica*; leaf	*Pseudomonas* sp.	45	94	“
EFPS-25	*S. persica*; stem	*Pseudomonas* sp.	46	69	“
EFPS-26	*S. persica*; root	*Pseudomonas* sp.	54	98	“
EFPS-27	*S. persica*; leaf	*Pseudomonas* sp.	61.6	68.3	”
EFPS-28	*S. persica*; root	*Pseudomonas* sp.	45	69.3	”
EFPS-29	*S. persica*; leaf	*Pseudomonas* sp.	53.3	96.6	”
EFPS-30	*S. persica*; stem	*Pseudomonas* sp.	46.6	78.3	”
EFPS-31	*S. persica*; root	*Pseudomonas* sp.	53.3	83.3	”
EFPS-32	*S. persica*; leaf	*Pseudomonas* sp.	41.6	71.6	”
EFPS-33	*S. persica*; stem	*Pseudomonas* sp.	54	74	”
EFPS-34	*S. persica*; leaf	*Pseudomonas* sp.	53.3	93.3	“
EFPS-35	*S. persica*; root	*Pseudomonas* sp.	41.6	71.6	”
EFPS-36	*S. persica*; leaf	*Pseudomonas putida*[2]	53.3	73.3	”
EFPS-37	*S. persica*; stem	*Pseudomonas* sp.	41.6	71.6	Malir
EFPS-38	*S. persica*; stem	*Pseudomonas* sp.	68.3	78.3	KU
EFPS-39	*S. persica*; root	*Pseudomonas* sp.	63.3	83.3	”
EFPS-40	*S. persica*; leaf	*Pseudomonas* sp.	41.6	76.6	”
$LSD_{0.05}$			4.3[1]	4.6[1]	

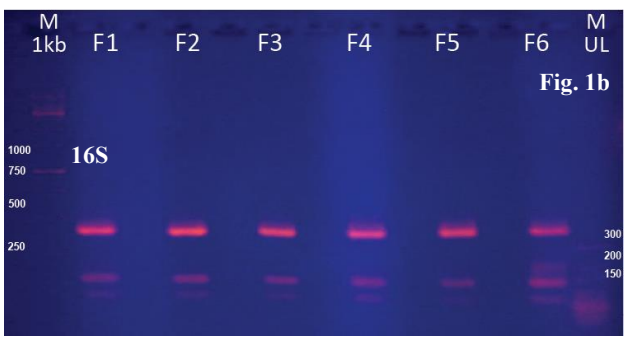
[1]Mean values in columns showing differences greater than LSD values are significantly different at p=0.05

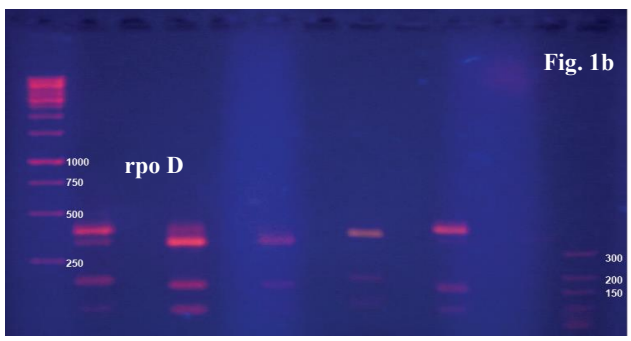
[2]*Pseudomonas* species were also identified up to species level by using Molecular Biology tools

