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ROLE OF *PSEUDOMONAS AERUGINOSA* IN ENHANCING THE GROWTH OF SUNFLOWER AND SUPPRESSION OF ROOT ROTTING FUNGI

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ABSTRACT

Treatment of Sunflower (*Helianthus annuus* L), with strains of *Pseudomonas aeruginosa* (PGPR) as a seed treatment and soil drench methods showed significant ($P<0.05$) result against root pathogenic fungi (*Rhizoctonia solani, Fusarium solani, Macrophomina phaseolina* and *F.oxysporum*). PGPR- (4, 11, 17, 28) were found promising strains of *P.aeruginosa* showed the highly significant reduction in infection of all examined fungi in both treatments. Complete control of *F. solani* was shown by PGPR-11 and PGPR-28. *P. aeruginosa* strain (PGPR-8, 11, 28) represented significant ($p<0.05$) improvement in root and shoot length while PGPR-(2, 3, 8, 11, 24 and 25) significantly ($p<0.05$) increased the weight of shoot by seed treatment. Soil was treated with *P. aeruginosa* strains PGPR-(11, 25, 26 and 28), completely controlled the infection of *F.solani*, PGPR-(3, 4, 17 and 28) controlled *R. solani* and PGPR-3 controlled *F. oxysporum* when applied in soil. Fresh shoot weight was recorded maximum with the use of PGPR-24 compared to PGPR-(4, 5).

Keywords: Root rot**,** pathogen, plant growth, soil, suppression, PGPR

INTRODUCTION

Rhizosphere is the best environment for microorganisms where they maintain the structure of soil, degrade the organic matter, and also enhanced nutrients uptake to plants (Xiao *et al.,* 2015). Microorganisms can increase the soil organic carbon losses as well as affect the soil CO_2 emissions (Carney *et al.,* 2007; Allison *et al.,* 2010). Microorganisms exert significant effect on the reduction of soil borne pathogens produced the plant diseases and enhance fertility of soil (Van Bruggen and Semenov, 2000). Rhizospheric microorganisms are ideal to use against soil-borne pathogens as a biocontrol agent (Weller, 1988; Liu *et al.,* 2018). A compound siderophore has been produced in iron-limiting situations by PGPB (Whipps, 2001). Siderophores are an iron chelating compound that is produced by PGPR (plant growth promoting rhizobacteria (Meyer, 2007). Different antibiotics also identified, such as oomycin A, hydrogen cyanide, 2,4-diacetylphloroglucinol (DAPG), amphisin, pyluteorin, cyclic lipopeptides, pyrrolnitrin, phenazinetropolone and tensin formed by different Pseudomonads strains (Nielsen *et al.,* 2003). PGPR Enhance the growth of plant (Shameer and Prasad, 2018; Mustafa, *et al.,* 2019) such features mark these species best applicants to practice as biocontrol agents. These strain of PGPR not only constrain the growth of rhizospheric pathogens also induce systemic resistance (ISR) (Liu *et al.,* 2018; Adrees *et al.,* 2019). Suspension of

PGPR suppressed *M. phaseolina*, significantly decreased rotting and damping-off in soybean plants (El-Barougy *et al.,* 2009). IAA produced in-vitro by *P. fluorescens* and suppressed the growth of *M. phaseolina* (Kumar *et al.,* 2007). PGPR show a vital role for the management of root diseases (Kumar *et al.,* 2009). PGPR enhanced 77% shoot and 30% root biomass compared to control and also increased the length of plant by constraining the mycelial development of *M. phaseolina* (Singh *et al.,* 2008).

The present study is proposed to suppress pathogens and enhance growth of sunflower plant by the application of PGPR like *P. aeruginosa*.

