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Multifaceted Beneficial Associations with *Pseudomonas* and Rhizobia on Growth Promotion of *Mucuna pruriens* L

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Experiments were conducted to isolate *Pseudomonas* compatible with both slow and fast growing rhizobia and assessed them for increased vegetative growth in *Mucuna pruriens*. Influence of cell-free culture filtrate of *P. fluorescens* PRS4 on growth promotion of *M. pruriens* in co-inoculation with *S. meliloti* RMP66^{rif+} and *Bradyrhizobium* sp. BMP17^{kan+} was statistically significant as compared to whole bacterial cultures and washed cells (71.7%, 64.2 % and 68.4 %, respectively, over control set of treatment), suggesting that plant growth promoting attributes in *P. aeruginosa* PRS4 are both intracellular and extracellular in nature. Synergistic rhizobacteria and rhizobia proved an effective strategy for co-inoculation which contributes multiple benefits such as increased germination, biomass and enhanced nodulation to improve plant health. Productivity of medicinal legumes has been ignored with respect to their use in indigenous and folk system of medicine; hence this study increases the scope of PGPR application from conventional fields.

Key words: Fluorescent pseudomonads, rhizobia, co-inoculation, microbial interaction

Rhizosphere harbors an extremely complex microbial community and several of them are supportive to plant growth, health and productivity. These form an important alternative to chemical based agriculture. A major group among them is one of the more aggressive root colonizers, belong to members of the genera *Pseudomonas*. Co-inoculation of fluorescent pseudomonads and rhizobia has been well documented for many legume species but their role in improving growth of medicinal legumes has been ignored. *Mucuna pruriens* L. (Kaunch), an annual herbaceous medicinal legume growing wildly in the Himalayan foothills has immense medicinal value, contains L-dopa (precursor to dopamine) as most active constituent, acts as green manure for soil fertility improvement and used in weed control (Buckles 1994).

The interaction between plant roots and organisms within their rhizosphere help them to acquire essential mineral nutrients and prevent the accumulation of toxic elements. Growth promotion of plants by soil microorganisms can be considered as a part of sustainable agro-industry in which interactions between plants and microorganisms range from deleterious (pathogens) to beneficial (PGPR). The beneficial effects of these bacteria on plant include profound effects on nodulation,

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nitrogen fixation, photosynthetic activities, overall plant productivity and yield which are attributed to production of various growth enhancing metabolites e.g. phytohormones, ACC deaminase, mineral and nitrogen availability, bacteriocins, production of antibiotics, siderophores, lytic enzymes, cyanogens, volatile organic compounds apart from induction of systemic resistance in host plant.

Successful colonization of the legume rhizosphere by rhizobial species require these bacteria to compete effectively with indigenous microorganisms, which grow rapidly in comparison to majority of rhizobia. These indigenous rhizospheric microorganisms may influence the survival of native as well as introduced rhizobia and also show synergism resulting in increased nodulation and nitrogen-fixing efficiency of rhizobia. Production of plant signaling compounds are key mechanisms known to direct compatibility between microbes, however, it needs to be evaluated because of possibility of antagonistic interactions among them (Dashti *et al*., 2000; Gupta *et al*., 2003). The process of compatibility and synergism establish communications that occur beneath the soil surface for achieving cooperation between microbes and result in multifaceted beneficial associations.

Screening, introducing and monitoring of rhizospheric bacteria having synergistic behaviour to rhizobia, are known to engineer increased productivity and yield but required to be assessed for potential hazardous effects on other crops before being used as inoculants. They should demonstrate the potential for root associated organisms to alter the dynamics of legume-rhizobia symbiosis, and to utilize different fractions of PGPR to increase plant growth promotion through co-inoculation. The addition of cell free culture filtrate of a PGPR is known to influence plant growth and nutrient uptake in comparison to the whole bacterial cultures or washed cells (Azcón-Aguilar and Barea, 1978; Azcon, 1993; Bai *et al*., 2002). In a series of experiments, we aimed to establish the proto-cooperation between pseudomonads and rhizobia and further, for growth promotion in *M. pruriens* L. PGPR fractions were applied as: cell-free culture filtrate, washed cells and whole bacterial culture in co-inoculation with slow and fast growing rhizobia.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Fast and slow growing rhizobia, *Sinorhizobium meliloti* RMP66^{rif+} and *Bradyrhizobium* sp. BMP17^{kan+}, respectively, were isolated earlier from root nodules of *M. pruriens* L. and obtained from culture collection of Department of Botany and Microbiology, Gurukul Kangri University, Haridwar, (India). Isolation of fluorescent pseudomonads was carried out from rhizosphere of *M. pruriens* on *Pseudomonas* isolation agar (Hi-Media) using dilution plate technique (Vincent 1970) and identified according to Bergey's Manual of Determinative Bacteriology (Holt *et al*. 1994), Enterobacteriaceae Identification Kit™ (Hi-Media) and Hi-carbo™ Kit (Hi-Media). Simultaneously, *Pseudomonas aeruginosa* MTCC 1934, *P. putida* MTCC 102 and *P. fluorescens* MTCC 103 were used as positive controls in all the experiments. All the pseudomonads were sub-cultured on Tryptic soy media (Hi-Media) slants while Yeast extract mannitol agar (Hi-Media) was used for rhizobial strains and maintained at 4^0C for further studies. *Escherichia coli* strain WA803 was used for *Tn5* Mutagenesis, as described (Kumar *et al*., 2003).

