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ROLE OF FLUORESCENT *PSEUDOMONAS* ASSOCIATED WITH ROOT NODULES OF MUNGBEAN IN THE INDUCTION OF NODULATION BY THE RHIZOBIA IN MUNGBEAN

RUBINA NOREEN[1], SYED ABID ALI[2], KHWAJA ALI HASAN[2], HABIBA[3], FAIZAH UROOJ[1], AMNA TARIQ[3] JAHAN ARA[3] AND SYED EHTESHAMUL-HAQUE[1]

[1] *Agricultural Biotechnology & Phytopathology Laboratory, Department of Botany, University of Karachi, Karachi-75270, Pakistan*
[2]*HEJ Research Institute of Chemistry & ICCBS, University of Karachi, Karachi-75270, Pakistan*
[3]*Postharvest & Food Biochemistry Laboratory, Department of Food Science & Technology, University of Karachi, Karachi-75270, Pakistan*
[*]*Corresponding author's email: sehaq@uok.edu.pk*

Abstract

Fluorescent *Pseudomonas* has been reported to associate with root nodules of a number of plants and play role in plant disease suppression and increased plant growth. However their role in nodule formation is still unclear. In a gnotobiotic experiment, combined effect of two isolates each of fluorescent *Pseudomonas* (NAFP-19 and NAFP-32) and rhizobia (*Rhizobium vignae* (NFB-103) and *R. vignae* (NFB-109) on nodules of mungbean was evaluated. The results showed an increase in the number of nodule primordia, mature nodules and the concentration of nitrogen and phosphorus in mungbean plants. In screen house experiment, garden soil which was naturally infested with root rotting fungi and rhizobia was used. Dual application of *Pseudomonas* and rhizobia in the garden soil produced greater number of nodules per plant and higher nitrogen fixation than when rhizobia were used alone. Dual application of bacteria also suppressed root rotting fungi. The results showed that nodule associated fluorescent *Pseudomonas* played a role in plant-rhizobia symbiosis.

Key words: Rhizobia, Fluorescent *Pseudomonas,* Nodulation, Biocontrol, Root rotting fungi, Mungbean.

Introduction

In Leguminous plants that associate with rhizobia and form nitrogen fixing symbiosis are an important part of agricultural system (Howieson *et al.,* 2008). Atmospheric nitrogen is fixed in nodules that develop on plant roots. The interaction of root nodule starts when rhizobia are attracted and attached to the tips of root hairs, causing curling and deformation (Chandra *et al.,* 2007). Rhizobia form infection threads while passing through epidermis. They reach cortical region of roots. The cortical cells undergo rapid cell division. As a result nodule primordia are formed which ultimately develop into mature nodules (Pueppke, 1986). Rhizobia absorb nutrients from leguminous plant in turn convert nitrogen to ammonia inside the root nodules of the host plant (Spaink, 2000). Association of non-rhizobia with root nodules have also been reported (Martinez-Hidalgo & Hirsch, 2017; Noreen *et al.,* 2015ab). Rhizobia were also found to suppress growth of root rotting fungi (Ehteshamul-Haque&Ghaffar, 1993; Noreen *et al.,* 2015ab; 2016).

In rhizosphere, certain free living bacteria are known to enhance plant growth, and are called as plant growth-promoting rhizobacteria (PGPR) (Kloepper *et al.,* 1980). Among (PGPR) fluorescent *Pseudomonas* are known as aggressive colonizers of the roots of a wide range of crop plants they show antagonistic activity against plant pathogens infecting plant roots (Bokhari *et al.,* 2014; Habiba *et al.,* 2016; Siddiqui *et al.,* 2000; Korejo *et al.,* 2017), They induce systemic resistance against pathogens infecting plant roots or shoots (Rahman *et al.,* 2016; Shafique *et al.,* 2015). Plant root associated fluorescent *Pseudomonas* are known to suppress soilborne fungal pathogens by the production of antifungal metabolites and with their biocontrol potential (Rahman *et al.,* 2016; Afzal *et al.,* 2013; Siddiqui & Ehteshamul-Haque, 2001). The biocontrol potential of root nodules associated with fluorescent *Pseudomonas* is also gaining attention (Noreen *et al.,* 2015a; Issar *et al.,* 2012). In our previous study we have reported the biocontrol potential of mungbean root nodules associated fluorescent *Pseudomonas* and rhizobia against root rotting fungi and root knot nematode (Noreen *et al.,* 2015ab). It was also reported that number of nodules in chickpea was greater in plants treated with both rhizobia and fluorescent *Pseudomonas* than treated with rhizobia alone (Noreen *et al.,* 2016). In the present report the role of fluorescent *Pseudomonas* associated with root nodules of mungbean, in nodule formation by the rhizobia in mungbean is described. Biocontrol potential of these bacteria against root rotting fungi has also been discussed.

