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EVALUATION OF BIOCONTROL POTENTIAL OF EPIPHYTIC FLUORESCENT *PSEUDOMONAS* ASSOCIATED WITH HEALTHY FRUITS AND VEGETABLES AGAINST ROOT ROT AND ROOT KNOT PATHOGENS OF MUNGBEAN.

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Abstract

Endophytic and rhizospheric fluorescent *Pseudomonas* have widely been used as biological control agents against soilborne plant pathogens. In this study, fifteen epiphytic fluorescent *Pseudomonas* isolated from the surfaces of citrus (grapefruit, orange and lemon) melon and tomato fruits were characterized for their *in vitro* activity against root rotting fungi viz., *Macrophomina phaseolina, Fusarium solani, F. oxysporum* and *Rhizoctonia solani* and nematicidal activity against the second stage juveniles of *Meloidogyne javanica.* Out of fifteen *Pseudomonas* isolates HAB-16, HAB-1 and HAB-25 inhibited the growth of all the test fungi and showed maximum nematicidal activity against second stage juvenile of *M. javanica.* Based on their effective *in vitro* activity nine epiphytic fluorescent *Pseudomonas* were evaluated for their growth promoting ability and biocontrol activity in screen house on mungbean. *Pseudomonas* isolates (HAB-13, HAB-2, HAB-4, HAB-1, HAB-14, HAB-9, HAB-7 and HAB-25) used as soil drench greatly reduced the root rot-root knot infection and thereby enhanced plant growth, root nodulation and yield in mungbean. Besides, rhizospheric and endophytic, epiphytic fluorescent *Pseudomonas* associated with healthy fruits may be used as biocontrol agent against root rotting fungi, besides, using for the mangemnet of postharvest diseases.

Key words: Epiphytic, *Pseudomonas,* Root rot, Root knot, Mungbean, Biocontrol.

Introduction

The surfaces of aerial plant parts provide a habitat for epiphytic micro-organisms, many of which also influence the growth of pathogens. Bacteria are generally the predominant initial inhabitants of newly expanded leaves, while yeasts and filamentous fungi dominate later in the growing season (Kinkel *et al*., 1987). Fluorescent Pseudomonads constitutes a major portion of indigenous microflora naturally present on surface of fresh vegetables and they are assumed to play the active role in maintaining the quality and safety of fresh cut fruits and vegetables (Nguyen-the & Carlin, 1994). Certain *Pseudomonas fluorescens* strains viz.CHA0 and Pf-5 have shown biocontrol properties and protected the roots of some plant species against pathogenic fungi such as *Fusarium* or *Pythium*, as well as some phytophagous nematodes (Haas & Keel, 2003). Besides siderophore production, induction of systemic resistance in plant and production of antifungal antibiotics are the mechanisms most commonly associated with the ability of plant growth-promoting bacteria to act as antagonistic agents against phytopathogens (De Meyer & Hofte, 1997; Ramamoorthy *et al*., 2001; Shafique *et al*., 2015ab; Siddiqui *et al*., 2000; Siddiqui & Ehteshamul-Haque, 2001). Fluorescent *Pseudomonas* have been reported to produce 2, 4-diacetyl phloroglucinol (Weller *et al*., 2007) active against soilborne pathogens including *Fusarium* (Srivastava & Shalini, 2008; Thangavelu & Mari, 2006) and *Verticillium dahlia* causes *Verticillium* wilt disease on cotton (Erdogan *et al*., 2011). Biocontrol potential of fluorescent *Pseudomonas* associated with the rhizosphere, rhizoplane (Ehteshamul-Haque *et al.,* 2007ab; Siddiqui *et al.,* 2000) and endo-root (Afzal *et al.,* 2013; Tariq *et al*., 2009) is well documented. However, the biocontrol role of fluorescent *Pseudomonas* associated with fruit surface has not been taken under consideration. The present report describes the isolation and characterization of epiphytic fluorescent *Pseudomonas* associated with the surfaces of citrus (grapefruit, orange and lemon) melon and tomato fruits and their biocontrol potential against root rotting fungi of mungbean.

Materials and Methods

Isolation and identification of fluorescent *Pseudomonas*

Sample collection: Fresh lemon, orange, grapefruit, tomato and melon free from any physical bruise or diseases were collected from field or supermarket (Metro; Cash and Carry). Samples were kept at 4°C until isolation was made within 24 hours.