MATERIAL AND METHODS

Isolation and differentiation of parasitic and saprophytic Fluorescent *Pseudomonas*

Roots with adhering soil were collected from some healthy plants of Kathor, Gharoo, Karachi University Campus, Memon Goth, Malir, Gumbat, Darsano Chano, Hub and Sacroo for the isolation and purification of varies strains of fluorescent *Pseudomonas* and processed according to the standard method (Gould, *et al.,* 1985; Noreen, *et al.,* 2015) kept all plates for three days at 28°C. Fluerescent pigments producing colonies of bacteria were picked at 366 nm, under UV light and preserved on medium of King's B agar (King *et al.,* 1954). Gram staining performed to check and differentiates the gram negative and positive bacteria (Schaad *et al.,* 1988). To separate the *Pseudomonas fluorescens, P. aeruginosa*, and *P. putida,* a trial of levan formation was performed by nutrient agar (NA), with 5% sucrose to grow the test bacterium .To distinguish between saprophytic and pathogenic strains of *Pseudomonas*, all examine bacteria was developed at 41°C (Krieg, 1984). According to the Krieg, (1984) Nutrient broth was used complemented with an Andrade's indicator and1% trehalose sugar for the growth of only *P. fluorescens.* Agar slant containing gelatin was inoculated by *P.aeruginosa* to check the liquefaction. Incubated for 3 days at 22°C, and kept in ice bath for twenty minutes. According to the Ayers *et al.,* (1919) nitrogen cannot be fermented by *Pseudomonas* but it can grow without O_2 and utilized nitrate

In vitro antifungal activity by direct and fraction method

Different strains of *P.aeruginosa* were used as direct dual plate method to check the antagonistic ability against root infecting fungi viz; *R.solani, F.solani, F.oxysporum,* and *M.phaseolina* (Drapeau *et al.,* 1973). One corner of the CDA containing plates were lined with the bacterial isolates of *P.aeruginosa* while other side actively pure growing culture of examine fungi like *F. oxysporum, F. solani, R. solani* and *M. phaseolina* were keep in Czapek's Dox agar, pH 7.2, incubated at 28°C for 5-7 days. Inhibitions in the form of zone formation were calculated in mm from fungal colony and disc of fungi. To find out the antifungal activity (zone of inhibition) of comprising stains in different solvent fractions, of CFC was taken out through organic solvents comprising *n*-Hexane, ethyl acetate and butanol in a separating/ fractionating funnel and concentrated on rotary vacuum evaporator. A 5mm sterilized disc was impregnated with each fraction. A technique (Dual Plate) used by Siddiqui *et al.* (2001) against both *Fusarium* species, *R.solani* and *M.phaseolina* and incubated at 28°C. Three replicates were made from each treatment after 7days of time period zone of inhibition was measured. While dried disc of all respective solvents were used in place of -ve control.

Pathogen suppressive siderophores production by *P. aeruginosa*

A method used to describe by Schwyn and Neilands, (1987) of universal chemical assay by this bacteria *P.aeruginosa* produced siderophores to suppress the pathogens.

POT EXPERIMENTS

Seed treatment

The naturally infested soil showed (5-10%) colonized by *R. solani*, 3-5 sclerotia per gram of *M. phaseolina* (sclerotia), 3000cfu/g soil infested with *Fusarium* species. Different strains of *P. aeruginosa* like, PGPR-(2,3,4,5,8,11,17,24,25,26 and 27) @ of (1.64×10^9, 6.0×10^9, 4.0×10^9, 1.12×10^9, 8.0×10^9, 1.0×10^9, 1.8×10^9, 2.4×10^9, 2.0×10^9, 2.2×10^9 and 2.8×10^9 cfu/mL) respectively, five days old cultures were used to treat the Sunflower (*Helianthus annuus* L.) seeds. 1% gum arabic used as sticky material, six seeds/plastic pot of 8 cm diam. were sown. Each pot contained 300 g soil and replicated 4 times. Finally, each pot kept with four seedlings and excess were eliminated after germination. The pots of experiment were arranged on bench of screen house as a CRB design with 50% water holding capacity.

Soil Drench Method

Five days old culture of different strains viz., PGPR-(2,3,4,5,8,11,17,24,25,26,27) were used at the rate of (1.0×10^8, 1.3×10^8, 8.25×10^8, 1.0×10^8, 9.0×10^8, 1.7×10^8, 1.1×10^8, 1.5×10^8, 1.53×10^8, 1.7×10^8 and 2.0×10^8 cfu/mL) respectively. A 25 mL cell suspension was added in plastic pots. 300g soil/pot having 8cm diam. Six seeds of sunflower were sown in each pot. Four seedlings were left and extra were eliminated after germination. Observations were noted after 30 days.