Materials and Methods

Bacterial cultures and molecular identification: Bacterial cultures used in present study were originally isolated from root nodules of mungbean and molecular identification of fluorescent *Pseudomonas* has been previously reported (Noreen *et al.,* 2015a). In this study16S rDNA amplification and amplified ribosomal DNA restriction analysis (ARDRA) were adopted as molecular tools for identification and taxonomic characterization of rhizobial isolates. *In-silico* restriction digest of 16S rDNA from *Rhizobium* sp. CCBAU 21244 (GenBank: AY555768.2) was used for restriction endo-nuclease selection aided by SnapGene Viewer 2.2.2. The electropherogram was analyzed by PyElph1.4 software (Pavel &Vasile, 2012).

Strains were cultured overnight in 1.5mL yeast mannitol broth and cells were centrifuged at 10,000g for 10min (Hanil, Korea) at room temperature. Pallets were washed and re-suspended in 250µL autoclaved de-ionized water. DNA was extracted by cell lysis at 100ºC on a heat block (thermo mixer comfort; Eppendorf Germany). The 16S rRNA gene was amplified with universal primer set BAC-27F (5'-AGA GTT TGA TCC TGG CTC AG- 3') and 1488R (5'-CGG TTA CCT TGT TAG GAC TTC ACC- 3'). PCR reactions were accomplished in a 20µL volume having 2.5 µL lysate, 0.5µL of 16µM for each primer, 6.5µL nuclease free water and 10µLof 2x Top-Taq master mix kit (Qiagen, Germany). Thermal cycling was performed in a master cycler *ProS* (Eppendorf, Germany) with the following conditions. Initial denaturation step: 3min at 94ºC followed by 35 cycles of denaturation; 30s at 94ºC, annealing; 30s at 58ºC, extension; 1min at 72ºC and with a final extension at 72ºC for 10 min (Rajesh *et al.,* 2012). A 10µL PCR product of the 16S rDNA gene was digested separately with fast digest *Hae-III* (*BsuRI*), (Fermentas, USA). 16S rDNA amplicons and ARDRA products were subjected to 2% agarose gels having 0.5µg/mL ethidium bromide. 100bp, ultralow and 1kb DNA marker were used as standards (GeneRuler DNA Ladder, Fermentas USA). For intra and inter-strain relationship between rhizobial isolates, the 16S rDNA-RFLP electropherogram was analyzed and a dendrogram was constructed by PyElph1.4 software as described by Pavel & Vasile (2012).

Germination of mungbean seeds, inoculation of young seedlings with *Pseudomonas* and rhizobia and count of nodule primordia formed in gnotobiotic condition: The method used by Fox *et al*., (2011) was used to study the initiation of nodules. Mungbean seeds were surface sterilized by soaking in 1% Ca $(OCl)_2$and were grown on 1.5% (w/v) water agar plates. The germinated seedlings were transferred into pots containing steam sterilized soil and were inoculated with a mixed culture of *Pseudomonas* and rhizobia as well as each microorganism separately. Non-inoculated N+ (5 mL of 0.5 M KNO_3 weekly) and N- served as control plants. The roots from each group of plants were harvested after 5 days intervals till 25 days. Roots were carefully rinsed with clean water. Roots were placed in potassium hydroxide solution (10% w/v)for 2 h at room temperature, rinsed with water and acidified in 0.25 M HCl for 5 min and stained in Brilliant green (0.1%) for 30 min. Roots were de-stained by placing them in water. Nodule primordia were counted under a dissecting microscope at 20X (Cheng *et al.,* 2002). The experiment was conducted twice with four replicates.

Combined effect of *Pseudomonas* and rhizobia on the growth of mungbean and incidence of root infecting fungi in screen house experiment: Sandy loam soil (pH 8.0) obtained from experimental field of the Botany Department, University of Karachi, was transferred into 12 cm diameter earthen pots with 1 kg of soil per pot. The soil had a natural infestation of *Macrophomina phaseolina* (4-13 sclerotia g^{-1} of soil) was determined by the wet sieving and dilution technique (Sheikh & Ghaffar, 1975), 5-12% colonization of *Rhizoctonia solani* on sorghum seeds used as bait (Wilhelm, 1955) and a mixed population of *Fusarium solani* and *Fusarium oxysporum* (3500 cfu g^{-1} of soil) as determined by soil dilution technique (Nash & Snyder, 1962). Six mungbean {*Vigna radiata* (L.) Wilczek} seeds were sown in each pots after applying 25 mL (10^8cfu/mL) bacterial suspension of NAFP-19 (3.3×10^8 cfu/mL), NAFP-32 (2.5×10^8 cfu/mL), NAFP-31 (1.4×10^8 cfu/mL), *Rhizobium vignae*(NFB-103) (3.6×10^8 cfu/mL), *R. vignae*(NFB-107) (3.4×10^8 cfu/mL) and *R. vignae*(NFB-109) (2.2×10^8 cfu/mL) into each pot. Plants that did not receive bacterial suspension served as control. Carbendazim (200 ppm) at 25 mL per pot functioned as positive control against root rotting fungi. Each treatment was replicated four times, randomized in block design and repeated once. After germination, four seedlings were retained in each pot and excess seedlings were uprooted. The experiment was terminated after 45 days and the plant height and fresh weight of the roots and shoots was recorded. Incidence of root infecting fungi were determined as described by Habiba*et al.,* (2016).