Microbial Interactions

Modified technique of Skidmore and Dickinson (1976) was used to study microbial interaction of various *pseudomonas* (abbreviated as PRS 1, PRS 2, PRS 3, PRS 4, PRS 5 and PRS6), with *S. meliloti* RMP66 and *Bradyrhizobium* sp. BMP17, respectively. Active culture of rhizobial strains (RMP66 and BMP17; 10^{7-} cfu/ml) were seeded into pre-sterilized YEM: NAM (1:1, w/w) agar. One loopful active culture (12h) of each strain of fluorescent pseudomonads pre-grown on TSM was spotted at center of plates. The resulting interaction between bacterial strains was noted after 24h of incubation at 30+1°C. Strains showing positive interaction observed as growth of test strains on plates pre-seeded with rhizobial strains, were further analyzed for proto-cooperation by metabolite cross-utilization test following the methodology of Homma *et al*. (1989). Briefly, rhizobial strains RMP66 and BMP17 were spot inoculated on Kings B medium pre-seeded with *P. fluorescens* PRS3 and PRS4 (strains selected on the basis of *in vitro* interaction result) under iron

deficient (Kings B medium treated with 8-hydroxyquinoline in 0.1% chloroform [100 ml]) and iron sufficient conditions (Kings B medium amended with 1mM $FeCl_3$) in triplicate. Further, to screen the nature of metabolite that influenced the compatibility between these strains, siderophore negative mutants of *P. fluorescens* PRS3 and PRS4 were constructed using *Tn5* delivery suicide vector pGS9 in donor *E. coli* strain WA803 (pGS9) as described (Kumar *et al*., 2003) and used for interaction studies as described above.

Plant growth promoting and antagonistic attributes

All strains of *Pseudomonas* along with *S. meliloti* RMP66 and *Bradyrhizobium* sp. BMP17 were assessed and quantified for growth promoting alongwith antagonistic attributes against plant pathogenic fungi obtained from Department of Botany and Microbiology, Gurukula Kangri University, Haridwar (*Macrophomina phaseolina*, *Fusarium udum* and *Fusarium oxysporum*) using standard methodology as adopted by Dey *et al*. (2004). Plant growth promoting and antagonistic attributes analyzed included solubilization of inorganic tricalcium phosphate (absorbance at 430nm), production of Indole acetic acid based on the reaction of indoles with the Salkowaski reagent and comparison of the color developed of the reaction mixture (absorbance at 535nm) and ACC deaminase using ACC as substrate (0.2 ml of enzyme extract, 50 mM ACC incubated in 0.2 ml of 0.1M Tris [Hydroxymethyl amino methane] buffer (pH 7.5) at 30^0 C for 30 min, reaction terminated by adding 1.8 ml of 0.56N hydrochloric acid (HCl). To it, 0.3 ml of 0.1% 2, 4-dinitrophenyl hydrazine solution was added and the mixture was kept at 30^0 C for 30 min. Thereafter, 2 ml of 2N sodium hydroxide was added in above mixture and absorbance was measured spectrophotometrically at 540 nm), secretion of siderophores (absorbance at 450/500nm; hydroxamate/catecholic type of Siderophore), chitinase assay using colloidal chitin as substrate and N-acetyl-d-glucosamine (NAGA) as standard (reaction mixture consisted of 1 ml of culture supernatant, 0.3 ml of 1 M sodium acetate buffer [pH 5], 0.2 ml of colloidal chitin incubated at 40°C for 1 h, centrifuged [12,225g; 5 min] at 4°C. After centrifugation, an aliquot of 0.75 ml of the supernatant, 0.25 ml of 1% solution of dinitrosalycilic acid in 0.7 M NaOH, and 0.1 ml of 10 M NaOH were mixed in 1.5-ml Eppendorf tubes and heated at 100°C for 5 min. Absorbance of the reaction mixture at 582 nm was measured after cooling it to room temperature), ^{2}-1,3-glucanase assay using laminarin (Sigma) as substrate, Roll towel assay for seedling vigour, spermosphere colonization to check colonization of legume seed (*M. pruriens*) by rhizospheric competent bacteria, ammonia production, dual culture and cup disc assay for study for antagonism using bacterial strains and its cell free culture filtrate, production of volatile antibiotics (cyanogens).

