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BIOCONTROL AND PLANT GROWTH PROMOTING POTENTIAL OF ENDO-NODULE FLUORESCENT *PSEUDOMONAS* AND RHIZOBIA ASSOCIATED WITH ROOT NODULES OF *LEUCAENA LEUCOCEPHALA*

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ABSTRACT

Rhizobia and fluorescent *Pseudomonas* associated with root nodules were studied for their *in vitro* activity against root rotting fungi and root knot nematode and their ability to promote plant growth by production of phytohormones. *Leucaena leucocephala* root nodules were collected from experimental field of University of Karachi and were used to isolate fluorescent *Pseudomonas* and rhizobia. Antifungal and nematicidal activity, cyanide and siderophore producing ability, phosphate solubilizing capacity and indole acetic acid and producing volatile antifungal compounds were tested. Antifungal volatile compounds were produced by all fluorescent *Pseudomonas. R*hizobia isolates inhibited growth of *Fusarium solani*, *Macrophomina phaseolina* and *F. oxysporum,* while fluorescent five isolates of *Pseudomonas* and one rhizobial isolate inhibited growth of *Rhizoctonia solani.* All isolates of bacteria showed activity against the second stage juveniles of *Meloidogyne javanica.* Four isolates of rhizobia and three isolates of fluorescent *Pseudomonas* produced siderophore. Cyanide was produced by two isolates of rhizobia and all isolates of fluorescent *Pseudomonas.* Phosphate solubilization was also observed in two isolates of rhizobia and three isolates of fluorescent *Pseudomonas.*

KEYWORDS: Volatile antifungal compounds, Rhizobia, Fluorescent *Pseudomonas,* siderophores, Phosphate solubilization, Cyanide, Root rotting fungi.

INTRODUCTION

Rhizobia are known to have association with leguminous plants' root nodules to fix nitrogen which plays a vital role in sustaining agricultural system (Howieson *et al.*, 2007). Other than rhizobia, some other bacteria are also found to be associated with the root nodules (Martinez-Hedalgo and Hirsch, 2017; Ibáňez *et al.*, 2009; Muresu *et al.*, 2008; Mahood and Athar, 2017) which are recognized as PGPR (plant growth promoting rhizobacteria). Plant growth promoting microorganisms are bacteria inhabiting the soil that colonizes roots and used to increase crop production as reviewed by Vessey (2003) and are known to support growth as well as health of plant by number of mechanism (Drogue *et al.,* 2012).

PGPR which are known to be associated with roots i.e., *Pseudomonas, Bacillus, Enterobacter* and *Microbacterium* are able to fix nitrogen (Park *et al.,* 2005), sequester

iron (Susilowatl *et al.,* 2011) phytohormones producer (Masciarelli *et al.,* 2014), inorganic phosphate solubilizer (Sharma *et al.,* 2012), and fungal or viral pathogens suppresser (Susilowatl *et al.,* 2011; Wahyudi *et al.,* 2010a,b; Khalimi and Suprata, 2011). Suppression of root rotting fungi by rhizobia has been frequently studied (Batool *et al.,* 2013; Ehteshamul-Haque and Ghaffar, 1993) along with endo-nodule fluorescent *Pseudomonas* (Noreen *et al.,* 2015; 2016). The effect of indigenous rhizobial effect can be overcome by using specific PGPR with rhizobia (Aung *et al*., 2013)**.**

Fluorescent *Pseudomonas* are acknowledged as active colonizers of roots belonging to extensive range of crop and have suppressive activity against root infecting fungi (Bokhari *et al.,* 2014; Habiba *et al.,* 2016; Rahman *et al.,* 2016; Siddiqui *et al.,* 2000; Siddiqui and Ehteshamul-Haque, 2001), and produce induced-systemic-resistance in plants (Verma *et al.,* 2013; Shafique *et al.,* 2015; Mishra *et al.,* 2014). Fluorescent *Pseudomonas* associated with plant roots are known to produce antifungal metabolites which cause reduction in soil associated fungal pathogens (Afzal *et al.,* 2013; Rahman *et al.,* 2016; 2017; Siddiqui *et al.,* 2000). Fluorescent *Pseudomonas* associated with root nodules is also attaining attention for its biocontrol property (Issar *et al.,* 2012; Batool *et al.,* 2013; Noreen *et al.,* 2015). In our previous study we have reported the biocontrol potential of nodules linked fluorescent *Pseudomonas* and rhizobia against root infecting fungi and root knot nematode (Noreen *et al.,* 2015). The present report describes the production of antimicrobial compounds such as cyanide, volatile antifungal compounds, phosphate solubilizing capability and plant growth regulators by *Pseudomonas* and rhizobia.