KU = Karachi University, Makl = Maklee,Thatta, Malir= Malir, Karachi









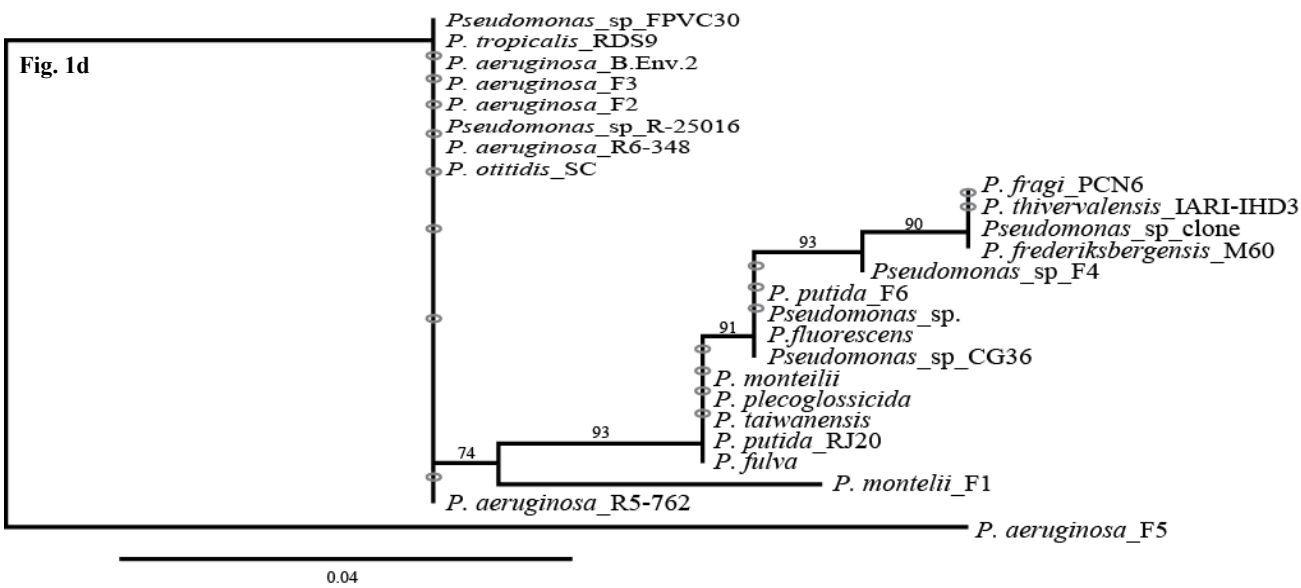


Fig. 1. Molecular basis of PGPR isolates identification. (a) PCR amplification of *16S rDNA* and *rpoD* gene. (b) RFLP analysis of 16S rDNA (top) *rpoD* (bottom) genes obtained by the restriction enzyme *Alu-I*. (c) BOX PCR analysis, where an asterisk indicates the genus related product (~500 and 740bp respectively). (d) Phylogenetictree constructed by multiple sequence alignment analysis. All reaction products were analyzed on 2% agarose gels and visualized by staining in ethidium bromide. F1 to F6 are PGPR isolates. M = 1kb and ultra-low molecular ladders (see "Materials and methods" for details).

Nematicidal activity: All the forty culture filtrates of *Pseudomonas* caused more than 50% juvenile mortality within 48 h (Table 1). Twenty-five isolates were able to kill more than 50% of the nematodes within 24 h.

Antifungal activity of endophytic fluorescent *Pseudomonas:* Out of forty isolates of fluorescent *Pseudomonas* tested all showed growth inhibition of all of the four test root infecting fungi *M. phaseolina*, *F. solani*, *F. oxysporum* and *R. solani* by producing a zone of inhibition (Table 2). Some isolates also caused lysis of fungal hyphae.

Screen-house experiment: In the experiments conducted in 2013 and repeated in 2014, *P. aeruginosa* (EFPS-20) and *P.aeruginosa* (EFPS-8) and *Pseudomonas* sp.(EFPS-22) were found effective against *F. oxysporum*, while *P.aeruginosa* (EFPS-8), *P.aeruginosa* (EFPS-20) and *P.monteilii* (EFPS,19) were found effective against *M. phaseolina* and *R. solani* in both years as compared to untreated control. Whereas, *P. aeruginosa* (EFPS-8) and *P. putida* (EFPS-36) were effective in both experiments against *R. solani* only (Table 3). A positive effect on sunflower growth was also observed in *P. aeruginosa* (EFPS-8, EFPS-20), *P. putida* (EFPS-36), and *P. monteilii* (EFPS-19) treatments. Plants were found significantly (p<0.05) taller with better fresh shoot weight compared to control plants in both experiments (Table 4).

Field experiment: After 40 days, a significant suppression of *M. phaseolina* and *R. solani* infection was observed in the *P. aeruginosa* (EFPS-8) treatment in both years, while *P. monteilii* (EFPS-19) and *Pseudomonas* sp. (EFPS-22) caused significant (p<0.0%) suppression of *F. solani* in both experiments, while against *M. phaseloina* and *R. solani* they showed inconsistent results (Table 5). *Pseudomonas* sp. (EFPS-22) was effective against both *F. oxysporum* and *F. solani*. *Pseudomonas* treated plants were found taller than untreated plants, however *P. aeruginosa* (EFPS-20) treated plants were found statistically taller (p=0.05) in both years (Table 6).