Analysis of early vegetative parameters of *M. pruriens*

For *in vivo* study, the inoculum was prepared by culturing rhizobial strains *S. meliloti* RMP66 and *Bradyrhizobium* sp. BMP17 and *P. fluorescens* PRS4 in respective broth medium and cultured up to exponential phase (10^8 cfu/ml; OD_{660nm} =1.0) for pot studies. Healthy seeds of *M. pruriens* L. were procured from Forest Seed Center, Dehradun, (U. K., India). Seeds of similar shape and size were soaked in lukewarm water for a period of 30 minutes, followed by surface sterilization using 4% sodium hypochlorite (NaOCl) for 5 minutes, 70% ethanol for 3-5 minutes and rinsed several times in sterile distilled water. Surface sterilized seeds of Kaunch (*M. pruriens*) were bacterized with *S. meliloti* RMP66 and *Bradyrhizobium* sp. BMP17, separately, using 1% (w/v) carboxymethylcellulose (CMC) following the methodology of Weller and Cook (1983). *P. fluorescens* PRS4 was added equivocally at the same time for co-inoculation and individual effects of strains in three forms, viz., (1) Whole bacterial culture (WBC), (2) Washed cells (pellets) and (3) Cell free culture filtrate (CFCF) (10000rpm, 15 min, 4°C). Seeds were allowed to air dry in aseptic conditions over night. The procedure yielded an average of 10^8cfu/ml per seed of each inoculated strain. Care was taken to avoid clumping of seeds. Seeds coated with 1% CMC slurry (without bacterial strains or its components) served as control.

Bacterial treatments for plant assay

To observe the effect of *P. fluorescens* PRS4 in different forms on *S. meliloti* RMP66 and *Bradyrhizobium* sp. BMP17 activity in *M. pruriens*, study was carried out in earthen pots (20 cm), having sterilized garden soil (sandy loam soil: sand 74%, silt14%, clay 12%, total organic

matter 0.035%, pH 7.4 and water holding capacity 35%). The pot experiment was conducted during the month of March-April in the year 2007 and 2008. Seeds were sown in a completely randomized block design with 12 seeds per pot in following treatments: (T1) Seeds bacterized with *S. meliloti* RMP66^{rif+}, (T2) seeds bacterized with *Bradyrhizobium* sp. BMP17^{kan+}, (T3) seeds bacterized with *S. meliloti* RMP66^{rif+} + whole bacterial culture of *P. fluorescens* PRS4, (T4) seeds bacterized with *Bradyrhizobium* sp. BMP17^{kan+} + whole bacterial culture of *P. fluorescens* PRS4, (T5) seeds bacterized with *E. meliloti* RMP66^{rif+} + washed cells of *P. fluorescens* PRS4, (T6) seeds bacterized with *Bradyrhizobium* sp. BMP17^{kan+} + washed cells of *P. fluorescens* PRS4, (T7) seeds bacterized with *S. meliloti* RMP66^{rif+} + cell free culture filtrate of *P. fluorescens* PRS4, (T8) seeds bacterized with *Bradyrhizobium* sp. BMP17^{kan+} + cell free culture filtrate of *P. fluorescens* PRS4, (T9) seeds bacterized with *P. fluorescens* PRS4, (T10) seeds bacterized with washed cells of *P. fluorescens* PRS4, (T11) seeds bacterized with cell free culture filtrate of *P. fluorescens* PRS4 and (T12) Control (non-bacterized seeds coated only with 1% CMC slurry).

Assessment of agronomic parameters

Seed germination (%) was recorded on 15th day after sowing (DAS). The early vegetative growth parameters including seedling vigour, weight of nodules, nodule number per plant, rate of nodulation (number of plants nodulated/days after inoculation) and seedling biomass was monitored 40 DAS. Percentage nodule occupancy was calculated using the formulae:

$$A = 100.X/Y,$$

where A is nodule occupancy (%), X = number of nodules occupied by introduced strain, Y= total number of nodules tested. Symbiotic nitrogenase activity in nodules was assayed by acetylene reduction assay (ARA) technique (Gibson 1976).

Statistical analysis

The data were analyzed statistically by using two way Analysis of Variance (ANOVA) for individual parameters on the basis of mean values to evaluate the efficiency of co-culture over mono-culture using different components of *P. fluorescens* PRS4 with fast and slow growing *S. meliloti* RMP66 and *Bradyrhizobium* sp. BMP17, respectively. Mean values were compared at significance levels of 1% and 5%.