After 30 days of plant to find out the incidence of fungi on roots, the plants of sunflower were dig up, washed with water and five root pieces of about 1cm long, sterilized the surface of root by 1% $Ca(OCl)_2$ and transferred on culture plates of PDA comprising streptomycin (0.2 g/litre) and penicillin (100,000 units/litre). Occurrence of root pathogenic fungi was noted after 5 days interval of time.

RESULTS

Isolation and differentiation of parasitic and saprophytic Fluorescent Pseudomonas

Twenty-eight different strains of *Pseudomonas* (fluorescent) collected from the rhizoplane and rhizospheres of 9 different plants and identified (Table 1). Due to presence of NH_3 *P. aeruginosa* showed red color (alkaline in nature) after 4 days, representing its saprophytic nature. To check the levan formation, *P. fluorescens* exhibited the occurrence of mucoid (white), convex colonies while *P. aeruginosa* and *P. putida* presented negative results after 3-5 days at 28 ^{o}C. It is revealing of levan formation only *P. aeruginosa* has ability to grow at 41°C temperature. During trehalose utilization test only *P. fluorescens* showed +ve results in nutrient broth. Only *Pseudomonas aeruginosa* showed positive results and liquefy the gelatin. Growth of *P. aeruginosa* was resulted as a +ve test for denitrification during nitrate reduction test.

Table 1. List of *Pseudomonas aeruginosa* strains isolated from different plant species.

P. aeruginosa strains	Plant Source	Locality
PGPR-1	*Achyranthus aspera*	Karachi University
PGPR-2	"	"
PGPR-3	"	"
PGPR-4	*Cynodon dactylon*	"
PGPR-5	*Ruellia patula*	"
PGPR-6	"	"
PGPR-7	*Tephrosia subtriflora*	"
PGPR-8	"	"
PGPR-9	"	"
PGPR-10	*Achyranthus aspera*	Malir
PGPR-11	*Ficus carica*	Karachi University
PGPR-12	"	"
PGPR-13	"	"
PGPR-14	"	"
PGPR-15	"	"
PGPR-16	"	"
PGPR-17	*Abutilon theophrasti*	"
PGPR-18	"	Malir
PGPR-19	"	"
PGPR-20	*Leucaena leucocephala*	Karachi University
PGPR-21	"	Hub
PGPR-22	"	Malir
PGPR-23	"	"

PGPR-24	*Achyranthus aspera*	Hub
PGPR-25	"	"
PGPR-26	*Lycopersicon esculentum*	"
PGPR-27	*Cynodon dactylon*	"
PGPR-28	*Zea mays*	"

Table 2. *In vitro* growth inhibition of *Macrophomina phaseolina, Rhizoctonia solani, Fusarium solani* and *F. oxysporum* by the strains of *Pseudomonas aeruginosa* plant growth promoting rhizobacterium.

P. aeruginosa Strains	*M. phaseolina*	*F. solani* Zone of Inhibition	*R. solani* (mm)	*F. oxysporum*
PGPR-2	3.0	9.6	4.6	3.0
PGPR-3	2.3	18.3	6.0	4.0
PGPR-4	5.6	5.0	8.5	5.5
PGPR-5	5.0	7.6	8.3	5.8
PGPR-8	5.7	9.0	7.5	6.0
PGPR-11	3.0	6.0	11.6	6.2
PGPR-17	2.5	5.66	6.0	2.5
PGPR-24	6.0	13.0	6.3	2.4
PGPR-25	00	9.0	5.3	2.0
PGPR-26	2.0	15.5	8.0	1.0
PGPR-27	5.0	9.6	5.0	1.0
PGPR-28	3.6	16.6	6.0	5.0

Table 3. *In vitro* growth inhibition of *Fusarium oxysporum* and *F. solani* by solvent fractions of culture filtrates of PGPR.