After 75 days no or less infection by *F. oxysporum*, *F. solani* and *R. solani* were found in most of the treatments. However, infection by *M. phaseolina* was found increased in most of the treatments as compared to observations recorded at 40 days. *Pseudomonas aeruginosa* (EFPS-20 & EFPS-8) and *P. putida* (EFPS-36) were able to significantly suppress *M. phaseolina* in both years (Table 7). *Pseudomonas* sp. (EFPS-22) treated plants showed no infection by *F. solani*, *F. oxysporum* and *R. solani* in both experiments. A positive effect of fluorescent *Pseudomonas* on plant growth was more obvious after 75 days where *P. aeruginosa* (EFPS-8 & EFPS-20) and *Pseudomonas* sp. (EFPS-22) treated plants were found taller than the control plants in both experiments. Maximum fresh shoot weight was recorded in the *P. aeruginosa* (EFPS-20) treatment (Table 8). Nematode's infection was also found significantly reduced in both years by the application of *Pseudomonas*. The efficacy of *Pseudomonas* was comparable with that of the commercial nematicide carbofuran (Table 9). The weight of flowers was significantly greater in bacterial treated plants than in control plants in both years. However, greatest flower weight per plant was observed in carbendazim treated plants in both years (Table 9).

Table. 2. *In vitro* growth inhibition of *Macrophomina phaseolina*, *Fusarium solani, F. oxysporum* and *Rhizoctonia. solani* by different isolates of endophytic fluorescent *Pseudomonas* in a dual culture plate assay.

Culture No.	Name of bacteria	Zone of inhibition (mm)			
		F. oxysporum	*F. solani*	*M. phaseolina*	*R. solani*
EFPS-01	*Pseudomonas* sp.	26*	23	27*	36
EFPS-02	*Pseudomonas* sp.	33*	24	24*	33
EFPS-03	*Pseudomonas* sp.	28	21*	19*	28*
EFPS-04	*Pseudomonas* sp.	23*	21	26	23
EFPS-05	*Pseudomonas* sp.	22*	20*	20*	25*
EFPS-06	*Pseudomonas* sp.	19	26	26*	23*
EFPS-07	*Pseudomonas* sp.	21	21	26	26
EFPS-08	*P. aeruginosa*	23	29	31*	26
EFPS-09	*Pseudomonas* sp.	20*	25*	24	28*
EFPS-10	*Pseudomonas* sp.	23*	24	25	29
EFPS-11	*Pseudomonas* sp.	25*	24	25	39
EFPS-12	*Pseudomonas* sp.	25	22	28*	26
EFPS-13	*Pseudomonas* sp.	19	25*	24*	29
EFPS-14	*Pseudomonas* sp.	24*	21	28	23
EFPS-15	*Pseudomonas* sp.	24	26	22	26
EFPS-16	*Pseudomonas* sp.	26	26	28	37
EFPS-17	*Pseudomonas* sp.	22	21	22	36
EFPS-18	*Pseudomonas* sp.	21	26	38	24
EFPS-19	*P. monteilii*	26	32	26	36
EFPS-20	*P. aeruginosa*	41*	28	23*	31
EFPS-21	*P. aeruginosa*	35	23*	23*	33
EFPS-22	*Pseudomonas* sp.	24*	24*	38	22
EFPS-23	*Pseudomonas* sp.	34*	34*	19	28
EFPS-24	*Pseudomonas* sp.	34	34	27	27
EFPS-25	*Pseudomonas* sp.	22*	22	26	25
EFPS-26	*Pseudomonas* sp.	23	23	34*	21
EFPS-27	*Pseudomonas* sp.	25*	29	24	24*
EFPS-28	*Pseudomonas* sp.	23*	23*	20*	38
EFPS-29	*Pseudomonas* sp.	36*	26	34*	26
EFPS-30	*Pseudomonas* sp.	20	20	16*	24
EFPS-31	*Pseudomonas* sp.	24	24	26*	31
EFPS-32	*Pseudomonas* sp.	23	20	27	26
EFPS-33	*Pseudomonas* sp.	24	24	26	30
EFPS-34	*Pseudomonas* sp.	22	22	29	41
EFPS-35	*Pseudomonas* sp.	25	25	25	23
EFPS-36	*P. putida*	23	23	25	21
EFPS-37	*Pseudomonas* sp.	10	27	21*	07
EFPS-38	*Pseudomonas* sp.	13	27	16*	12
EFPS-39	*Pseudomonas* sp.	16	21*	22*	24
EFPS-40	*Pseudomonas* sp.	12*	14*	13	13

*Fungal hyphae lysed

Table 3. Effect of endophytic fluorescent *Pseudomonas* associated with *Salvadora* species on the infection of sunflower by the root infecting fungi *Fusarium oxysporum, F. solani, Macrophomina phaseolina,* and *Rhizoctonia solani* in a screen house experiment.