Isolation of fluorescent *Pseudomonas* from fruit surface: Fruit sample were washed with sterilized water and two gram sample were taken from fruit surface in 20 ml of 0.05 M phosphate buffer (pH 6.5) and crushed in thistle mortar, then 0.1 ml of each sample was transferred on Petri dishes containing Gould's S1 medium (Gould *et al.,* 1985; Bashan *et al.,* 1993). Dishes were incubated for 1-2 days and bacterial colonies fluoresced under UV light at 366 nm were purified on King B agar medium (King *et al.,* 1954). The presumptive *Pseudomonas* spp. was initially identified according to the Bergey's Manual (Brenner *et al.,* 2005) and the selected isolates were further confirmed using established molecular biology techniques recently described by us (Noreen *et al*., 2015) and reported elsewhere.

***In vitro* juvenile mortality test:** *Pseudomonas* were grown on King's B broths at 30°C for 72 hours in dark and centrifuged at 3000 rpm for 20 minutes. The pellets were discarded and the culture filtrate was collected in the beaker for use. One ml of freshly hatched second stage juvenile suspension (20 juveniles) and 1 ml of cell free culture filtrate of bacterial strains were transferred in glass cavity blocks and kept at 26 ± 5°C. There were three replicates of each treatment and juvenile mortality was recorded after 48 hours. The nematodes were considered dead if they did not move when probed with needle (Cayrol *et al*., 1989).

Screen house experiment: Non- sterilized sandy loam; pH 8.0, with moister holding capacity of 40% was obtained from the field of Department of Botany, University of Karachi and transferred into 15 cm diameter earthen pots at 1 kg of soil per pot. The soil had natural infestation of 3-6 sclerotia of *Macrophomina phaseolina* g^{-1} of soil, as determined by wet sieving and dilution technique (Shiekh & Ghaffar, 1975), 5-10% colonization of sorghum seeds was used as bait for *Rhizoctonia solani* (Wilhelm, 1955) and 3000 $cfu.g^{-1}$ of soil of a mixed population of *Fusarium solani* and *F. oxysporum* as determined by a soil dilution technique (Nash & Synder, 1962). Six mungbean seeds were sown in each per pots after applying 25ml bacterial suspension (cfu 10^8) of *Pseudomonas* isolates viz., HAB-13, HAB-2, HAB-4, HAB-16, HAB-1, HAB-14, HAB-9, HAB-7 and HAB-25 into each pot. Plant not received bacterial suspension served as control. While carbendazim (200 ppm) at 25 ml per pot served as positive control against root rotting fungi. The experiment was conducted in complete randomized block design with four replicates. After germination, four seedlings were kept in each pot and excess were removed.

The experiment was terminated after 45 days and data on plant height and fresh weight of the roots and shoots were recorded. To determine the root-infecting fungi, the roots were washed in running tap water, surface sterilized in 1% $Ca(OCl)_2$ and five, 1cm long root pieces were inoculated onto PDA plates containing penicillin (100,000 units/L) and streptomycin sulphate (0.2g/L). The plates were incubated at room temperature (26 ± 5°C) and the incidence of root infecting fungi was recorded as follows;

$$\text{Infection (\%)} = \frac{\text{No. of plant infected by fungi}}{\text{Total number of plants}} \times 100$$

Data analysis: For fungal infection two way ANOVA was used to compare the means among the treatments and also among different fungal pathogens. The follow up of ANOVA include least significant difference (LSD) at ($p<0.05$) to compare the means. Whereas for plant growth parameters one way ANOVA was used and LSD at ($p<0.05$) was calculated (Gomez & Gomez, 1984).

Results

In vitro* antifungal activity of epiphytic fluorescent *Pseudomonas: All fifteen test isolates of epiphytic fluorescent *Pseudomonas* tested against four root rotting fungi i.e., *M. phaseolina*, *R. solani, F. solani* and *F. oxysporum* caused growth inhibition of all the test fungi and produced zone of inhibition, except HAB-10, which was not effective against *R. solani* (Table 1). Most of the *Pseudomonas* isolates produced larger zone of inhibition against *F. solani, F. oxysporum* and *M. phaseolina* than *R. solani.*

Table 1. *In vitro* inhibition of *Fuasrium solani, F. oxysporum, Macrophomina phaseolina* and *Rhizoctonia solani* by epiphytic fluorescent *Pseudomonas* and juvenile mortality of *Meloidogyne javanica* by cell free culture filtrates.