Fractions (50 µg/disc)	Treatments	*F. solani*	*F. oxysporum*
		Zone of Inhibition (mm)	
Control		0	0
n-Hexane	PGPR-4	*	*
Ethyl-acetate	PGPR-4	21.6	16.3
n-Hexane	PGPR-11	12.5	45
Ethyl-acetate	PGPR-11	12.3	15
n-Hexane	PGPR-27	11.6	14.3
Ethyl-acetate	PGPR-27	15	20
n-Butanol	PGPR-27	16.6	20

Antifungal activity of *P.aeruginosa* by direct and fraction method

Different strains of *P.aeruginosa* were used as direct dual plate method to check the antagonistic ability against root decaying test fungi (*F.solani, M.phaseolina, R.solani* and *F.oxysporum*) during laboratory test PGPR–(2, 4, 11, 27) produced maximum zone of inhibition (Table 2). PGPR-(4, 11 and 27) collected in bulk amount and examine for fungicidal activity, Ethyl acetate of PGPR-4 made maximum inhibition zone (21.6mm)

against *F. solani* while FractionsPGPR-27 (*n*-hexane, ethyl acetate and butanol) formed maximum inhibition zone (20mm) against *F. oxysporum* (Table 3).

Table 4. Siderophore production by some selected strains of *Pseudomonas aeruginosa*.

***P. aeruginosa* strains**	**Siderophore Production**
PGPR-2	-
PGPR-3	-
PGPR-4	***
PGPR-5	-
PGPR-11	***
PGPR-17	-
PGPR-24	**
PGPR-27	***
PGPR-28	-

*** Large size halo present around bacterial culture.
** Medium size halo present around bacterial culture
* Small size halo present around bacterial culture
- No halo present around bacterial culture

Table 5. Effect of *Pseudomonas aeruginosa* used as seed treatment on infection of *Fusarium solani*, *F. oxysporum*, *Macrophomina phaseolina* and *Rhizoctonia solani* on sunflower roots.

Treatments	***F. solani***	***F. oxysporum***	***M. phaseolina***	***R. solani***
		INFECTION %		
Control	37.5	62.5	87.5	12.5
PGPR-2	18.7	18.7	87.5	00.0
PGPR-3	25.0	31.2	43.7	06.2
PGPR-4	18.7	12.5	62.5	06.2
PGPR-5	12.5	18.7	81.2	00.0
PGPR-8	12.5	43.7	68.7	00.0
PGPR-11	00.0	31.2	50.0	00.0
PGPR-17	18.7	43.7	56.2	00.0
PGPR-24	31.2	12.5	68.7	00.0
PGPR-25	06.2	18.7	62.5	00.0
PGPR-26	06.2	25.0	31.2	12.5
PGPR-27	00.0	31.2	50.0	06.2
$LSD_{0.05}$	Treatments = 16.2[1],	Pathogens = 9.3[2]		

[1] Mean values for treatments in columns showing differences greater than the LSD value are significantly different at $p<0.05$

[2] Mean values for pathogens in rows showing differences greater than the LSD value are significantly different at $p<0.05$

Pathogen suppressive siderophores production by *P.aeruginosa*

PGPR strains were tested, four strains of PGPR-(4, 11, 24 and 27), and produced siderophore by creating halo formation (Table 4).

