CHAPTER 4

4.0 STUDIES ON THE INTERACTIONS OF FLUORESCENT PSEUDOMONADS WITH RHIZOBIA

4.1 INTRODUCTION

4.1.1 Rhizobia as a N_2 fixer:

Organisms that can fix nitrogen, i.e., convert the stable nitrogen gas in the atmosphere into a biologically useful form; all belong to prokaryotes and do so with the aid of an enzyme complex, nitrogenase. A wide range of specific organisms have the ability to fix nitrogen, about 87 species totally in 2 genera of archaea, 38 genera of bacteria, and 20 genera of cyanobacteria have been identified as diazotrophs or organisms that can fix nitrogen. These are further classified as symbiotic, associative or free-living depending on the intimacy of their interaction with plants. Atmospheric N_2 fixed symbiotically by the association between *Rhizobium* species and legumes represents a renewable source of N for agriculture. Values estimated for various legume crops and pasture species commonly fall in the range of 200 to 300 kg of N ha^{-1} $year^{-1}$ (Peoples et al., 1995).

4.1.1.1 Taxonomy of rhizobia:

The three rhizobial genera, *Rhizobium, Bradyrhizobium, and Azorhizobium*, have for many years been grouped with the agrobacteria and phyllobacteria into one family, the *Rhizobiaceae* (Jordan, 1984). It is now widely accepted that *Rhizobium* and *Bradyrhizobium* are only distantly related. Each of these genera has close relatives that are not plant symbionts and are placed in different families (Young, et al., 1991). The phylogenetic relationship of the rhizobia is shown in Fig. 4.1.

The species name of the microsymbionts reflects in most cases the corresponding host plant nodulated and suggests that symbiosis is a species-specific process. Some strains have a very narrow host range, for example *Rhizobium leguminosarum* bv. *trifolii*, while others, like *Rhizobium* sp. strain NGR234, have a very broad host range. The introduction of new rhizobia inocula into soil containing native *Rhizobium* populations frequently results in only a small proportion of nodules containing the introduced strain. This is due to competition with ineffective indigenous strains and other rhizospheric bacteria (McLoughlin et al., 1985). Plant growth and nodulation by rhizobia are

promoted by certain rhizobacteria (Dahsti et al., 1998; Bai et al., 2002; Rao & Pal, 2003). Similarly, coinoculation of *Pseudomonas* spp. with *Rhizobium* spp. has been reported to enhance nodulation and N fixation, plant biomass and grain yield in various leguminous crops such as alfalfa (Knight and Langston-Unkefer 1988), pea (Bolton et al., 1990; Kumar et al., 2001).