RESULTS AND DISCUSSION

All the six isolates were identified as *Pseudomonas* spp. based on morphological and biochemical data (data not given). Iron induced and protein-based anti-microbial compounds are known to confer a selective advantage in terms of saprophytic competition and competitive ability in nodulation (Wilson *et al*. 1998). Such compounds might have caused the inhibition in agar overlay plate based assay in *in vitro* interaction studies that revealed *S. meliloti* RMP66^{rif+} and *Bradyrhizobium* sp. BMP17^{kan+} were compatible to *P. fluorescens* PRS3 and PRS4 (Table 1A; Fig 1: g, h). This is supported by the fact that each individual within a population will be in direct competition with all other adjacent individuals, whether for oxygen, metabolites or nodulation sites, with identical requirements have the ability to inhibit the sensitive strains in surrounding population where fierce competition can reduce the pressure and allow the bacteriocin producing strain to survive and multiply (Wilson *et al*. 1998). *P. fluorescens* PRS4 showed no antagonism even under iron supplemented conditions towards *S. meliloti* RMP66^{rif+} and *Bradyrhizobium* sp. BMP17^{kan+}. However, strong antagonistic relationship was observed between *P. fluorescens* PRS3 and *Bradyrhizobium* sp. BMP17^{kan+} in low iron conditions (Table 1B). In case of *P. fluorescens* PRS3 pre-seeded plates, *S. meliloti* RMP66^{rif+} survived and showed no signs of inhibition but slow growing *Bradyrhizobium* sp. BMP17^{kan+} was present with a clear zone of inhibition indicating metabolite produced by *P. fluorescens* PRS3 under iron stress was inhibitory to slow growing rhizobia. It was observed that some isolates of *Pseudomonas* decreased the growth of rhizobia whereas other had synergistic effect on rhizobial growth quite similar to the way *P. fluorescens* PRS4 showed indifferent compatibility with both slow and fast growing rhizobial strains as also observed by many workers (De freitas *et al*., 1993; Nautiyal 1997; Rao *et al*. 1999). Further, PRS3^{sid-} strain did not antagonize fast growing rhizobium which suggests the involvement of non-siderophore metabolite in the inhibition of slow growing rhizobia while there

was no effect of PRS4^{sid-} strain on growth of rhizobia (De La Fuente *et al*. 2002). Other iron induced metabolites such as HCN and phenazines are known to cause antagonism, although the strain PRS3 did produced HCN, but volatile mediated inhibition in interaction between slow growing rhizobium and PRS3 can be ruled out in a diffusible plate assay. Alternatively, a possibility of compatibility between PRS4 and BMP17^{kan+} could be excretion of some growth inducing substances into the media that were utilized by slow growing *Bradyrhizobium* sp. BMP17^{kan+} to survive in low iron induced conditions in interaction with PRS4 as opposed to in interaction with PRS3 but contrary to it, cross-utilization of siderophores produced by rhizobacteria were observed in rhizobial strains (Geetha *et al*. 2008). These siderophore helped in stimulating the growth of the rhizobia and thus facilitated the plant growth while addition of sterile spent medium of *P. fluorescens* increased the growth of *B. japonicum* in yeast mannitol broth (Chebotar *et al*. 2001).

All the strains solubilized insoluble tricalcium phosphate with lowering in pH indicating production of organic acids. Solubilization of insoluble phosphate to make it available for uptake by plants is one of the key mechanisms rhizobia and rhizobacteria are known to exhibit. Thus, continuous supply of available phosphorus required for sustaining enhanced biological nitrogen fixation, was made possible by phosphate solubilization ability of all three strains (Dey *et al*., 2004). In fact ability of all the strains to solubilize phosphate caused increased nodulation as observed in our experiments and this could be further facilitated by increase in root growth due to production of ACC deaminase by *S. meliloti* RMP66^{rif+}. *S. meliloti* RMP66^{rif+} also produced IAA, which is known to influence early vegetative growth parameters in plants. Production of IAA by rhizobia and rhizobacteria has been substantiated in earlier findings (Patten and Glick, 2002). This resulted in more active root-zone, since plant roots are able to offer a specialized niche for the proliferation of soil bacteria that thrive on root exudates and lysates. Production of low molecular weight compounds with high affinity for iron is known to increase iron competition in rhizosphere and starves the pathogens by chelating iron from surroundings and in turn providing it to plants. Such activity could be possible due to *S. meliloti* RMP66^{rif+} and *P. fluorescens* PRS4, both of which

Table 1(a). Interaction study of fast growing *S. meliloti* RMP66^{rif+} and slow growing *Bradyrhizobium* sp. BMP17^{kan+} with fluorescent Pseudomonads