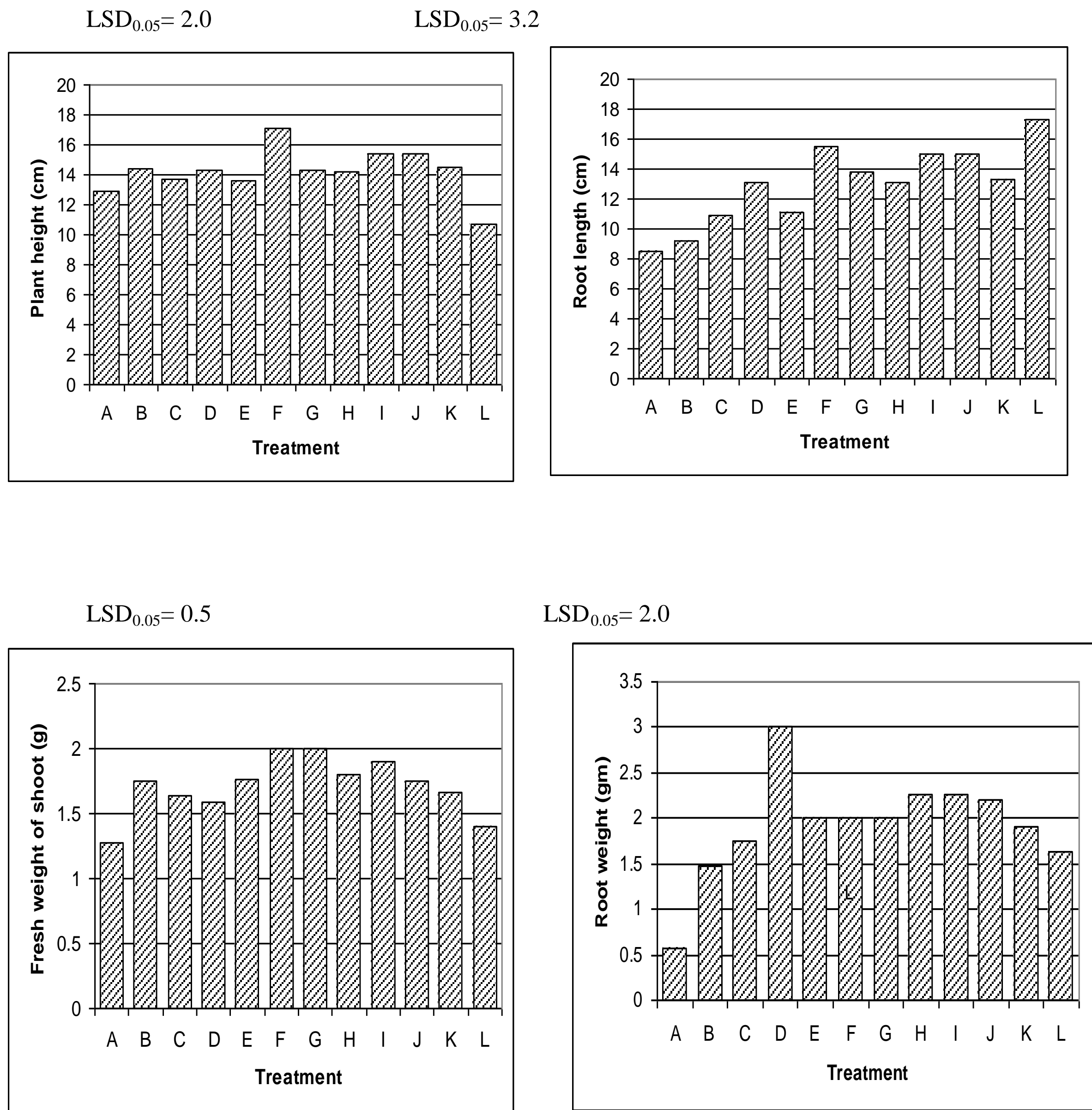

Fig.1. Effect of seed dressing with different strains of *Pseudomonas aeruginosa* on growth of sunflower plants.