Nitrogen estimation: Nessler's method was used to estimate nitrogen content in plant material (Singh, 1982). 0.2 gm of oven dried, powdered leaf material was digested in 2 mL H_2SO_4 (conc.) andH_2O_2 (30%) was poured drop wise until a colorless solution appeared at the base of the flask. The sample was processed till 1-2 mL of it remained. The volume of solution was adjusted to 100 mL with distilled water and filtered. 1 mL of digested sample was taken in a 50mL flask along with 1 mL of NaOH (10%), 1 mL of Na-silicate (10%) and 15 mL of Nessler's reagent. The volume was brought to 50 mL with distilled water followed by incubation for 20 min at room temperature. Absorbance was read on a spectrophotometer at 410 nm against the blank.

A (ppm Nitrogen) = Nitrogen value (ppm) from standard curve × Total dilution factor (T.D.F)

Phosphorus estimation: A modified method of Barton reagent was used to estimate phosphorus content presented by Rayan *et al.,* (2001). 0.2 g of oven dried, powdered leaf material was digested in 2 mL H_2SO_4 (conc.) to which 30% solution of H_2O_2 was poured drop wise until a colorless solution appeared at the base of the flask. The sample was processed until 1-2 mL of digest remained. Then the volume was adjusted to 100 mL with distilled water and filtered. In 100 mL conical flask, 10 mL of digested sample was added along with10 mL Barton reagent. The volume was brought to 100 mL with distilled water followed by incubation for 30 min at room temperature. Absorbance was read on a spectrophotometer at 420 nm against the blank.

A (ppm Phosphorus) = Phosphorus value (ppm) from standard curve × Total dilution factor (T.D.F)

Analysis of data

For plant growth parameters one way ANOVA was used while two way anova was used to determine the mean of infection of fungal pathogens and among the treatments. Least significant difference (LSD) test at (p=0.05) was measured to compare the means as the follow up of ANOVA (Gomez & Gomez, 1984).

Results

Molecular identification of rhizobial isolates: Rhizobial isolates were identified by amplification of 16S rDNA and the estimated amplicons lengths were in the range of approximately 1300-1500bp when compared with the standard DNA ladder. The variation in gene lengths of 16S rDNA in between rhizobial isolates reveals promising separation value and power of discrimination among the isolated strains (Fig. 1a). RFLP analysis of the 16S rDNA gene products reveals the phylogenetic relations between isolates when digested with *Hae-III* (*BsuRI*) restriction enzyme (Fig. 1b). The restriction fragment weights were estimated manually and PyElph1.4 software in comparison with standard DNA markers. The restriction patterns were applied to construct a dendrogram and infer relationships between rhizobial isolates by the neighbor joining method representing the clustering of the various isolates on the basis of similarity index of the 16S rRNA gene. A dendrogram constructed from amplified ribosomal DNA restriction analysis (ARDRA) confirms that our isolates share a common ancestral and taxonomic relationship (Fig. 1c).

Effect of co-inoculation of fluorescent *Pseudomonas* and rhizobia on nodule initiation and development in gnotobiotic condition: Co-inoculation of mungbean with fluorescent *Pseudomonas* and rhizobia increased the shoot and root length as well as root hair curling and nodule initiation (Fig. 2) as well as nitrogen fixation and phosphorus uptake by the plant (Tables 1-5). Root nodules were not formed in plants which were not inoculated with bacteria (Fig. 2A). Nodule initials were evident in roots of inoculated plants after inoculation of 15 days (Table 1). After 25 days (Table 5) there was significant rise in number of nodule initials as well as nodules on plants. Plants co-inoculated with NAFP-32+*R. vignae* (NFB-109) produced significantly greater number of nodules compared to those plants which were inoculated with NAFP-19, NAFP-32, *R. vignae* (NFB-103) and *R. vignae* (NFB-109) individually (Table 5; Fig. 2). The nodule development was enhanced by co-inoculation of mungbean with fluorescent *Pseudomonas* and rhizobia. A significant increase in nitrogen fixation as well as phosphorus uptake by plants inoculated with fluorescent *Pseudomonas* and rhizobia was also observed.