Test strainsindicator strains	RMP66^{rif+}	BMP17^{kan+}	PRS1	PRS2	PRS3	PRS4	PRS5	PRS6
RMP66^{rif+}	+	-	-	-	+	+	-	-
BMP17^{kan+}	-	+	-	-	-	+	-	-
PRS1	-	-	+	-	-	-	-	-
PRS2	-	-	-	+	-	-	-	-
PRS3	+	-	-	-	+	-	-	-
PRS4	+	+	-	-	-	+	-	-
PRS5	-	-	-	-	-	-	+	-
PRS6	-	-	-	-	-	-	-	+

+, positive interaction (Growth without zone); -, no interaction (Growth with zone)

Table 1(b). Metabolite cross utilization study of fast growing *S. meliloti* RMP66^{rif+} and slow growing *Bradyrhizobium* sp. BMP17^{kan+} with *P. fluorescens* PRS3 and PRS4 in iron deficient and sufficient conditions

Test strainsindicator strains	Conditions	*S. meliloti* RMP66^{rif+}	*Bradyrhizobium* sp.BMP17^{kan+}
P. fluorescens PRS3	Iron deficient	+	-
	Iron sufficient	+	-
P. fluorescens PRS4	Iron deficient	+	+
	Iron sufficient	+	+

+, positive interaction (Growth without zone); -, no interaction (Growth with zone)

Table 2. Plant growth-promoting and antagonistic properties of *Pseudomonas* and rhizobial strains (*M. pruriens*)

M. pruriens	IAA[A] (µg/ml)	P[B] (µg of P/ml)	HCN[C] (OD at 625nm)	S[D] (U/ml/h)	C[E] (U/ml)	ACC[F] (nm α-ketobuty rate/mg/h)	VA[G] (% increase over control)	SC[H]	Antagonism[I] (%)					
									1		2		3	
									DC	CFCF	DC	CFCF	DC	CFCF
Pseudomonas spp. PRS1	+	++	-	-	-	-	+	-	-	-	-	-	-	-
	(31)	(75)					(22)							
P. aeruginosa PRS 2	++	+	+	+++	-	-	+	-	-	-	+	+	-	-
	(46)	(55)	(0.8)	(29)			(32)				(45)	(46)		
P. fluorescens PRS 3	+	+	+	+	+	-	+	++	+	+	+	-	-	-
	(32)	(51)	(0.7)	(17)	(0.85)		(55)		(49)	(57)	(48)			
P. fluorescens PRS 4	++	+	-	++	-	+++	++	+++	+++	++	+		+++	+
	(48)	(56)		(22)		(78)	(49)		(81)	(79)	(44)		(80)	(48)
P. puitda PRS5	-	+	-	-	-	-	+	-	-	-	-	-	-	-
		(58)					(24)							
Pseudomonas spp. PRS6	-	-	-	-	+	+	+	-	-	-	-			-
					(06)	(41)	(19)							
Rhizobial strains														
S. meliloti RMP66^{rif+}	+++	+++	-	+++	-	++	+	+	+++	+++	+	-	-	-
	(48)	(61)		(21)		(65)	(21)		(78)	(76.6)	(55)			
Bradyrhizobium sp.	+++	+++	-	+++	-	-	+	+	+++	+++	++	+	-	-
BMP17^{kan+}	(40)	(65)		(23)			(31)		(71.9)	(61.7)	(59)	(40)		

Abbreviations: A -, IAA negative, +, IAA positive; B -, Phosphate solubilization negative; +, phosphate solubilization positive, C-, HCN negative, +, HCN positive; D Siderophore: -, Absence of halo formation; +, small halos <0.5 cm wide surrounding colonies; ++, medium halos > 0.5 cm wide surrounding colonies; +++, large halos >1.0cm wide surrounding colonies; E- chitinase production, F ACC 1-aminocyclopropane-3-carboxylic acid , - ACC negative; + ACC positive; G vigour assay using roll towel method; H spermosphere colonization using seed bacterization in sterile Petriplates; I ,- antagonism negative, +, antagonism present;1: *M. phaseolina*, 2: *F. oxysporum*, 3: *F. udum*; DC dual culture; CFCF cell free culture filtrate; (%) pathogen inhibition percentage (control – treatment/control x 100); PGP attributes were repeated at least thrice with three replicates each. Parenthesis value represents quantitative estimation of individual PGP attributes with an average value of three independent experiments each having three replicates

Table 3. Effect of PGPR *P. fluorescens* PRS4 and its fractions in co-inoculation with *S. meliloti* RMP66^{rif+} and *Bradyrhizobium* sp. BMP17^{kan+} on vegetative parameters of *M. pruriens* (40 DAS)