MATERIALS AND METHODS

Collection of plant material for the isolation of fluorescent *Pseudomonas* and Rhizobia: In recent study, culture of fluorescent *Pseudomonas* and rhizobia were isolated from nodules of *Leucaena leucocephala* (Lam.) de Wit roots grown-up in experimental fields at the University of Karachi.

Isolation and antifungal and nematicidal activity of fluorescent *Pseudomonas* and rhizobia from root nodules: Isolation and purification of bacterial cultures was done as explained in Noreen *et al.* (2015). The antifungal activity of bacterial strains was determined by dual culture plate method (Ji *et al.,* 2014), while nematicidal activity was done by method explained by Cayrol *et al.* (1989).

Determination of pathogen suppressive siderophores by *Pseudomonas* and rhizobia: Production of pathogen suppressive siderophores by root nodules associated fluorescent *Pseudomonas* and rhizobia of *Leucaena leucocephala* as determined by universal chemical assay for siderophores (Schwyn and Neilands, 1987). Each test bacterium was streaked onto a plate containing Chrome Azurol s medium, kept at 26±5°C in completely randomized design and replicated four times. Experiment was repeated three times. A clear orange/red halo formation after 3-4 days indicated the production of siderophore.

Determination of indole acetic acid production by *Pseudomonas* and rhizobia: Test bacteria were grown on King's B broth (*Pseudomonas*), yeast extract mannitol broth (Rhizobia) and 10mM phosphate glucose broth at 28±1ºC for 48 h in triplicates and was repeated twice. The broths were taken and centrifuged for 5 min at 10000 rpm. One mL supernatant was mixed with 1 mL of Salkowski's reagent and permitted to stand at room

temperature for 30 min. Optical density was measured spectroscopically at 530 nm (Patten and Glick 2002; Tariq *et al.,* 2009). The concentration of IAA in each sample was determined from standard curve of IAA (5-50 µg/mL).

Assay for cyanide production of *Pseudomonas* and rhizobia: Cyanide was detected by the procedure presented by Bakker and Schippers (1987). Petri plates having 10% Trypticase soy agar (TSA. Difco, Franklin Lakes NJ USA) accompanied with 4.4 g of glycine per liter were inoculated with bacteria and upturned after a piece of filter paper, infused with 0.5% picric acid ($C_6H_3N_3O_7$) and 2% sodium carbonate (Na_2CO_3) was positioned on the cover of each Petri dish, each bacterium was replicated four times while experiment was repeated twice. The plates were incubated at 28^{o}C for 3 to 5 days. Change in color of filter paper from yellow to orange-brown indicated cyanide production.

Phosphate solubilization ability of *Pseudomonas* and rhizobia: The bacteria were streaked onto Pikavskaya agar medium in order to determine the solubilization of phosphate (Pikovskaya, 1948). These plates were incubated at 28^{o}C for 5 days. The colonies forming clear halos were recorded as phosphate solubilizers. Each test was replicated four times while experiment was repeated two times.

Production of volatile antifungal compounds by *Pseudomonas* and rhizobia: The production of volatile antifungal compounds was assessed by a sealed plate method as designated by Fiddman and Rossal (1993). Cultures of rhizobia and *Pseudomonas* were grown on YEM broth and KB broth for 72 h, 20 µL were poured on YEM and KB medium in a petri dish. For 24 h at 37^{o}C plates were incubation, another Petri dish having PDA was inoculated with a 5 mm plug of the test fungus in the middle of the plate and upturned over the bacterial culture. Parafilm was used to seal two plates together and further incubated for 5 days at 25^{o}C. This guaranteed that both organisms were developing in the same atmosphere through physically separated. As a control, a petri dish having agar medium without bacteria was positioned over the PDA medium inoculated with the fungal pathogen. Fungal growth was determined as increase in radial growth of the test fungus for a period of 5 days after 24 h intervals. Each test bacterium was replicated three times.

Analysis of data: For concentration of IAA and volatile antifungal compounds, one way ANOVA was used. Least significant difference (LSD) test at (p=0.05) was measured to compare the means as the follow up of ANOVA (Gomez and Gomez, 1984).