Effect of co-inoculation of fluorescent *Pseudomonas* and rhizobia under screenhouse condition: Number of nodules per plant were found higher in plants received mixed application of rhizobia and fluorescent *Pseudomonas* compared when they were used singly. Although plants treated with rhizobia or *Pseudomonas* separately produced greater number of nodules compared to control plants but it was non-significant. Maximum number of nodules was observed in plants treated with combination of NAFP-31 and *R. vignae* (NFB-109). Combined treatment by NAFP-32 and *R. vignae* (NFB-103) resulted in the maximum plant height, while maximum shoot weight was observed in plants treated with NAFP-19 followed by combined treatment of NAFP-19 and *R. vignae* (NFB-109). Maximum root length was observed in plants treated with *R. vignae* (NFB-109) used with NAFP-31 (Table 6).

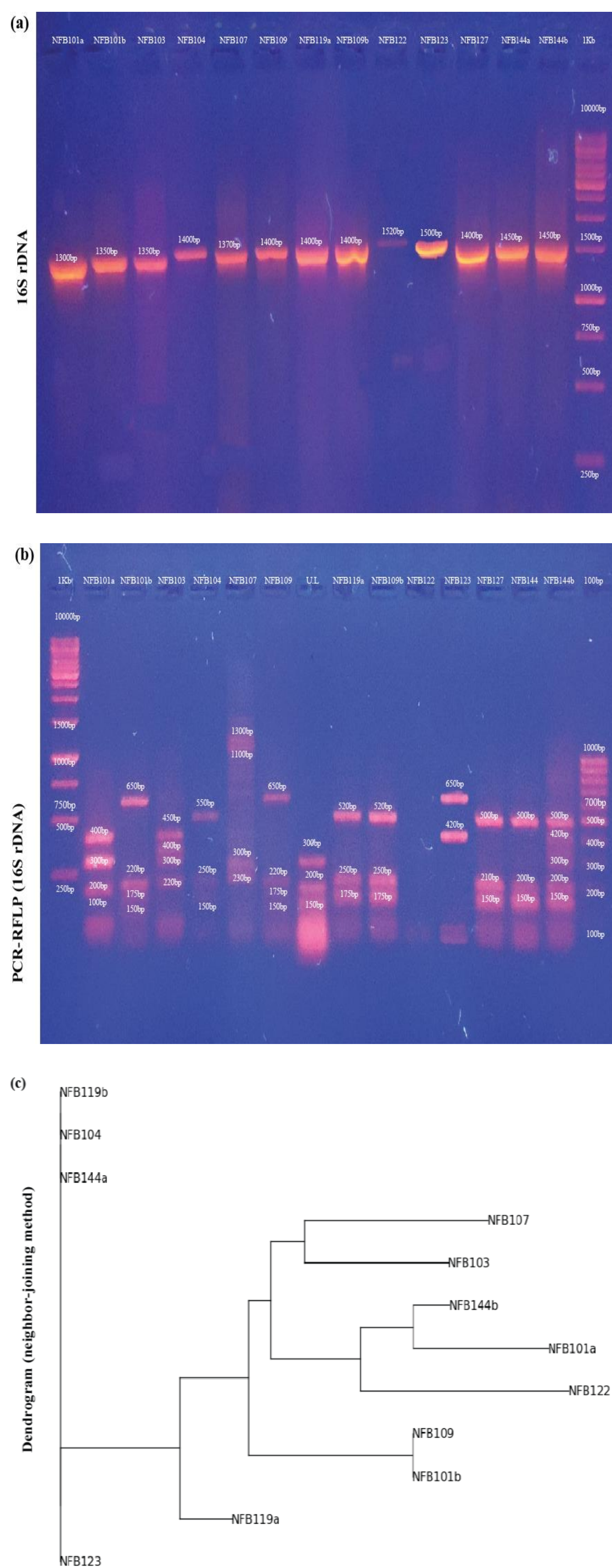

Fig. 1. Molecular identification of rhizobialisolates. (a) PCR amplification of 16S rDNA as a molecular marker for identification. (b) Amplified ribosomal DNA restriction analysis (ARDRA) of 16S rDNA gene by the restriction enzyme *Hae-III* (Fermentas, USA). (c) Inferred relationships of rhizobial isolates using the neighbor-joining method by PyElph1.4 software. The reaction products were analyzed on 2% agarose gels containing 0.5µg/mL ethidium bromide. NFB numbers represents rhizobial isolates subjected in this study. 100 bp=100 bp, U.L = ultra low range and 1kb DNA ladders, respectively (Fermentas, USA).