Treatments	Seed Germination (%)	Seedling Vigour	Seedling biomass (gm)	Nodule Number/plant	Nodule fresh weight/plant (mg)	Nodule Occupancy (%)	Nitrogenase activity µM C_2H_4/hr/plant
T1	77.3**	896.4**	11.5 ^{ns}	22.66**	34.13**	88**	9.8**
T2	73.2**	831.3**	12.1*	20.0**	30.01**	85**	11.39**
T3	83.1** ^{c}	932.1** ^{b}	11.0 ^{ns} ^{c}	35.33** ^{c}	43.6** ^{c}	89** ^{c}	12.37** ^{c}
T4	85.5** ^{b}	953.1** ^{b}	12.9* ^{c}	25.0** ^{c}	35.33** ^{c}	91** ^{c}	12.56** ^{c}
T5	81.8** ^{c}	923.2** ^{b}	13.1* ^{c}	31.33** ^{c}	38.78** ^{c}	93** ^{c}	13.89** ^{c}
T6	87.3** $^{a\ b}$	1482.2** $^{a\ b}$	12.7* ^{c}	32.66** ^{c}	39.7** ^{c}	87** ^{c}	16.4** ^{c}
T7	89** $^{a\ b}$	1233.0** $^{a\ b}$	14.5* ^{c}	33.6** ^{c}	39.05** ^{c}	86** ^{c}	16.9** ^{b}
T8	87.9** $^{a\ b}$	1269.3** $^{a\ b}$	13.8* ^{c}	34.15* ^{c}	40.01** ^{c}	89** ^{c}	18.06** ^{b}
T9	88.2**	1132.2**	11.9*	Nl	Nl	Nl	Nl
T10	78.3**	1002.3**	10.2 ^{ns}	Nl	Nl	Nl	Nl
T11	78.3**	989.9**	10.6^{ns}	Nl	Nl	Nl	Nl
T12	65.6	790.0	10.9	Nl	Nl	Nl	Nl
SEM	0.2707	0.2103	0.223	1.547	1.056	0.233	1.989
Cd 1%	1.063	0.365	0.877	6.08	4.14	0.865	8.8
Cd 5%	0.786	0.235	0.65	4.49	3.06	0.156	5.7

Data presented is mean of Two Trials. Values are mean of Ten replicates; ** = Significant at 0.01 level of LSD as compared to control (ANOVA); * = Significant at 0.05 level of LSD as compared to control (ANOVA). ; ^{ns} = Not Significant at 0.05 level of LSD as compared to control(ANOVA); Nl, Nil; ^{a} Values are statistically significant over the respective PGPR strain alone; ^{b} Values are statistically significant over the rhizobial strain alone; ^{c} non-significant over PGPR/