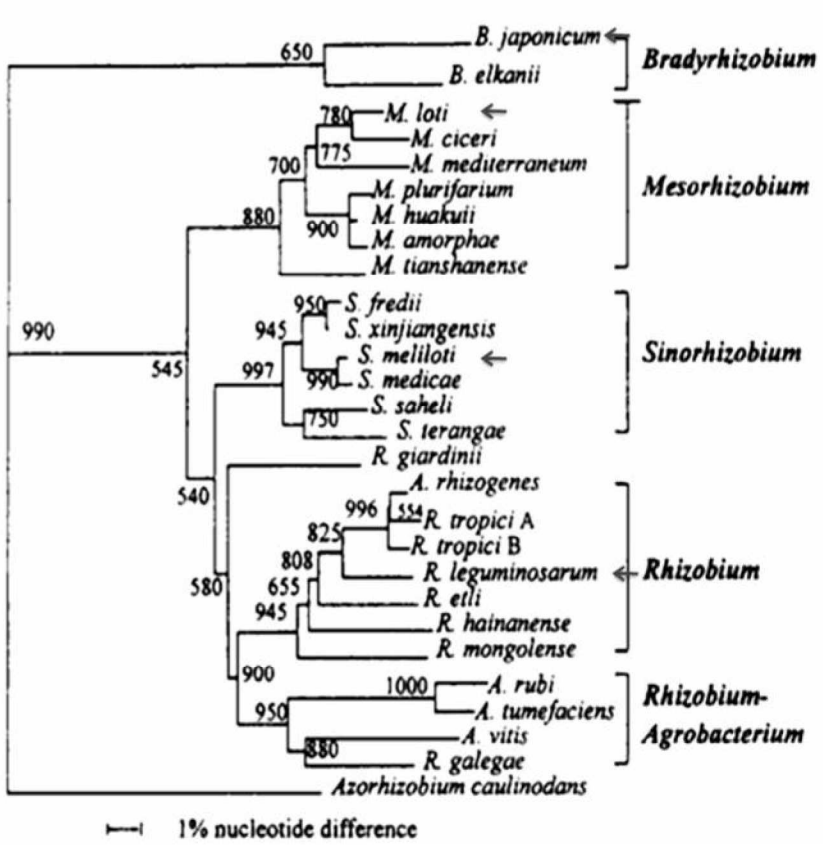


Fig.4.1 Evolutionary relationship between standard rhizobial strains used in study (Young et al., 1991)

Soybean (Dashti et al., 1998), green gram (Sindhu et al., 1999) and chickpea (Goel et al., 2002) (Table 4.1).The commonly studied plant growth promoting bacteria (PGPB) are usually of rhizospheric origin (i.e. PGPR), while another class of endophytic bacteria with plant growth-promoting abilities exist (Hallmann et al., 1997).

4.1.2 Microbe–microbe interactions for sustainable agro-ecosystem development

4.1.2.1 Positive interactions in the rhizosphere:

Interactions between microbial populations in rhizosphere often result in a positive effect on plant health. As they share common microhabitats in the root–soil interface, rhizobia and PGPR interact during their processes of root colonization. Some PGPRs can improve nodulation and N2-fixation in

legume plants (Polenko et al., 1987; Fuhrmann and Wollum, 1989; Andrade et al., 1998; Lucas-Garcia et al., 2004).Three types of interactions are found to be relevant for the development of sustainable agro-ecosystems. These are: (i) microbial antagonism for the biocontrol of plant pathogens; (ii) interactions between rhizosphere microbes and AM fungi to establish a functional mycorrhizosphere and (iii) the co-operation between Plant Growth Promoting Rhizobacteria (PGPR) and *Rhizobium* for improving N_2-fixation. As they have common microhabitats at root–soil interface, rhizobia and PGPR interact during root colonization. PGPRs have been reported to enhance the nodulation and N_2-fixation in legume plants (Polenko et al., 1987; Fuhrmann and Wollum, 1989; Andrade et al., 1998; Lucas-Garcia et al., 2004) and also under field conditions (Dashti et al., 1998; Bai et al., 2002, 2003, Mishra et al., 2009, 2012). Research on the mechanisms by which PGPR enhance nodule formation implicates their production of phytohormones among the co-inoculation.

Table 4.1: Rhizobial strains and their host plants (Kers et al , 2003)

Rhizobium	**Host plant(s)**
Sinorhizobium meliloti	*Medicago, Melilotus, and Trigonella spp.*
R. leguminosarum bv. viciae	*Pisum, Vicia, Lathyrus, Lens spp.*
R. leguminosarum bv. trifolii	*Trifolium spp.*
R. leguminosarum bv. phaseoli	*Phaseolus vulgaris*
R. loti	*Lotus spp.*
R. huakuii	*Astragalus sinicus*
R. ciceri	*Cicer arietinum*
Rhizobium sp. strain NGR234	*Parasponia spp.(nonlegume)*
R. tropici	*Phaseolus vulgaris,., Macroptilium spp.*
R. etli	*Phaseolus vulgaris*
R. galegae	*Galega officinalis, G.orientalis*
R. fredii	*Glycine max, G. soja*, and other legumes
B. japonicum	*Glycine max, G. soja*, and other legumes
B. elkanii	*Glycine max, G. soja*, and other legumes
Bradyrhizobium sp. strain Parasponia	*Parasponia* spp.
Bradyrhizobium sp. strain caulinodans	*Sesbania* spp. (stem nodulating)