Treatments	*F. oxysporum*		*F. solani*		*M. phaseolina*		*R. solani*	
	Infection %							
	2013	2014	2013	2014	2013	2014	2013	2014
Control	56.2	50	31.2	31.2	68.7	75	93.7	87.5
Control (carbendazim)	50	50	43.7	37.5	81.2	62.5	62.5	43.7
P. aeruginosa (EFPS-8)	31.2	56.2	62.5	50	75	50	50	56.2
P. monteilii (EFPS-19)	43.7	37.5	50	43.7	56.2	56.2	62.5	37.5
Pseudomonas sp. (EFPS-22)	31.2	31.2	37.5	31.2	56.2	62.5	56.2	43.7
P. aeruginosa (EFPS-20)	18.7	18.7	0	37.5	50	50	62.5	50
P. putida (EFPS-36)	62.5	25	50	43.7	87.5	56.2	62.5	50
$LSD_{0.05}$	Treatments = 5.9[1], Pathogens = 4.5[2], Year= 3.2[3]							

[1]Difference less than LSD value among treatments are not significantly different (p=0.05)
[2]Difference less than LSD value among pathogens are not significantly different (p=0.05)
[3]Difference less r than LSD value between year are not significantly different (p=0.05)

Table 4. Effect of endophytic fluorescent *Pseudomonas* associated with *Salvadora* species on the growth of sunflower in a screen house experiment.

Treatments	Plant height (cm)		Fresh shoot wt. (g)		Root length (cm)		Fresh root wt. (g)	
	2013	2014	2013	2014	2013	2014	2013	2014
Control	26.2	31.2	2.25	2.13	13.28	9.8	1.26	1.6
Control (carbendazim)	25.3	35.1	3.13	2.95	7.18	9.18	0.05	0.5
P. aeruginosa (EFPS-8)	35.7	41.8	3.89	3.11	7.40	9.9	0.41	0.69
P. monteilii (EFPS-19)	43.2	47.9	3.12	3.48	9.93	9.4	0.69	0.41
Pseudomonas sp. (EFPS-22)	27.1	37.1	3.23	3.3	12.65	11.5	1.27	1.3
P. aeruginosa (EFPS-20)	39.9	43.9	4.45	3.8	8.02	8.6	1.68	0.52
P. putida (EFPS-36)	35.1	45.7	5.52	4.47	6.66	8.02	0.52	1.8
$LSD_{0.05}$	8.8[1]	10.4[1]	0.76[1]	ns	ns	ns	ns	ns

[1]Difference less than LSD value among treatments are not significantly different (p=0.05)

Table 5. Effect of endophytic fluorescent *Pseudomonas* associated with *Salvadora* species on the infection of sunflower by the root infecting fungi *Fusarium oxysporum, F. solani, Macrophomina phaseolina,* and *Rhizoctonia solani* in a field experiment after 40 days.

Treatments	*F. oxysporum*		*F. solani*		*M. phaseolina*		*R. solani*	
	Infection %							
	2013	2014	2013	2014	2013	2014	2013	2014
Control	18.7	25	25	18.7	56.2	62.5	62.5	25
Control (carbendazim)	25	31.2	31.2	18.7	37.5	56.2	68.7	31.2
Control (carbofuran)	6.2	56.2	12.5	43.7	56.2	56.2	68.7	37.5
P. aeruginosa (EFPS-8)	18.7	12.5	25	25	31.2	50	43.7	18.7
P. monteilii (EFPS-19)	25	18.7	12.5	12.5	62.5	25	62.5	18.7
Pseudomonas sp. (EFPS-22)	12.5	6.2	6.2	6.2	50	37.5	31.2	56.2
P. aeruginosa (EFPS-20)	18.7	31.2	6.2	25	31.2	56.2	50	56.2
P. putida (EFPS-36)	25	6.2	31.2	18.7	68.7	56.2	68.7	31.2
$LSD_{0.05}$	Treatments = 6.7[1], Pathogens = 4.7[2], Year= 3.3[3]							

[1]Difference less than LSD value among treatments are not significantly different (p=0.05)
[2]Difference less than LSD value among pathogens are not significantly different (p=0.05)
[3]Difference less than LSD value between year are not significantly different (p=0.05)

Table 6. Effect of endophytic fluorescent *Pseudomonas* associated with *Salvadora* species on the growth of sunflower in a field experiment after 40 days.