RESULTS

***In vitro* antifungal activity of endo-nodule fluorescent *Pseudomonas* and rhizobia:** Five isolates of fluorescent *Pseudomonas* and five isolates of rhizobia were isolated from root nodules of *Leucaena leucocephala*. All isolates of endo-nodule fluorescent *Pseudomonas* and rhizobia caused growth inhibition of all the four test fungi *M. phaseolina, R. solani, F. solani* and *F. oxysporum* and produced zones of inhibition. Several isolates also caused the lysis of fungal hyphae (Table 1).

Table 1. *In vitro* growth inhibition of *Macrophomina phaseolina, Rhizoctonia solani, Fuasrium solani* and *F. oxysporum* by endo-nodule fluorescent *Pseudomonas* (NAFP) and rhizobia (NFB) isolated from *Leucaena leucocephala* and juvenile mortality of *Meloidogyne javanica* by cell free culture filtrates.

Name of bacteria	Zone of inhibition (mm)				Juvenile mortality (%)	
	M. phaseolina	*R. solani*	*F. solani*	*F. oxysporum*	24 hours	48 hours
NAFP-34	14*	0	21	14*	100	100
NAFP-37	0	2	20	25	91.6	95
NAFP-40	13*	5	11	20	100	100
NAFP-43	0	5	27	23	100	100
NAFP-45	0	10	27	25	90	100
Rhizobium leucaenae (NFB-117)	13*	5	8	13	91.6	100
R. leucaenae (NFB-119)	27	5	11	10	96.6	100
R. leucaenae (NFB-122)	8	4	23	26	100	100
R. leucaenae (NFB-123)	5	4	25	20	100	100
R. leucaenae (NFB-127)	10*	0	25	28	100	100
$LSD_{0.05}$	**2.01**	**0.76**	**2.21**	**0.77**	**1.99**	**2.21**

* = Lysis of mycelium

***In vitro* nematicidal activity of endo-nodule fluorescent *Pseudomonas* and rhizobia:** Culture filtrates of fluorescent *Pseudomonas* showed significant activity against nematodes by killing the second stage juvenile of *M. javanica* to inconsistent degrees. Isolates of fluorescent *Pseudomonas* namely NAFP-34, NAFP-40, NAFP-43 and NAFP-45 showed 100% death of second stage juveniles of *M. javanica*. While all isolates of rhizobia showed 100% death of second stage juveniles (Table 1).

***In vitro* siderophore production by endo-nodule fluorescent *Pseudomonas* and rhizobia:** Out of five isolates tested, three isolates of endo-nodule fluorescent *Pseudomonas* showed positive result by the development of an orange/red halo around the bacterial colony of isolates NAFP-37 and NAFP-43 which produced larger halos than the other isolates. Of 5 isolates of rhizobia, 4 isolates produced an orange/red halo which were *R. leucaenae* (NFB-117) and *R. leucaenae* (NFB-127) which produced larger halos than other isolates of rhizobia (Table 2; Fig. 1).

Cyanide production and phosphate solubilization activity of endo-nodule fluorescent *Pseudomonas* and rhizobia: All the isolates of endo-nodule fluorescent *Pseudomonas* tested for the production of cyanide showed a positive result by changing the color of filter paper from yellow to orange. Among the 5 isolates of rhizobia, 2 isolates changed the color of filter paper (Table 1; Fig. 2). Of the 5 isolates of endo-nodule fluorescent *Pseudomonas* tested for phosphate solubilizing ability, 3 isolates formed a clear halo around the bacterial colony, which indicated their ability to solubilize phosphate. Similarly, among the 5 isolates of rhizobia, 2 isolates formed a clear halo around the bacterial colony (Table 2).

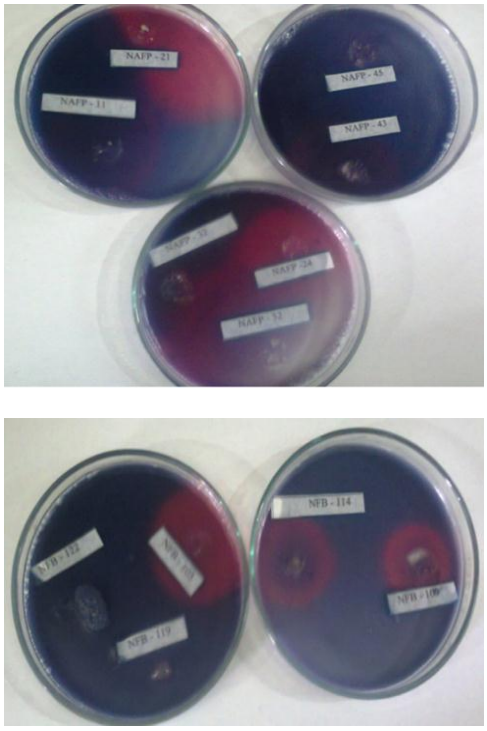

Fig. 1. Siderophore production by isolates of fluorescent *Pseudomonas* and R*hizobia*: orange/red halo around bacterial colony indicates the siderophore production.