Chebotar et al., (2001) have reported that when some *Pseudomonas* strains co-inoculated with *Badyrhizobium japonicum* increased nodule number and acetylene reduction in soybean. Using both cell-free supernatants of PGPR cultures and pure chemicals, it was demonstrated that plant-growth-regulating substances produced by PGPR affected nodulation and nitrogen fixation (Barea and Azcon, 2003). Metabolites other than phytohormones, such as siderophores, phytoalexins and flavonoids, might enhance nodule formation has been proposed (Lucas-Garcia et al., 2004). Inoculation of phosphate-solubilizing bacteria (PSB) enhanced nodulation and nitrogen fixation by alfalfa plants along with an increase in the P content of plant tissues (Toro et al., 1998). It was thought that an improvement in P nutrition of the plant resulting from the presence of PSB was responsible for increased nodulation and N_2-fixation, as these processes are P-dependent (Barea et al., 2005). In addition, an increase in soil enzymatic activities (phosphatase, β-glucosidase and dehydrogenase) and of auxin production by PGPR strains was also thought to be involved in the PGPR effect on nodulation. Chebotar et al., (2001) demonstrated that some *Pseudomonas* strains, but not all, increased nodule number and acetylene reduction in soybean plants inoculated with *B. japonicum*. The possibility that metabolites other than phytohormones, such as siderophores, phytoalexins, and flavonoids, might enhance nodule formation has also been proposed (Lucas-Garcia et al., 2004).

Ecological interactions between rhizobia and other soil bacteria have been of interest in recent years because of their agronomical implications. Many papers have reported enhancement of nodulation and growth of a wide variety of forage and grain legumes, due to a positive interaction between *Rhizobium* spp. and other PGPRs present in soil. The coinoculation of non-rhizobial plant growth promoting strains to improve the nodulation and N-fixing potential of the inoculated rhizobial strains has received much attention. Dual inoculation of *Azotobacter vinelandii* or *Azospirillum lipoferum* with Rhizobium strains showed a synergistic effect on nodulation, yield and N uptake in soybean, clover, peanut and *Cajanus cajan* (Burns et al., 1981; Raverker and Konde, 1988, Dardanelli et al., 2008, Tilak et al., 2006, Qureshi et al., 2009).

4.1.2.2 Negative interactions in rhizosphere:

Although most of the times plant growth-promoting rhizobacteria may interact synergistically with root nodulating rhizobia but sometimes can be antagonistic to each other.Several researchers have identified microbial populations in the rhizosphere as a first barrier to pathogen infection. It is well known that some soils are naturally suppressive to soil-borne plant pathogens including *Fusarium,*

Gaeumannomyces, Rhizoctonia, Pythium, and *Phytophthora*(Weller et al., 2002).. Although this suppression relates to both physicochemical and microbiological features of the soil, in most systems the biological elements are the primary factors in disease (Weller et al., 2002). The most widely studied bacteria by far in relation to biocontrol are *Pseudomonas* spp., such as *P. aeruginosa* and *P. fluorescens*, which are amongst the most effective root colonizing bacteria. Pathogen suppression by antagonistic micro-organisms can result from one or more mechanisms depending on the antagonist involved. Direct effects on the pathogen include competition for colonization or infection sites, competition for carbon and nitrogen sources as nutrients and signals, competition for iron through the production of iron-chelating compounds or siderophores, inhibition of the pathogen by antimicrobial compounds such as antibiotics and HCN, degradation of pathogen germination factors or pathogenicity factors and parasitism. These effects can be accompanied by indirect mechanisms, including improvement of plant nutrition and damage compensation, changes in root system anatomy, microbial changes in the rhizosphere, and activation of plant defence mechanisms, leading to enhanced plant resistance. An effective biocontrol agent often acts through the combination of several different mechanisms (Whipps, 2001). Rhizobacteria from the genus *Pseudomonas* provide an excellent example of a combination of multiple mechanisms for effective bio-control including direct antagonism and induction of plant resistance. Rhizobia and fluorescent pseudomonads form an important part of the PGPR group of microorganisms. It is already reported that the fluorescent *Pseudomonas* strains promote plant growth by suppressing plant pathogenic microorganisms through production of antibiotics but the action of these antibiotics is non-specific and thus may also harm the other plant beneficial microorganisms in the vicinity.Fluorescent pseudomonas produces spectrum of antibiotics viz. DAPG,pyrrolnitrin,pyoluteorin and phenazine and some of the antibiotics have shown the bacteriocidal effect (Table 4.2) and mechanism of action of these antibiotics are also described in Table 4.2.