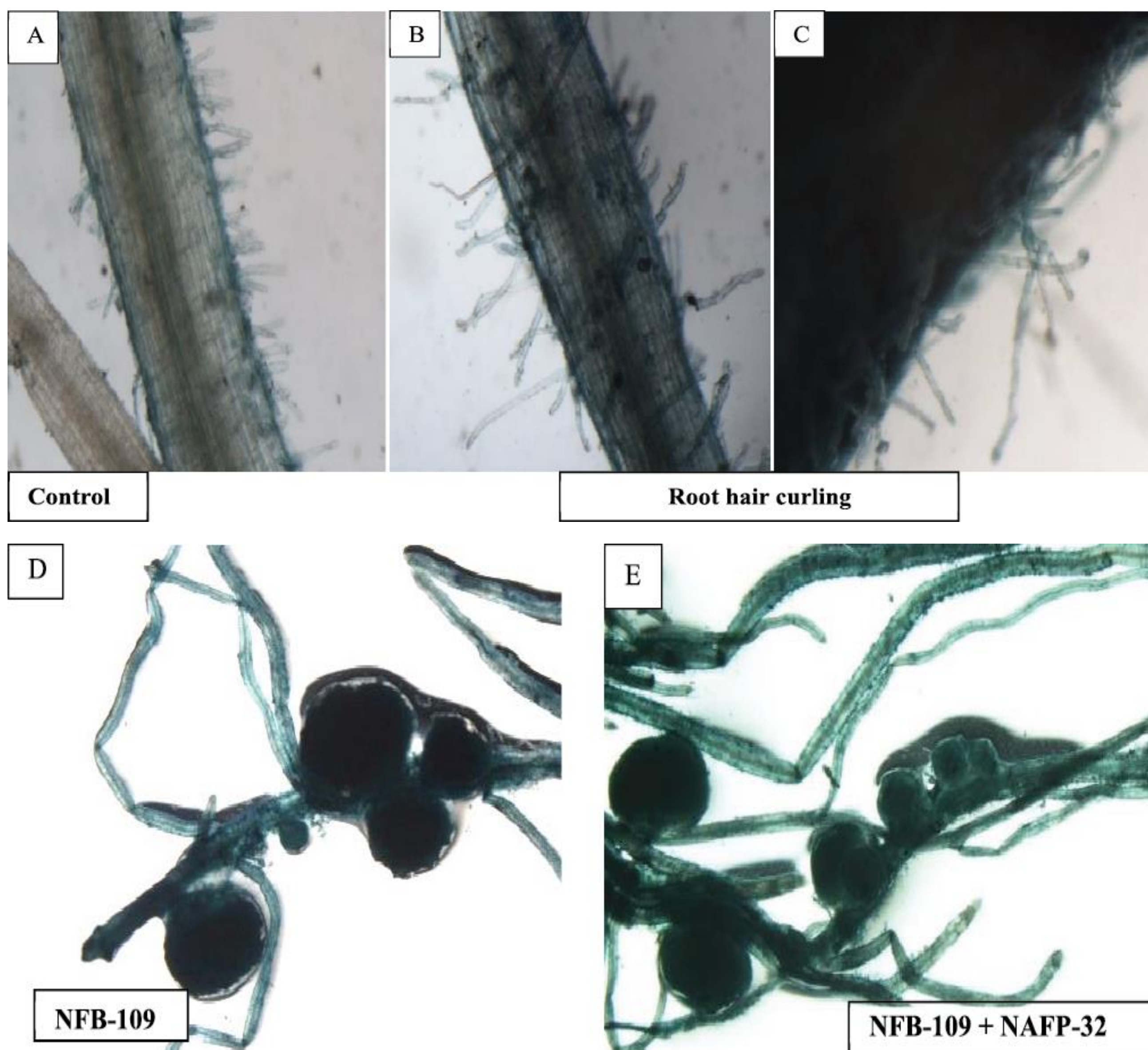

Fig. 2. Effect of co-inoculation of fluorescent *Pseudomonas* and rhizobia on nodule initiation under gnotobiotic condition A= Root hairs of control plants, B&C= Root hair curling in plants treated with rhizobia and fluorescent *Pseudomonas*, D= Nodule formation in plants treated with rhizobia alone, E= nodule formation in plants treated with rhizobia + fluorescent *Pseudomonas.*

Table 1. Effect of fluorescent *Pseudomonas* and rhizobia on vegetative growth, nodulation, nitrogen and phosphorus content of mungbean after 5 days of inoculation.

Treatments	Shoot length (cm)	Shoot mass (gm)	Root length (cm)	Root mass (gm)	Nodule number	Mineral content	
						Nitrogen (ppm)	Phosphorus (ppm)
Control	6.4	0.16	3.37	0.07	0	5.11	0.52
Nitrogen added	5.47	0.12	2.72	0.04	0	7.07	0.87
NAFP-19	8.87	0.17	2.32	0.07	0	6.3	1.22
NAFP-32	7.3	0.15	2.62	0.06	0	6.88	0.39
R. vignae (NFB-103)	6.87	0.13	3.87	0.08	0	5.37	0.88
R. vignae (NFB-109)	7.12	0.12	4	0.07	0	7	0.80
NAFP-19 + *R. vignae* (NFB-103)	6.75	0.12	3.37	0.08	0	5.31	1.14
NAFP-19 + *R. vignae* (NFB-109)	7	0.12	3.5	0.06	0	5.07	1.89
NAFP-32 + *R. vignae* (NFB-103)	6.5	0.13	3.5	0.04	0	6.74	1.84
NAFP-32 + *R. vignae* (NFB-109)	6.5	0.12	4.37	0.09	0	6.21	1.87
$LSD_{0.05}$	1.48[1]	0.07[1]	1.32[1]	0.04[1]	-	0.76[1]	0.67[1]