Treatments	Plant height (cm)		Fresh shoot wt. (g)		Root length (cm)		Fresh root wt. (g)	
	2013	2014	2013	2014	2013	2014	2013	2014
Control	82.6	85.5	55.8	49.0	10.3	11.2	5.9	5.7
Control (carbendazim)	90.3	89.3	46.3	47.2	11.0	11.6	5.3	4.8
Control (carbofuran)	83.3	87.3	46.0	44.5	12.6	13.7	7.8	5.6
P. aeruginosa (EFPS-8)	91.0	95	45.6	50.5	14.2	22.3	3.6	7.6
P. monteilii (EFPS-19)	95.0	90.1	48.1	43.3	12.3	13.1	6.6	3.8
Pseudomonas sp. (EFPS-22)	93.3	92.3	37.4	39.4	13.8	14.2	4.6	5.5
P. aeruginosa (EFPS-20)	100.6	96	45.7	44.9	11.5	10.7	5.0	4.4
P. putida (EFPS-36)	96.3	93	45.9	43.7	10.7	11.5	4.4	5.0
$LSD_{0.05}$	16.0[1]	6.8[1]	ns	9.0[1]	ns	ns	ns	ns

[1]Difference less than LSD value among treatments are not significantly different(p=0.05)
ns = Non significant

Table 7. Effect of endophytic fluorescent *Pseudomonas* associated with *Salvadora* species on the infection of sunflower by the root infecting fungi *Fusarium oxysporum, F. solani, Macrophomina phaseolina* and *Rhizoctonia solani* in a field experiment after 75 days.

Treatments	*F. oxysporum*		*F. solani*		*M. phaseolina*		*R. solani*	
	Infection %							
	2013	2014	2013	2014	2013	2014	2013	2014
Control	0	0	18.7	18.7	93.7	75	0	6.2
Control (carbendazim)	0	6.2	25	12.5	93.7	56.2	0	6.2
Control (carbofuran)	0	0	0	6.2	93.7	43.7	0	12.5
P. aeruginosa (EFPS-8)	0	6.2	0	12.5	81.2	56.2	0	6.2
P. monteilii (EFPS-19)	0	0	25	0	93.7	50	0	12.5
Pseudomonas sp. (EFPS-22)	0	0	0	0	87.5	75	0	0
P. aeruginosa (EFPS-20)	0	12.5	0	12.5	75	62.5	0	0
P. putida (EFPS-36)	0	6.2	0	12.5	75	62.5	0	12.5
$LSD_{0.05}$	Treatments = 5.4[1], Pathogens = 3.8, Year= 2.7[3]							

[1]Difference less than LSD value among treatments are not significantly different (p=0.05)
[2]Difference less than LSD value among pathogens are not significantly different (p=0.05)
[3]Difference less r than LSD value between year are not significantly different (p=0.05)

Table 8. Effect of endophytic fluorescent *Pseudomonas* associated with *Salvadora* species on the growth of sunflower in a field experiment after 75 days.

Treatments	Plant height (cm)		Fresh shoot wt. (g)		Root length (cm)		Fresh root wt. (g)	
	2013	2014	2013	2014	2013	2014	2013	2014
Control	110.0	107.0	40.4	38.7	12.0	11.8	7.9	8.2
Control (carbendazim)	118.2	113.6	38.7	37.3	16.1	15.3	6.2	7.1
Control (carbofuran)	118.7	112.7	45.4	35.6	10.8	14.5	4.4	7.3
P. aeruginosa (EFPS-8)	136.1	132.3	52.5	49.8	12.0	13.7	7.8	8.3
P. monteilii (EFPS-19)	127.1	121.1	48.3	46.4	15.4	14.8	6.2	7.1
Pseudomonas sp. (EFPS-22)	143.5	139.4	62.9	59.5	11.8	13.2	9.6	8.9
P. aeruginosa (EFPS-20)	137.4	134.7	81.9	83.0	13.0	11.8	7.1	7.8
P. putida (EFPS-36)	121.9	119.7	46.3	48.6	14.5	13.8	5.1	6.2
$LSD_{0.05}$	18.9[1]	17.2[1]	26.5[1]	23.4[1]	ns	ns	ns	ns

[1]Difference less than LSD value among treatments are not significantly different (p=0.05)
ns = Non significant

Table 9. Effect of endophytic fluorescent *Pseudomonas* associated with *Salvadora* species on the infection of root knot nematode and flower weight of sunflower in field experiments after 75 days.