***In vitro* indole acetic acid (IAA) production by endo-nodule fluorescent *Pseudomonas* and rhizobia:** All the 5 isolates of endo-nodule fluorescent *Pseudomonas* and rhizobia each showed production of IAA on KB, YEM and phosphate glucose broth. The highest concentration of IAA was produced by NAFP-45. The concentration of IAA ranged from 5.25-10.25 µg/mL for fluorescent *Pseudomonas* while for rhizobia the concentration ranged from 4.25-10 µg/mL. The concentration of IAA in phosphate glucose broth ranged from 5.62-14.25 µg/mL for fluorescent *Pseudomonas* and for rhizobia, it ranged from 8.5-14.25 µg/mL (Table 2).

Production of volatile antifungal compounds by endo-nodule fluorescent *Pseudomonas* and rhizobia: All the isolates of endo-nodule fluorescent *Pseudomonas* tested for production of antifungal volatile compounds were able to diminish the growth of plant pathogenic fungi *M. phaselonia, R. solani, F. solani* and *F. oxysporum.* Similarly, all the isolates of rhizobia tested for production of antifungal volatile compounds were able to reduce the growth of plant pathogenic fungi *M. phaselonia, F. solani* and *F. oxysporum.* Out of the 5 isolates of rhizobia tested, only one isolate was able to diminish the growth of *R. solani* while all the isolates were able to cause degradation of the mycelial growth of *R. solani* and causing the thinning of mycelial growth of fungi (Table 3).

Table 2. *In vitro* production of siderophore, cyanide, indole acetic Acid (IAA) and phosphate solubilization by nodule associated fluorescent *Pseudomonas* (NAFP) and rhizobia isolated from *Leucaena leucocephala*.

Culture # of *Pseudomonas*	Siderophore production	Cyanide production	Phosphate solubilization	Concentration of IAA (µg/mL)	
				King's B broth	Phosphate glucose broth
NAFP-34	*	+	+	5.25	5.62
NAFP-37	***	++	-	6	8.35
NAFP-40	-	+	-	5.25	6.75
NAFP-43	***	++	+	6.75	7.42
NAFP-45	-	++	+	10.25	14.25
				0.53[1]	0.39[1]
Rhizobium leucaenae (NFB-117)	***	-	-	7.5	18
R. leucaenae (NFB-119)	-	+	-	5.5	10.25
R. leucaenae(NFB-122)	*	++	+	4.25	14.25
R. leucaenae (NFB-123)	**	-	+	10	15
R. leucaenae (NFB-127)	***	-	-	5.75	8.5
$LSD_{0.05}$				**0.23[1]**	**0.39[1]**

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

For siderophore production

*** Large size of halo present around bacterial culture

** Medium size of halo present around bacterial culture

* Small size of halo present around bacterial culture

- No halo present around bacterial culture

For cyanide production

++ = Brown color

+ = Orange color - = no change in color

For Phosphate Solubilization

+ = Clear zone formation

- = No zone

DISCUSSION

In the current study, 5 isolates each of fluorescent *Pseudomonas* and rhizobia isolated from root nodules of *Leucaena leucocephala* were tested for production of siderophore, cyanide, indole acetic acid and phosphate solubilizing capacity because, bacteria possessing these traits are known to able increase plant growth and suppress plant disease (Bakker and Schippers 1987; Glick 1995; Glick *et al.*, 1995; Farhat *et al*., 2017; Iqbal *et al*., 2017). PGPR are known to produce siderophore which confiscate most of available Fe^{3+} in the rhizosphere and causes the starvation of iron for the pathogens, hence it is a main contributor that causes suppression of pathogen (O'Sullivan and O'Gara, 1992). In our study, three isolates of fluorescent *Pseudomonas* out of five formed orange/red halo around the colony (+ve result) while in case of rhizobia four isolates out of five showed positive result for siderophore production. Carson *et al.* (2000) reported that synthesis of siderophores is limited to a specific range of rhizobial strains rather than wide distribution. Inoculation of soybean