A=Control B=PGPR-2 C=PGRP-3 D=PGPR-4 E=PGPR-5
F=PGPR-8 G=PGPR-11 H=PGPR-17 I=PGPR=24 J=PGPR=25
K=PGPR=26 L=PGPR-27

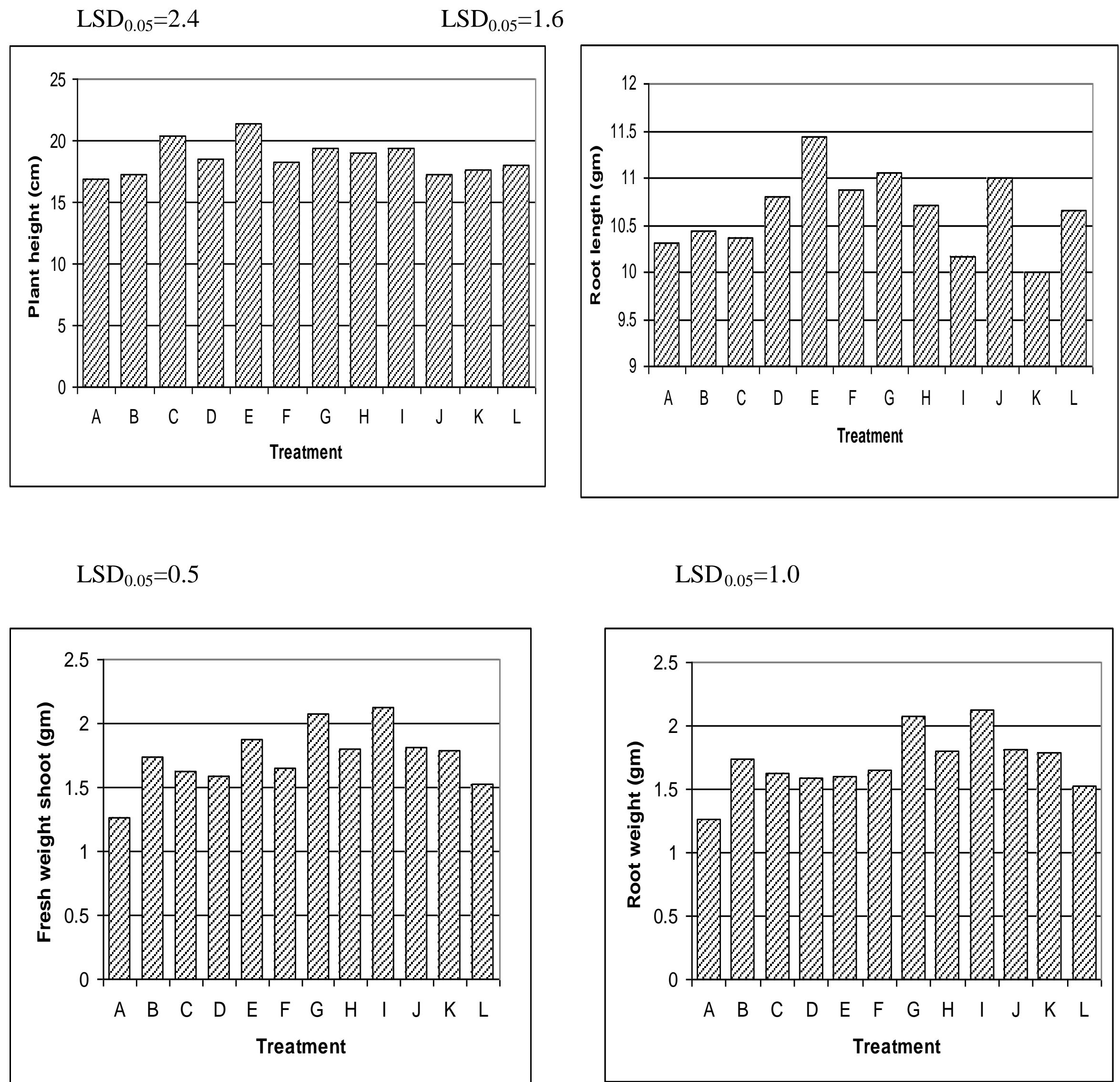

Fig.2. Effect of soil drench with different strains of *Pseudomonas aeruginosa* on growth of sunflower plants.

A=Control	B=PGPR-2	C=PGRP-3
D=PGPR-4	E=PGPR-5	F=PGPR-8
G=PGPR-11	H=PGPR-17	I=PGPR=24
J=PGPR=25	K=PGPR-26	L=PGPR-27

Table 6. Effect of *Pseudomonas aeruginosa* used as soil drench on infection of *Fusarium solani*, *F. oxysporum*, *Macrophomina phaseolina* and *Rhizoctonia solani* on sunflower roots.

Treatments	*F. solani*	*F. oxysporum*	*M. phaseolina*	*R. solani*
		INFECTION %		
Control	25.0	75.0	18.7	31.2
PGPR-2	12.5	37.5	25.0	06.2
PGPR-3	31.2	00.0	12.5	00.0
PGPR-4	12.5	50.0	06.2	00.0
PGPR-5	06.2	18.7	06.2	06.2
PGPR-8	06.2	56.2	25.0	06.2
PGPR-11	00.0	31.2	62.5	06.2
PGPR-17	12.5	50.0	50.0	00.0
PGPR-24	06.2	50.0	62.0	12.5
PGPR-25	00.0	37.5	62.5	06.2
PGPR-26	00.0	18.7	68.7	12.5
PGPR-27	00.0	25.0	62.5	00.0
$LSD_{0.05}$	Treatments = 15.0[1], Pathogens = 8.6[2]			