Overproduction of antibiotics by the use of genetically modified organism and effect of over concentration of antibiotics on the microbial community associated with different plant rhizosphere was studied extensively by different researchers as described in Table 4.3.

Table 4.2 Bactericidal effect of antibiotics produced by fluorescent pseudomonads

	2,4- DAPG	Pyrrolnitrin	Pyoluteorin	Phenazine
Antimicrobial	Bacteriocidal effect against multi drug resistant S.aureus	10 µg/ml inhibits S. cerevisiae and P. atrovenetum	Bactericidal	?
Mode of action	Membrane damage, inhibitory to zoospores of oomycete	Terminal electron transport in fungus	?	Interfers normal ETS and Induces overproduction of O_2 radicals.
Other functions	Intercellular signal at time of pathogen attack	Persists actively for 30 days in soil and it is photosensitive	Intercellular signal between distinct bacterial populations	Helps in microbial competition in rhizosphere, survival and competence

Table 4.3 Geneticaly modified PGP strains of fluorescent pseudomonads and their impact on microbial community (Source: Bais et al., 2006)

Peudomonas WT strain	GM trait (GM derivative)	Impact on non microbial communities in the rhizosphere	References
***Pseudomonas putida* WCS358**	Produces Phz and Phl	Transient shift in composition of bacterial and fungal communities in wheat rhizosphere. No effect on soil metabolic activity	Glandorf et al., 2001; Viebahn et al., 2003
***Pseudomonas fluorescens* F113**	Overproduce Phl	No influence on microbial population in sugar beet rhizosphere	Barea et al., 1998
***Pseudomonas fluorescens* CHA0**	Overproduces Phl and Plt	Wild type strain and GM derivative altered the structure of culturable fungal community in mungbean rhizosphere	Shaukat & Siddiqui, 2003
***Pseudomonas fluorescens* CHA0**	Overproduces Phl and Plt	GM derivative had a detectable influence on fungal community in the cucumber rhizosphere at 32 d	Girlanda et al., 2001
***Pseudomonas fluorescens* CHA0**	Overproduces IAA	Increased root mass in natural soil however in autoclaved soil WT increases root and shoot yield while GM derivative caused root stunting	Beyler et al., 1998
***Pseudomonas fluorescens* CHA0**	Overproduces Phl and Plt	WT and GM derivative inoculation decreased the population size of nitrogen fixing symbiotic *S. meliloti* in soil microcosm with alteration in nodulation efficiency of Alfalfa.	Niemann et al., 1997

Pseudomonas putida WCS358 which produces Phz and Phl which causes the transient shift in composition of bacterial and fungal communities in wheat rhizosphere (Glandorf et al., 2001; Viebahn et al., 2003). As per observation by Niemann et al., (1997) that GM *Pf*CHA0 has decreased the population of nitrogen fixing symbionts S.meliloti to Alfalfa root.So, it was hypothesized that there could be the negative effect of antibiotics produced by biocontrol strains of fluorescent pseudomonads , on the rhizobia spp. present in the rhizosphere.