and chickpea (*Cicer arietinum* L.) seeds with a siderophore producing fluorescent *Pseudomonas* resulted in increased plant growth, yield and seed germination (Kumar and Dube, 1992). In this study, all fluorescent *Pseudomonas* isolates showed positive result for cyanide production while two rhizobial isolates were able to produce cyanide in *in vitro* testing. Voisard *et al.* (1989) reported, *Pseudomonas fluorescens* CHAO strain has the cyanide production characteristic which showed antagonistic activity against *Thielaviopsis basicola* causing tobacco's black root rot. Bhatia *et al.* (2003) found that volatile cyanide production exhibited the sclerotial germination of *M. phaseolina*, but some of the researchers have observed detrimental consequence of cyanide on the plant growth (O'Sullivan and O'Gara, 1992).

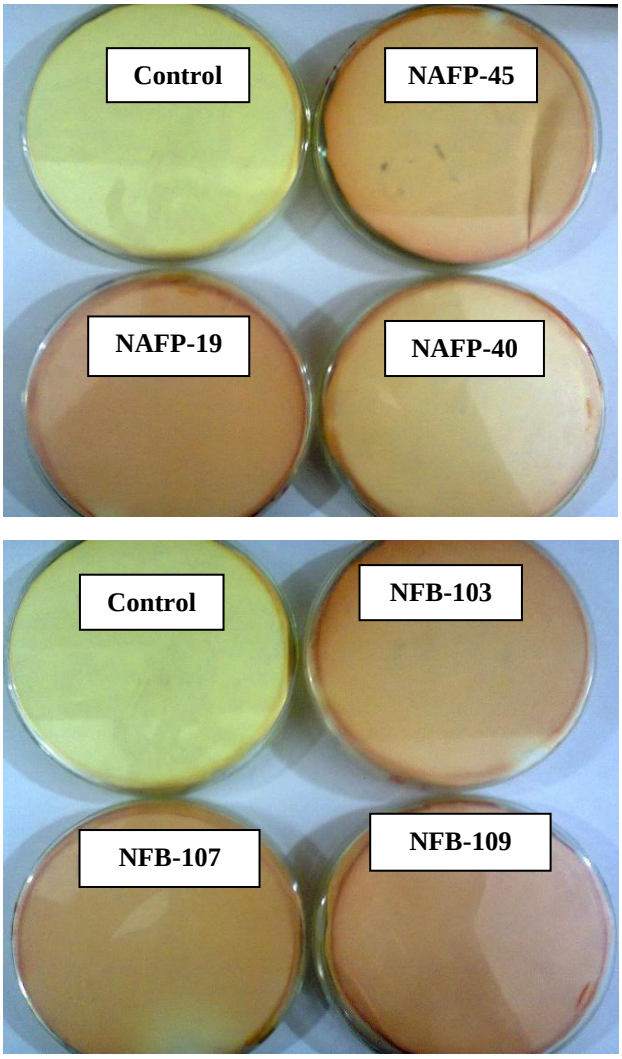

Fig. 2. Cyanide production by isolates of fluorescent *Pseudomonas* and *Rhizobia* on TSA (Trypticase soy Agar).

In this study, 40% fluorescent *Pseudomonas* and 60% rhizobial isolates have the phosphate solubilizing ability. Arora *et al.* (2001) stated that several rhizobia can solubilize inorganic phosphate from the soil. Yield of crop increased significantly because of the presence of phosphorous which was solubilized by the soil bacteria (Brown, 1974). In this study, the concentration of IAA ranged from 5.25-10.25 µg/ml for fluorescent *Pseudomonas* while for rhizobia the concentration ranged from 4.25-10 µg/ml which is similar to the result found by Zafar *et al.* (2012). In phosphate glucose broth, concentration of IAA ranged from 5.62-14.25 µg/ml for fluorescent *Pseudomonas* and for rhizobia it ranged from 8.5-14.25 µg/ml. Mehnaz *et al.* (2010) reported that PGPR from the maize (*Zea mays* L.) rhizospheric region produced IAA in the range of 0.28–5.49 µg mL^{-1}. Among different strains of bacteria, the amount of IAA production varies which can be ascribed to numerous regulatory sequences, biosynthetic pathways and occurrence of enzymes to transform active free indole acetic acid into conjugated forms (Patten and Glick, 1996).

Table 3. Percentage growth inhibition of fungi by volatile compounds by selected isolates of fluorescent *Pseudomonas* and rhizobia.