Culture # of *Pseudomonas*	Source	Zone of inhibition (mm)				Nematode mortality (%)	
		F. oxysporum	*F. solani*	*R. solani*	*M. phaseolina*	24 hrs.	48 hrs.
Control	KB broth	--	--	--	--	0	24
HAB-13	Lemon	17	20	8.5	16	66.6	93.3
HAB-2	Lemon	19.3	20.3	8.5	17.3	90	91.6
HAB-4	Lemon	19.5	21.3	10.5	20	66.6	96.6
HAB-16	Lemon	18	19.6	7.5	22.3	75	100
HAB-1	Lemon	21	21.3	6.5	15.6	90	100
HAB-14	Lemon	16.5	20	9.5	19.6	73.3	91.6
HAB-9	Tomato	20	24	7.5	18.6	81.6	96.6
HAB-7	Tomato	20	21.6	5.5	19.6	75	96.6
HAB-25	Tomato	18.6	22	10	23	96.6	100
HAB-24	Orange	16	17	6.5	14	93.3	100
HAB-10	Orange	18	19.5	0	13.5	100	100
HAB-8	Grapefruit	18.5	19	5.5	17	95	100
HAB-15	Melon	19.5	21	8.5	16.5	75	100
HAB-20	Melon	19	20.5	10	14.5	90	100
HAB-21	Tomato	18.5	21.5	7.5	15	100	100

***In vitro* juvenile mortality test:** Cultural filtrate of *Pseudomonas* spp. showed significant nematicidal activity by killing the second stage juvenile of *M. javanica* to the varying degree. Maximum mortality of juvenile (100%) was caused by HAB-16, HAB-1, HAB-24, HAB-25, HAB-10, HAB-8, HAB-15, HAB-20, and HAB-21 within 48 hours (Table 1). HAB-10 and HAB-21 were found to kill 100% juveniles within 24 hours.

Screen house experiment: In the screen house test, some isolates of *Pseudomonas* viz., HAB-13, HAB-2, HAB-4, HAB-16, HAB-1, HAB-14, HAB-9, HAB-7 and HAB-25 were used as biocontrol agent against root infecting fungi. HAB-2, HAB-13, HAB-4, HAB-1, HAB-9, HAB-7 and HAB25 significantly ($p<0.05$) suppressed *R. solani* as compared to control (Table 2). While Carbendazim caused significant reduction of all root infecting fungi except *F. solani* in mungbean. Maximum inhibition of *M. phaseolina* was observed in HAB-25 treated plants. Whereas HAB-13, HAB-2, HAB-1, HAB-9 and HAB-7 also caused significant control of *M. phaseolina* (Table 2). Maximum suppression of *F. solani* was observed in HAB-7 treated plants, while complete suppression of *F. oxysporum* was found in HAB-13, HAB-16, and HAB-1and HAB-25 treated plants (Table 2). Application of HAB-7 resulted in the maximum shoot length followed by HAB-16 as compared to control, whereas, HAB-7 also produced maximum shoot weight. All the test *Pseudomonas* showed an improvement in root nodulation in mungbean as compared to untreated control (Table 3). Highest number of nodules was found in HAB-14 treated plants followed by HAB-2, HAB-1 and HAB-7 treatments (Table 3).

Table 2. Effect of different isolates of *Pseudomonas* isolated from fruit surface on root infection by *Macrophomina phaseolina, Fusarium solani, F. oxysporum* and *Rhizoctonia solani* on mungbean roots.

Treatments	Infection %			
	M. phaseolina	***R. solani***	***F. solani***	***F. oxysporum***
Control	100	100	100	31.2
Carbendazim	43.7	37.5	100	6.2
HAB-13	81.2	12.5	100	0
HAB-2	62.5	25	93.7	18.7
HAB-4	93.7	75	100	6.2
HAB-16	93.7	93.7	100	0
HAB-1	68.7	75	93.7	0
HAB-14	87.5	93.7	100	6.2
HAB-9	75	75	87.5	6.2
HAB-7	75	81.2	50	6.2
HAB-25	50	50	68.7	0
	Treatments = 13.9[1]		**Pathogens = 8.4[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 3. Effect of soil drench with different isolates of *Pseudomonas* isolated from fruit surface on the growth and nodulation of mungbean plants.