With the above background, the objectives of the present study were to study the effect of antagonistic metabolites produced by fluorescent pseudomonads on the growth of rhizobia. In this chapter the interaction between rhizobia and *Pseudomonas* spp. was studied in sequential manner with following steps e.g. a) *In vitro* studies for the effect of pure antifungal metabolites on rhizobia; b) interaction of biocontrol strains of fluorescent *Pseudomonas* with rhizobia spp.in dual culture experiments; c) *In vitro* studies for the effect of extracellular metabolites of fluorescent *Pseudomonas* strains on rhizobial spp.; d) Interactions of fluorescent *Pseudomonas* strains and rhizobia under biocontrol supportive nutritional amendments in the presence/absence of fungal phytopathogen.

4.2. MATERIALS AND METHODS

4.2.1 Bacterial strains used in the study:

Standard rhizobial strains and lab rhizobial strains are described in Table 4.4).

Table 4.4 Bacterial strains used in the study

Organism used	Strains	References/Source
Rhizobial strains	*R. leguminosarum* bv. viciae *3841*	ATCC (AM236080)
	Sinorhizobiu.meliloti (Ensifer meliloti)	ATCC9930
	M. loti	Maff 303
	B. japonicum 61A152	M. L.Guerinot, Dartmouth college, New Hampshire
	IC 3123 (Pigeon pea nodulating)	IARI, NewDelhi; Rajendran et al., 2007; 2008; Arif et al., 2012
	IC 3169 (Pigeon pea nodulating)	IARI, NewDelhi
	IC 3109 (Pigeon pea nodulating)	IARI, NewDelhi
	ST1 (Pigeon pea nodulating)	Laboratory isolate , Rajendran et al., 2007; Geetha et al., 2009; Arif et al., 2012.
Model *Pseudomonas fluorescens* strains	*Pf* CHA0	Rezzonico Fabio, ACW,Switzerland
	Q2-87	Rezzonico Fabio, ACW
	Pf-5	J. E. Loper ,Oregon university,USA
Fluorescent *Pseudomonas* isolates	C2, C7,G2,G5,G13, G16, G20, G25,G29,G31, G44,G45,H4, H9,P1	This study
Fungal pathogen	*R. bataticola*	Anand Agriculture University, Gujarat

4.2.2 Effect of pure antifungal metabolites on rhizobia:

For the study of the effect of pure antifungal metabolites known to be produced by fluorescent *Pseudomonas* on rhizobial cultures, Yeast Extract Mannitol Agar (YEMA) plates were prepared and were spread with 0.1 ml of overnight grown rhizobial culture using a glass spreader. YEMA composition: (Ingredients (g/100ml) – Mannitol- 1 g, Sodium glutamate- 0.2 g, KH_2PO_4-0.02g, MgSO4-0.05g,Sodium chloride-0.01g,Yeast extract-0.1g, Agar 3%).Autoclaved at 10 psi for 20 minutes. 5 mm wells were bored on these plates using sterile 1 cm cork borer. Various dilutions of pure antifungal compounds- DAPG, pyoluteorin and pyrrolnitrin prepared using sterile distilled water except for phenazine which was prepared using 1N NaOH. Various concentrations (80 µl) of pure antifungal compounds, which were dissolved in methanol/NaoH, were loaded in the wells on the

YEMA plates. Plates were incubated in upright positions overnight at 28^{0}C. HPLC grade methanol and 1N NaOH were used in the experiment.

4.2.3 Dual culture test for compatibility of bio-control fluorescent pseudomonads with rhizobia:

Fluorescent pseudomonad strains were streaked on *Pseudomonas* agar plates with mannitol to support rhizobial strain growth. Rhizobial strains were streaked side by side to fluorescent pseudomonas strains and the plates were incubated at 28 ^{o}C for 7-8 d and observed for their growth.

4.2.4 Rhizobial inhibition by ethyl acetate extracts of fluorescent pseudomonad strains:

Yeast Extract Mannitol Agar (YEMA) plates were prepared and were spread with 0.1 ml of overnight grown rhizobial culture using a glass spreader. Using a sterilized 1 cm diameter cork borer, wells were bored on these plates at four places on plate periphery and 80 µl of the ethyl acetate extracts of *Pseudomonas* strains were loaded in the well. The plates were incubated at 28^{o}C overnight in upright position. HPLC grade methanol was used as control in these experiments .The inhibition was recorded based on the zone of inhibition observed around the wells after overnight incubation.