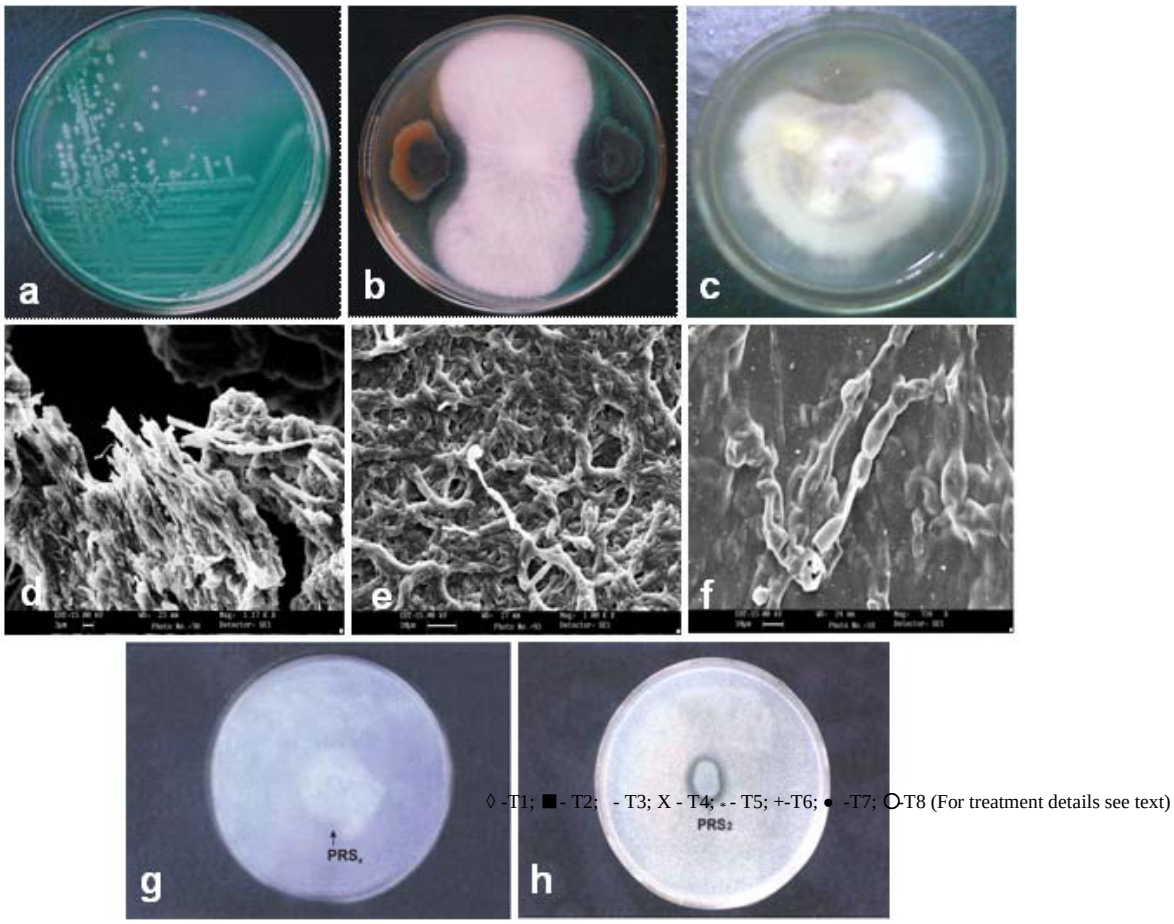

Fig. 1. (a) Colony growth of *P. fluorescens* PRS4 on King's B Agar (b) Interaction of *P. fluorescens* PRS4 with *F. udum* (c) Interaction of *P. fluorescens* PRS4 with *F. oxysporum*; SEM images of interaction of *P. fluorescens* PRS4 (d) with *M. phaseolina* (e) with *F. udum* (f) with *F. oxysporum* (g) Synergism between *P. aeruginosa* PRS4 and *S. meliloti* RMP66^{amp+}; (h) Antagonism between *P. aeruginosa* PRS2 and *Bradyrhizobium* sp. BMP17^{kan+}.

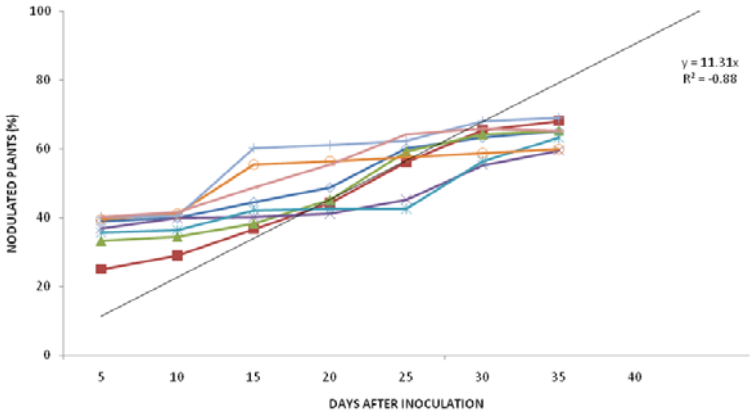

Fig. 2. Rate of nodulation on *M. pruriens* under influence of different treatments

were able to chelate iron from CAS dye as indicated by change in colour surrounding these two strains producing a distinct zone on CAS agar plate. None of the strains produced ammonia neither these emit volatile cyanogens except PRS2 and PRS3. Enzymes chitinase was produced by the strain PRS3 and PRS6 but ²-1,3-glucanase activity was absent in all the strains, such activity are obvious causes of plant pathogen mortality *in vitro* (Arora *et al*., 2007). When tested against some common pathogens PRS4 was the most active of all the strains with inhibition of all three pathogens, *M. phaseolina*, *F. udum* and *F. oxysporum* followed by PRS3 evidenced by SEM images showing shredding of mycelium, coagulation of cytoplasm and breakage of mycelium (Table 2; Fig 1: b, c, d, e, f). Since the bacterium PRS4 failed to produce any lytic enzyme the observed antagonism must be due to either production of siderophore or some antibiotics. The siderophore negatitve mutant failed to inhibit the pathogen a combined activity of siderophore and antibiotics (CFCF) was responsible for pathogen inhibition. Antibiotic producing PGPR, known to antagonize plant pathogen can co-exist with rhizobia and provide healthy environment for nodulation by rhizobia and increased plant growth (De La Fuente *et al*., 2002).