Treatments	Growth parameter					
	Shoot length (cm)	**Shoot weight (g)**	**Root length (cm)**	**Root weight (g)**	**Number of Nodules**	**Number of Fruits**
Control	29.31	6.49	18.25	1.61	4	2
Carbendazim	29.75	6.23	13.13	3.79	10	2
HAB-13	28.56	6.97	16.37	1.05	18	3
HAB-2	27.5	6.25	18.43	1.14	20	3
HAB-4	30.25	6.31	17.12	1.17	18	3
HAB-16	33.31	7.73	20.37	1.32	19	2
HAB-1	32.13	5.8	15.75	0.97	20	3
HAB-14	31.68	6.18	18.62	1.05	23	4
HAB-9	31.06	7.49	21.37	1.79	15	4
HAB-7	35.5	8.69	21.25	1.00	20	4
HAB-25	31.0	7.44	18.25	0.94	19	3
$LSD_{0.05}$	**6.17[1]**	**2.81[1]**	**ns**	**ns**	**6.1[1]**	**1.1[1]**

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

Discussion

Mungbean, *Vigna radiata* (L.) Wilczek is an important pulse crop of Pakistan cultivated on 140.8 million hectares with an annual production of 93.0 million tons (Anon, 2012). The crop is infected by soil borne root infecting fungi such as *Fusarium* spp., *Rhizoctonia solani* Kühn, *Macrophomina phaseolina* (Tasi) Goid and root knot nematode, *Meloidogyne* spp., which produce a root-rot and root-kot disease complex which results in the substantial death of the plant and consequent decrease in yield (Ehteshamul-Haque & Ghaffar, 1994; Bokhari *et al.,* 2013; 2014). In this study, fifteen isolates of *Pseudomonas* isolated from healthy surface of tomato, lemon, orange and grapefruit inhibited the radial growth of the root infecting fungi such as *M. phaseolina, F. solani, F. oxysporum* and *R. solani.* It was noted that different isolates of *Pseudomonas* have different effect of the growth of the test fungi. All the 15 isolates (100%) were effective against *M. phaseolina*, *F. solani* and *F. oxysporum* while against *R. solani*, 14 out of 15 isolates (93.33% of the total isolates) were antagonistic. All the 15 isolates showed antagonistic activity against *M. javanica.*

Bacteria-fungal pathogen interactions is now gaining interest in the area of biocontrol (Haggag, 2008, Rachid & Ahmed, 2005). Such methods involve either biological control or use of plant defense elicitors (Mohamed *et al.*, 2007). Many of the antifungal interactions involved *Pseudomonas* sp. In the present study, epiphytic fluorescent *Pseudomonas* isolated from the healthy fruits and vegetables surface showed significant antifungal and nematicidal activity *in vitro* and *in vivo*. These epiphytic fluorescent *Pseudomonas* were further used as soil drench in mungbean plant, which showed increased in plant growth as well as decrease the infection caused by root infecting fungi, viz; *M. phaseolina, F. solani, F. oxysporum* and *R. solani*. *Pseudomonas* have been reported to produce antibiotic and siderophore which is vital for bio-controlling of diseases. The involvement of substances like siderophore, HCN or antibiotics, seems to play a role in the active inhibition of four major root infecting systematic fungi, viz., *M. phaseolina, F. solani, F. oxysporum* and *R. solani*. Whereas, direct mechanism in plant growth promotion involves the production of plant growth hormones, or improvement in plant nutrient uptake (Glick, 1995; Kloepper, 1993). Raaijmakers & Weller (1998) reported the role of 2, 4-diacetylphloroglucinol an antifungal metabolite from species of fluorescent *Pseudomonas* in plant root disease suppression. Ganeshan & Kumar (2005) reported control of root rot disease complex caused by *M. phaseolina* and cyst nematode, *Heterodera cajani* in *Vigna mungo*.

In this study, the epiphytic fruit *Pseudomonas* greatly increased the plant height and produced better root growth. Increased root development means increased nutrient uptake by the plant and some PGPB are known to increase the root growth by the production of indole-3-acetic acid (Barbiei & Galli, 1993; Srinivasan *et al*., 1996). In this study, application of *Pseudomonas* also improved root nodulation in mungbean, which is in conformation with our previous study (Noreen *et al*., 2015; Izhar *et al.*, 1995). The study has revealed that epiphytic fluorescent *Pseudomonas* of healthy fruits and vegetables can inhibit the mycelium growth of pathogenic fungi and could be used as biocontrol agent against root infecting fungi and root knot nematode. These epiphytic *Pseudomonas* could be developed into a valuable crop management tool against soil borne root infecting fungi and parasitic nematodes.

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