Bacterial isolates	Growth of fungi (mm)			
	Macrophomina phaseolina	***Rhizoctonia solani***	***Fusarium solani***	***Fusarium oxysporum***
Control	9^{a}	9^{a}	8 ± 0.1^{a}	8.4^{a}
NAFP-34	8.33 ± 0.15^{cde} (7.44)	8.9 ± 0^{b} (1.1)	6.53 ± 0.05^{bcd} (18.37)	7.43 ± 0.20^{efg} (11.54)
NAFP-40	8.06 ± 0.11^{fg} (10.44)	8.76 ± 0.15^{c} (2.66)	6.2 ± 0.2^{d} (22.5)	$7.8\pm0b^{c}$ (7.14)
NAFP-37	8.53 ± 0.05^{bc} (5.22)	8.9 ± 0^{b} (1.1)	6.5 ± 0.5^{bcd} (18.75)	7.43 ± 0.11^{efg} (11.54)
NAFP-43	8.6 ± 0.1^{bc} (4.4)	8.9 ± 0^{b} (1.1)	6.9 ± 0.2^{bc} (13.75)	7.63 ± 0.37^{bcdef} (9.16)
NAFP-45	8.7 ± 0.1^{b} (3.3)	8.9 ± 0^{b} (1.1)	6.2 ± 0.1^{d} (22.5)	7.76 ± 0.15^{bcd} (7.61)
$LSD_{0.05}$	**0.23^{1}**	**0.06^{1}**	**0.44^{1}**	**0.27^{1}**
Control	9^{a}	9	7.86 ± 0.25^{a}	8.4^{a}
Rhizobium leucaenae (NFB-117)	8.5 ± 0.17^{bcd} (5.5)	9 ± 0^{a} (0)	6.53 ± 0.25^{bc} (16.92)	7.73 ± 0.20^{cdef} (7.97)
R. leucaenae (NFB-119)	8.5 ± 0^{bcd} (5.5)	8.76 ± 0.05^{c} (2.66)	6.5 ± 0.17^{bc} (17.30)	7.86 ± 0.05^{bcde} (6.42)
R. leucaenae (NFB-122)	8.6 ± 0^{bc} (4.4)	9 ± 0^{a} (0)	7 ± 0.5^{b} (10.92)	7.83 ± 0.20^{bcde} (6.78)
R. leucaenae (NFB-123)	8.5 ± 0.1^{bcd} (5.5)	9 ± 0^{a} (0)	6.33 ± 0.28^{bc} (19.46)	7.9 ± 0^{bcde} (5.95)
R. leucaenae (NFB-127)	8.6 ± 0.2^{bc} (4.4)	9 ± 0^{a} (0)	6.76 ± 0.37^{b} (13.99)	7.3 ± 0.1^{g} (13.09)
R. vignae (NFB-144)	8.56 ± 0.56^{bcd} (4.88)	9 ± 0^{a} (0)	6.66 ± 0.47^{b} (15.26)	7.93 ± 0.05^{bcd} (5.59)
$LSD_{0.05}$	**0.30^{1}**	**0.05^{1}**	**0.66^{1}**	**0.26^{1}**

Each value is mean ± S. D (Standard deviation) of 3 replicates.
Mean values in column showing differences greater than LSD values are significantly different at $p< 0.05$
[1]Mean values in column bearing same subscript are not significantly different at $p<0.05$ different according to Duncan's multiple range test
Values within **Parenthesis** represent percent decrease with respect to control

In the current study, all isolates of fluorescent *Pseudomonas* were able to produce volatile antifungal compounds which subdued the growth of root infecting fungi *M. phaseolina, F. solani, F. oxysporum* and *R. solani*. All isolates of rhizobia showed an inhibitory effect on *M. phaseolina, F. solani* and *F. oxysporum*; while only one isolates of rhizobia showed inhibition of *R. solani* mycelium. Quite a few studies have revealed the significance of volatiles in the biocontrol potential of bacteria against different plant diseases (Gagne *et al.,* 1991; Shafique *et al*., 2017). The present study confirmed the biocontrol potential of fluorescent *Pseudomonas* associated with root nodules of *L. leucocephala* against root rotting fungi and root knot nematode.

CONCLUSION

In conclusion, this study has revealed that root nodule associated fluorescent *Pseudomonas* and rhizobia not only have suppressive effect on root knot nematode and root infecting fungi but also have the ability to produce siderophores, cyanide, indole acetic acid, volatile antifungal compounds as well as have the ability to solubilize phosphate. On the basis of these *In vitro* testing these cultures can be developed into a valuable crop management tool against soilborne root infecting fungi and parasitic nematodes.

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