Seed bacterization with combination of *Bradyrhizobium* sp. BMP17^{kan+}+ CFCF of *P. fluorescens* PRS4 (T8) resulted in significant increase in seed germination (38%) over control and the same combination was also responsible for maximum increase in nodule number and nodule fresh weight in all treatments (Table 3). Seedling vigour showed a significant increase (43%) over control when combination of *Bradyrhizobium* sp. BMP17^{kan+} + washed cells of *P. fluorescens* PRS4 (T6) was used while highest increase in seedling biomass (13.7%) was observed with combination of *S. meliloti* RMP66^{rif+} + CFCF of *P. fluorescens* PRS4 (T7), suggesting that co-inoculation not only increase nodule number but also nodule mass (Table 3). Biomass of host plant was found to increase in all treatments over control, and this increment was statistically significant in all treatments with co-culture as opposed to mono-culture. These observations are in agreement with Seneviratne (2003) and Pandey and Maheshwari (2007) who stated that co-inoculation and co-culture of microbes perform tasks better than the individual microbes. Co-inoculation of rhizobia with PRS4 and its fractions improved early plant growth parameters but when CFCF of PRS4 was applied, it was observed that seed germination, nodulation and nodule fresh weight was relatively better to that of control which can be attributed to production of plant growth regulating substances as also evidenced by Manero *et al*. (2003) and Humphry *et al*. (2007). Stimulatory effect of rhizobacteria *P. fluorescens* PRS4 on the efficacy of rhizobial isolates were also confirmed by the reports of De frietas *et al*. (1993) and Kumar *et al*. (2001) who observed that strains of fluorescent pseudomonas and rhizobia that enhanced the growth and nodulation in pea and field bean, respectively.

Nodulation and the subsequent nitrogen fixation are important factors that determine the productivity of legumes. Nodule occupancy increased when *P. fluorescens* PRS4 was used as co-inoculant with both slow and fast growing rhizobia. The value of nodule occupancy was 88% and 85% when treated with rhizobia alone but increased to that of 89% and 91% when *P. fluorescens* PRS4 was used as a co-inoculant (T3 and T4). Although maximum nodule occupancy (93%) was observed with combination of *S. meliloti* RMP66^{rif+} + washed cells of *P. fluorescens* PRS4 (T5). The rate of nodulation was evaluated to determine if the presence of *P. fluorescens* PRS4 or its components affects nodulation in *M. pruriens*. Nodulation was indeed effective when different fractions of PRS4 were used and about 69% of total plants were nodulated by 40th day when combination of *Bradyrhizobium* sp. BMP17^{kan+} + CFCF of *P. fluorescens* PRS4 (T8) were used for seed bacterization but presence of *P. fluorescens* PRS4 in co-inoculation with both rhizobia had an effect on nodulation as the number of nodulated plants are comparatively higher in comparison to the individual treatment by rhizobia. A variation was observed in rate of nodulation and nitrogenase activity across different treatments with rhizobia and co-inoculation using fractions of PRS4 (Fig. 2/Table 3). Maximum symbiotic nitrogenase activity was recorded with combination of *Bradyrhizobium* sp. BMP17^{kan+} + CFCF of *P. fluorescens* PRS4 (T8) at 18.06 µ mole C_2H_4/hour/ weight of nodule. Similar results were obtained in

three independent experiments (Table 3). Earlier, differential effects of co-inoculation of PGPR and rhizobia, increase in nodulation and subsequent ARA activity was observed by Garcia *et al*. (2004) and Valverde *et al*. (2006) citing different mechanisms for such activity by Gram-positive and Gram-negative bacteria such as siderophore, lipolysaccharide (LPS), flavonoids and phytoalexins. A common attribute, although, was efficient colonization of roots by PGPR strain to reduce the ethylene concentration inside the plant, if it is able to utilize ACC as sole nitrogen source, also observed in PRS4 thereby increasing the root surface in contact with soil.

In vitro, findings revealed that strains *P. fluorescens* PRS3 and PRS4 had synergistic effect with *S. meliloti* RMP66^{rif+} and *Bradyrhizobium* sp. BMP17^{kan+}. Co-inoculation of *S. meliloti* RMP66^{rif+} and *Bradyrhizobium* sp. BMP17^{kan+} with *P. fluorescens* PRS4 enhanced germination and increase in seedling biomass of inoculated plants, respectively in comparison to that of either *S. meliloti* RMP66^{rif+} and *Bradyrhizobium* sp. BMP17^{kan+} when applied individually. Co-inoculation of *S. meliloti* RMP66^{rif+} and *Bradyrhizobium* sp. BMP17^{kan+} with *P. fluorescens* PRS4 raised the nodule number in all three forms but increase was more effective with CFCF of *P. fluorescens* PRS4 as the fraction in combination with *Bradyrhizobium* sp. BMP17^{kan+} The work done in the paper showed that application of CFCF of PGRR *P. fluorescens* PRS4 had equivalent role to washed cells (3.62%), but was better than whole bacterial culture (10.9%), for growth promotion of *M. Pruriens*.

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