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

Table 2. Effect of fluorescent *Pseudomonas* and rhizobia on vegetative growth, nodulation, nitrogen and phosphorus content of mungbean after 10 days of inoculation.

Treatments	Shoot length (cm)	Shoot mass (gm)	Root length (cm)	Root mass (gm)	Nodule number	Mineral content	
						Nitrogen (ppm)	Phosphorus (ppm)
Control	8.75	0.30	3.9	0.13	0	5.14	1.09
Nitrogen added	12.5	0.30	3.9	0.17	0	5.51	1.01
NAFP-19	13.25	0.28	4.5	0.13	0	6.8	1.60
NAFP-32	12.75	0.27	5.5	0.14	0	5.25	1.55
R. vignae (NFB-103)	13.75	0.30	5.25	0.19	0	6.02	1.13
R. vignae (NFB-109)	10.5	0.22	5.25	0.14	0	7.48	1.67
NAFP-19 + *R. vignae* (NFB-103)	11.5	0.21	3.25	0.17	0	6.27	1.39
NAFP-19 + *R. vignae* (NFB-109)	11.25	0.21	4.3	0.18	0	6.2	1.28
NAFP-32 + *R. vignae* (NFB-103)	11.25	0.35	6.25	0.18	0	7.61	1.25
NAFP-32 + *R. vignae* (NFB-109)	12.5	0.25	6.75	0.22	0	6.18	1.24
$LSD_{0.05}$	2.35[1]	0.10[1]	2.33[1]	0.09[1]	--	1.71[1]	0.76[1]

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

Table 3. Effect of fluorescent *Pseudomonas* and rhizobia on vegetative growth, nodulation, nitrogen and phosphorus content of mungbean after 15 days of inoculation.

Treatments	Shoot length (cm)	Shoot mass (gm)	Root length (cm)	Root mass (gm)	Nodule number	Mineral content	
						Nitrogen (ppm)	Phosphorus (ppm)
Control	9.25	0.30	3.75	0.03	0	2.48	1.69
Nitrogen added	13.2	0.40	3.25	0.15	0	2.6	2.16
NAFP-19	13.87	0.31	5.5	0.09	0	3.28	2.32
NAFP-32	13.5	0.25	7.75	0.09	0	3.02	2.40
R. vignae (NFB-103)	12.12	0.30	5.5	0.10	1.20	4.35	3.23
R. vignae (NFB-109)	13	0.36	5.9	0.08	0.5	4.44	4.31
NAFP-19 + *R. vignae* (NFB-103)	13.75	0.32	4.0	0.11	1	4.81	4.53
NAFP-19 + *R. vignae* (NFB-109)	12	0.275	4.5	0.19	1.25	4.57	3.21
NAFP-32 + *R. vignae* (NFB-103)	11.25	0.30	8.5	0.10	1.25	4.02	3.90
NAFP-32 + *R. vignae* (NFB-109)	12.5	0.325	8.25	0.10	1.25	3.8	2.16
$LSD_{0.05}$	2.50[1]	0.179[1]	2.29[1]	0.087[1]	ns	0.95[1]	0.72[1]

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

Table 4. Effect of fluorescent *Pseudomonas* and rhizobia on vegetative growth, nodulation, nitrogen and phosphorus content of mungbean after 20 days of inoculation

Treatments	Shoot length (cm)	Shoot mass (gm)	Root length (cm)	Root mass (gm)	Nodule number	Mineral content	
						Nitrogen (ppm)	Phosphorus (ppm)
Control	11.5	0.31	5	0.05	0	3.72	2.10
Nitrogen added	13.5	0.57	3.85	0.18	0	4.31	2.62
NAFP-19	15.37	0.39	5.75	0.11	0	3.45	2.41
NAFP-32	14.75	0.40	8.12	0.11	0	3.62	5.79
R. vignae (NFB-103)	13.47	0.32	6.87	0.11	1.25	4.81	4.78
R. vignae (NFB-109)	14.22	0.48	6.12	0.11	1.5	4.91	4.38
NAFP-19 + *R. vignae* (NFB-103)	14.02	0.36	4.35	0.18	1.75	4.94	4.63
NAFP-19 + *R. vignae* (NFB-109)	12.12	0.355	5.37	0.75	1.5	4.98	4.14
NAFP-32 + *R. vignae* (NFB-103)	12.37	0.35	9.5	0.11	1.45	4.8	5.30
NAFP-32 + *R. vignae* (NFB-109)	13.87	0.39	9.12	0.12	1.5	3.91	5.29
$LSD_{0.05}$	2.08[1]	0.135[1]	2.27[1]	0.06[1]	2.70[1]	1.35[1]	1.34[1]