Treatments	No. of knots per root system		Flower weight/plant (g)	
	2013	2014	2013	2014
Control	33.5	37.8	13.8	12.8
Control (carbendazim)	24.3	28.7	31.3	32.8
Control (carbofuran)	18.9	15.6	33.5	21.1
P. aeruginosa (EFPS-8)	20.5	23.5	24.6	17.5
P. monteilii (EFPS-19)	14.3	17.6	19.3	23.0
Pseudomonas sp. (EFPS-22)	11.6	13.8	22.3	21.4
P. aeruginosa (EFPS-20)	10.2	12.3	25.1	17.9
P. putida (EFPS-36)	13.6	16.7	18.7	26.9
$LSD_{0.05}$	3.6[1]	8.9[1]	3.5[1]	2.4[1]

[1]Difference less than LSD value among treatments are not significantly different (p=0.05)

Discussion

Bacteria living in soil, may colonize root surface for nutrients, but sometimes they enter inside the plant tissues (Prieto *et al.,* 2011; Rosenblueth & Mattinz-Romero., 2006) and even colonize leaves (Romero *et al*., 2014). Of the various soil bacteria, fluorescent *Pseudomonas* occupy the space around the plant roots (rhizosphere) may enter inside the roots (Mercado-Blanco & BakkerKER, 2007; Tariq *et al*., 2009; 2014). In this study, fluorescent *Pseudomonas* were isolated from stems and leaves besides roots of *S. persica* and *S. oleoides.* Significant antagonistic activity against root rot pathogens and root knot nematode was observed by the most of the isolates. Endophytic bacteria have been reported to have some positive impact on host plant including suppression of diseases and enhancement of plant growth (Afzal *et al*., 2013; Hallmann *et al*., 1997; Tariq *et al*., 2009).

The root colonizing bacteria have been reported to improve plant growth by producing growth regulators or by suppressing the pathogens (Inam-ul-Haq *et al.,* 2012; Raaijmakers *et al*., 2002; Siddiqui & Ehteshamul-Haque, 2001; Weller *et al*., 2002; Weller, 2007). Application of endophytic fluorescent *Pseudomonas* not only suppressed infection of sunflower by root rotting fungi and the root knot nematode but also improved plant growth. The plant growth promoting bacteria may improve plant growth by direct or indirect modes of action (Beauchamp, 1993; Kloepper, 1993; Lazarovits & Nowak, 1997). Production of stimulatory phytohormones, lowering of the ethylene level, liberation of phosphates and micronutrients are considered as direct mechanisms of plant growth (Anton & Prevost, 2005). Whereas production of certain antibiotics (Raaijmakers *et al.,* 2002) and siderophores (De Meyer & Hofte, 1997) has been reported as mechanisms involved in disease suppression. Raaijmakers & Weller (1998) reported the role of 2, 4-diacetyl-phloroglucinol, an antifungal metabolite from species of fluorescent *Pseudomonas* in plant root disease suppression. Like *P. fluorescens* and *P. putida, P. aeruginosa* has also been reported as endophytic bacteria showing activity against plant pathogenic fungi and parasitic nematodes (Afzal *et al.,* 2013; Kumar *et al.,* 2013 Tariq *et al.,* 2009). Due to their ability to colonize plant tissues internally, endophytic bacteria are nowgaining importance (Prieto *et al.,* 2011), an ecological niche similar to that of phytopathogens (Berg *et al.,* 2005). In this study, endophytic *Pseudomonas* isolated from facultative halophytic *Salvadora* plants have shown significant biocontrol potential against soilborne pathogens affecting sunflower. Although endophytes are now being used in various fields, but their potential in crop protection is seems enormous. It is suggested that besides healthy field crops, endophytic bacteria with biocontrol potential should also be evaluated from tree plants, particularly those found in different ecological conditions and able to tolerate different stresses.

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