[1] Mean values for treatments in columns showing differences greater than the LSD value are significantly different at p<0.05

[2] Mean values for pathogens in rows showing differences greater than the LSD value are significantly different at p<0.05

POT EXPERIMENTS

Seed Treatment

Different strains of PGPR - (2,3,4, 5, 8, 11,17, 24, 25, 26 and 27) represented significant (p<0.05) controlled of *F.oxysporum*, *F.solani* totally suppressed by PGPR-(11 and 27). Although a significant (p<0.05) reduction of the infection of *F. solani* was produced with the use of PGPR-(2, 4, 5, 8, 17, 25 and 26). Maximum reduction of *M. phaseolina* caused by PGPR-26, whereas infection was significant (p<0.05) decreased with the use of PGPR-(3, 4, 11, 17, 25, 26 and 27). Isolates of *P. aeruginosa,* PGPR-(2, 5, 8, 11, 17, 24 and 25) recorded complete reduction of *R. solani* (Table 5).

Maximum height of plant was recorded by PGPR-8. Application of PGPR-(2, 3, 8, 11, 24 and 25) enhanced the fresh shoot weight significantly (p<0.05). Similarly, significant (p<0.05) improved the length and weight of root by PGPR-11. While, PGPR-28 caused highly significant effect on root length (Fig. 1).

Soil Drench Method

P. aeruginosa strains PGPR-(11, 25, 26 and 28) completely controlled infection of *F. solani*. *P. aeruginosa* strains PGPR-(5, 8 and 24) significantly (p<0.05) inhibited the infection of *F. solani*. *P. aeruginosa* strains PGPR-(2, 4, 5, 8, 11, 17, 24, 25, 26, and 27) significantly (p<0.05) decreased the inoculum of *F. oxysporum*. Whereas, complete control was recorded by PGPR-3 in the inoculum of *F. oxysporum*. Inoculum of *R. solani* was complete reduced via *P. aeruginosa* strains PGPR-(3, 4, 17 and 28), *R. solani*

infection was controlled with the used of strains of PGPR-(2, 5, 8, 11, 24, 25 and 26) (Table 6).

Maximum length of the plant recorded by PGPR-5. While PGPR-24 produced maximum fresh shoot weight. *P. aeruginosa* strain (PGPR-5) recorded maximum length of root and PGPR-24 produced highest weight of root (Fig. 2).

DISCUSSION

Twenty-eight different strains of fluorescent *Pseudomonas* were isolated, while 12 were stabled and resulted inhibition zone against pathogenic root attacking fungi like *F. solani, M. phaseolina, R. solani* and *F. oxysporum*. Variability were found among various isolates of *P.aeruginosa* against different pathogenic fungi (Ehteshamul-Haque and Ghaffar, 1993) showed different ability to antagonized (Durairaj *et al.,* 2018). Different function performed by the antagonist, when applied in soil it kills the inoculum of pathogens, provide protection to the seeds germination against the pathogens and induced systemic resistant (Cook and baker, 1983; Adrees *et al.,* 2019). At the time of sowing, antagonist direct applied to the soil resulted a great potential in gardens, commercial glass house operations and in large field such as strawberries and grown tomatoes field (Cook and baker, 1983; Jisha, *et al.,* 2019). When antagonists or beneficial microorganisms used as treatment of seed, it controls infections and increase the plant's growth and yield (Compant *et al.,* 2005; El-Hadad *et al.,* 2010). Used of *P. aeruginosa* as soil drench or seed dressing prevented the sunflower plants from the root infected fungi like *F.oxysporum*, *R. solani, F. solani,* and *M. phaseolina*. Such antagonistic bacteria that colonized the root and cause beneficial effects are called plant growth promoting bacteria (Kloepper *et al.,* 1980). They have ability to produce growth regulator (Mia *et al.,* 2010). A siderophore an iron chelating compound produced by *P. aeruginosa* (De Meyer and Höfte, 1997), antibiotics production (Levy *et al.,* 1992). An antifungal metabolite 2,4-diacetylphloro-glucinol, suppressed the root disease of crop plants (Raaijmakers and Weller, 1998).

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