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

Table 5. Effect of fluorescent *Pseudomonas* and rhizobia on vegetative growth, nodulation, nitrogen and phosphorus content of mungbean after 25 days of inoculation

Treatments	Shoot length (cm)	Shoot mass (gm)	Root length (cm)	Root mass (gm)	Nodule number	Mineral content	
						Nitrogen (ppm)	Phosphorus (ppm)
Control	12.17	0.4	5.5	0.11	0	5.46	4.43
Nitrogen added	13.75	0.32	4.37	0.09	0	6.27	5.18
NAFP-19	15.75	0.45	6.5	0.15	0	7.90	3.87
NAFP-32	15.72	0.46	8.62	0.17	0	7.11	3.52
R. vignae (NFB-103)	13.45	0.41	7.12	0.14	1.25	7.17	3.00
R. vignae (NFB-109)	13.95	0.40	8	0.17	4.75	7.52	2.81
NAFP-19 + *R. vignae* (NFB-103)	13.2	0.47	6	0.15	2	7.40	5.74
NAFP-19 + *R. vignae* (NFB-109)	14.25	0.36	6.12	0.13	2	7.04	4.18
NAFP-32 + *R. vignae* (NFB-103)	15.4	0.43	9.5	0.17	2	7.42	4.50
NAFP-32 + *R. vignae* (NFB-109)	15.47	0.48	10.5	0.40	6.25	7.09	4.39
$LSD_{0.05}$	3.21[1]	0.18[1]	3.46[1]	0.13[1]	3.79[1]	1.04[1]	1.30[1]

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

Table 6. Effect of soil drench with different isolates of *Pseudomonas* and rhizobia on growth of mungbean plants in screen house experiment.

Treatments	Growth parameter				
	Shoot length (cm)	Shoot weight (g)	Root length (cm)	Root weight (g)	Number of Nodules
Control	11.68	0.31	7.68	0.10	2.75
Carbendazim	10.98	0.37	8.52	0.21	5.25
NAFP-19	12.43	0.88	10.75	0.28	6
NAFP-32	9.16	0.41	10.58	0.24	8.5
NAFP -31	11.10	0.43	11.25	0.24	6.5
R. vignae(NFB-107)	8.37	0.37	8.43	0.12	7.75
R. vignae(NFB-109)	10.25	0.50	12.33	0.17	6.75
R. vignae(NFB-103)	11.06	0.43	8.25	0.20	7
NAFP-19 + *R. vignae*(NFB-107)	9.58	0.45	8.82	0.14	7.5
NAFP-19 + *R. vignae*(NFB-109)	12.03	0.82	8.65	0.26	10.5
NAFP-19 + *R. vignae*(NFB-103)	11.85	0.41	7.16	0.12	11.5
NAFP-32 + *R. vignae*(NFB-107)	10.75	0.49	8.82	0.23	9.75
NAFP-32 + *R. vignae*(NFB-109)	11.62	0.51	8.52	0.21	12.75
NAFP-32 + *R. vignae*(NFB-103)	12.62	0.67	10.08	0.43	10.5
NAFP-31 + *R. vignae*(NFB-107)	7.60	0.26	7.56	0.13	11.25
NAFP-31 + *R. vignae*(NFB-109)	12.08	0.79	9.64	0.21	15.5
NAFP-31 + *R. vignae*(NFB-103)	18.16	0.57	15.38	0.07	10.25
$LSD_{0.05}$	3.04[1]	0.43[1]	4.25[1]	0.24[1]	3.23[1]

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

In this experiment biocontrol potential of these isolates was also evaluated against root rotting fungi. Results showed complete suppression of *M. phaseolina* in plants treated with NAFP-32, *R. vignae* (NFB-107), *R. vignae* (NFB-109) and *R. vignae* (NFB-103) individually as well as in combined treatment of NAFP-31 with rhizobia in comparison to control. Significant suppression of *R. solani* was observed by individual treatment of NAFP-19, NAFP-32 as well combined treatments in comparison to control. Maximum inhibition of *F. solani* was observed in *R. vignae* (NFB-103) and NAFP-19 with *R. vignae* (NFB-103) while *F. oxysporum* was completely suppressed by individual as well as combined treatment of fluorescent *Pseudomonas* and rhizobia (Table 7).

Table 7. Effect of different isolates of *Pseudomonas* and rhizobia on root infection by *Macrophomina phaseolina, Rhizoctonia solani, Fusarium solani* and *F. oxysporum* on mungbean roots in screen house experiment.

Treatments	Infection %			
	M. phaseolina	***R. solani***	***F. solani***	***F. oxysporum***
Control	62.5	43.7	100	56.2
Carbendazim	62.5	31.2	93.7	0
NAFP-19	6.25	0	68.7	0
NAFP-32	0	0	87.5	0
NAFP -31	25	6.2	93.7	12.5
R. vignae(NFB-107)	0	25	62.5	31.2
R. vignae(NFB-109)	0	6.2	50	0
R. vignae(NFB-103)	0	6.2	43.7	12.5
NAFP-19 + *R. vignae*(NFB-107)	18.7	25	56.2	18.7
NAFP-19 + *R. vignae*(NFB-109)	18.7	6.2	68.7	0
NAFP-19 + *R. vignae*(NFB-103)	12.5	0	43.7	0
NAFP-32 + *R. vignae*(NFB-107)	6.2	18.7	68.7	0
NAFP-32 + *R. vignae*(NFB-109)	6.2	12.5	56.2	37.5
NAFP-32 + *R. vignae*(NFB-103)	6.2	0	56.2	0
NAFP-31 + *R. vignae*(NFB-107)	0	0	50	0
NAFP-31 + *R. vignae*(NFB-109)	0	0	62.5	12.5
NAFP-31 + *R. vignae*(NFB-103)	0	6.2	43.7	6.2
$LSD_{0.05}$	Treatments = 15.0[1]		Pathogens = 7.2[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

Discussion

In the current study, co-inoculation of mungbean with fluorescent *Pseudomonas* and rhizobia, NAFP-19 with*R. vignae* (NFB-109), NAFP-19 with *R. vignae* (NFB-103), NAFP-32 with *R. vignae* (NFB-109) and NAFP-32 with *R. vignae* (NFB-103) improved the frequency of nodule initiation and development in screen house as well as in gnotobiotic condition in comparison to the plants inoculated with rhizobia alone. The plants treated with fluorescent *Pseudomonas*as well as rhizobia produced nodules earlier than plants treated with fluorescent *Pseudomonas* and rhizobia alone. Some former studies suggested that combined treatment of rhizobia along with plant growth promoting rhizobacteria (PGPR) increases the amount of nitrogen and phosphorus contents in plants (Osman *et al.,* 2010; Egamberdiyeva, 2008; Zafar *et al.,* 2012), our results confirm these early findings. There are also reports that combined inoculation of PGPR and rhizobia can induce early initiation of nodules in soybean (Nishijima *et al.,* 1988) and *Phaseolus vulgaris* (Srinivasan *et al.,* 1996). In this study, combined treatment of fluorescent *Pseudomonas* and rhizobia showed an increased root and shoot mass and total nitrogen accumulation and phosphorus uptake in plants, similar results have been reported by Egamberdiyeva (2008) and Egamberdiyeva *et al.,* (2010).

Increased rate of nitrogen uptake and total nitrogen content in plants reported in present study could be due to fixation of nitrogen and activity of nitrate reductase or by the absorbance of amino acids as well as NH^{+4}produced by PGPR (Osman *et al.,* 2010). Microbes isolated from rhizosphere of legumes, were found more proficient in phosphate solubilizing capacity than those isolated from root zone or from non-rhizospheric regions or from rhizosphere of non-leguminous plants (Hameed *et al.,* 2004). The strains of PGPR showed distinctive property to solubilize phosphate and convert it into an accessible form in situations where phosphorus is a preventive reason for production of crop. It has been generally suggested that the number of nodules is not a suitable trait for selection of the most effective nitrogen fixing rhizobium-legume connotations and the selection of such symbiotic connotations should be made on the basis of dry weights of shoot and root (Hefny *et al.,* 2001). In this study biomass of plants was also increased in treatments which received both *Pseudomonas* and rhizobia. This improvement in plant growth may be due to solubilization of phosphate and production of indole acetic acid by the root nodule associated bacteria (Noreen *et al*., 2015b).

In this study plants grown in soil naturally infested with root rotting fungi showed less infection. Both rhizobia and PGPR have been reported to produce hydrogen cyanide, antibiotics and sidrophores that adversely affect plant pathogens (Noreen *et al.,* 2015ab; 2016). Besides, PGPR rhizobia have also been reported to suppress root rotting fungi and root knot nematode on both leguminous and non-leguminous crops (Ehteshamul-Haque & Ghaffar, 1993). The *Pseudomonas* and rhizobial isolates used in this study have potential to increase plant growth and suppressed soilborne root infecting fungi. The present study confirmed the beneficial role fluorescent *Pseudomonas* associated with root nodules in facilitating the process of nodule formation by the rhizobia. Role of root nodule associated fluorescent *Pseudomonas* in plant-rhizobia symbiosis needs further investigation.

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