4.2.5 Interaction of fluorescent pseudomonads with rhizobial strains in the presence of fungal pathogen:

The effect of metabolites produced by fluorescent *Pseudomonas* strains on the rhizobial strains in the presence of the fungus. Live interaction study between fungus, biocontrol strains of fluorescent *Pseudomonas* and rhizobial cultures was performed by triple culture plate assay (Fig. 4.2).

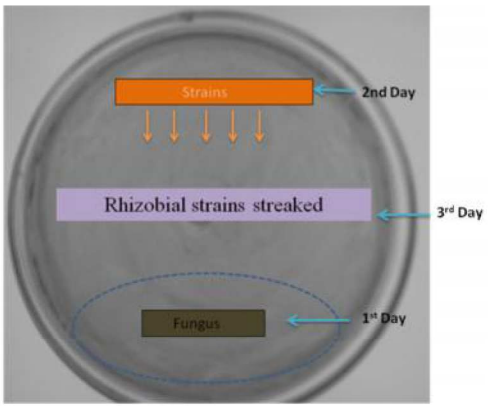


Fig.4.2 Representative diagram of the plate assay for the triple interaction of rhizobia, fluorescent pseudomonads and *R.bataticola*

For this experiment, a nutrient agar plate was prepared supplemented with 0.5% mannitol which supports the growth of rhizobia. On the first day, *R. bataticola* was inoculated on the NBM agar plates on one end of the plate. This was done by cutting a piece of agar from a PDA plate containing

previously grown fungus and placing it on the plate. The plate was incubated overnight and the next day the opposite end of the plate, a thick and heavy inoculum of fluorescent *Pseudomonas* strain was inoculated and the plates incubated at 28^0C overnight.After overnight which the inhibition of the fungus by the fluorescent *Pseudomonas* cultures was recorded. A second set of experiment also included the rhizobial cultures. For the second set of experiment, sterile NBM plates were prepared and the fungus as well as fluorescent *Pseudomonas* cultures were inoculated in the same way as mentioned above. The plates were incubated at 28^0C overnight until inhibition of the fungus by the fluorescent *Pseudomonas* cultures was apparent. Overnight grown rhizobial cultures were inoculated in the space between the fungus and the fluorescent *Pseudomonas* cultures. The distance between the fluorescent *Pseudomonas* strain and rhizobial strain was maintained at around 2 mm. The plates were incubated at 28^0C, the growth/inhibition of the rhizobial cultures were recorded after 48-72 h incubation. Growth of rhizobial strains was compared with without fungal control and also without fluorescent pseudomonads control.

4.2.6 Rhizobial culture inhibition by fluorescent *Pseudomonas* strains under various nutritional conditions

To test the growth of the fluorescent *Pseudomonas* strains as well as fungal cultures under these conditions of nutrition, 1/10 diluted nutrient agar plates were prepared containing the required supplements according to Table 3.4 of Chapter 3. In the combinations wherein both glucose and citrate were to be amended, they were autoclaved separately. Zn^{2+} and Mo^{2+} salt solutions were autoclaved separately and then added. Fe^{2+} salt solution was filter sterilized and added. The basal 1/10 nutrient broth along with 3% agar was autoclaved separately and the amendments were added according to the media composition provided in Table 3.4 of Chapter 3. The fungal culture was inoculated in the plates by cutting a piece of agar from a PDA plate containing previously grown fungus and inoculating it on the plate. Overnight grown fluorescent *Pseudomonas* strains were inoculated on the plates. The plates were incubated over night at 28^0C. The growth was observed and recorded. As per the results obtained for the previous experiments, it was observed that those combinations containing iron, molybdenum and zinc in the concentration of 0.5mM, 0.5mM and 0.35 Mm respectively showed decreased growth and hence to confirm that the reduced growth was the effect of presence of metal ions in concentrations higher than that the cultures can tolerate the following experiment was performed.

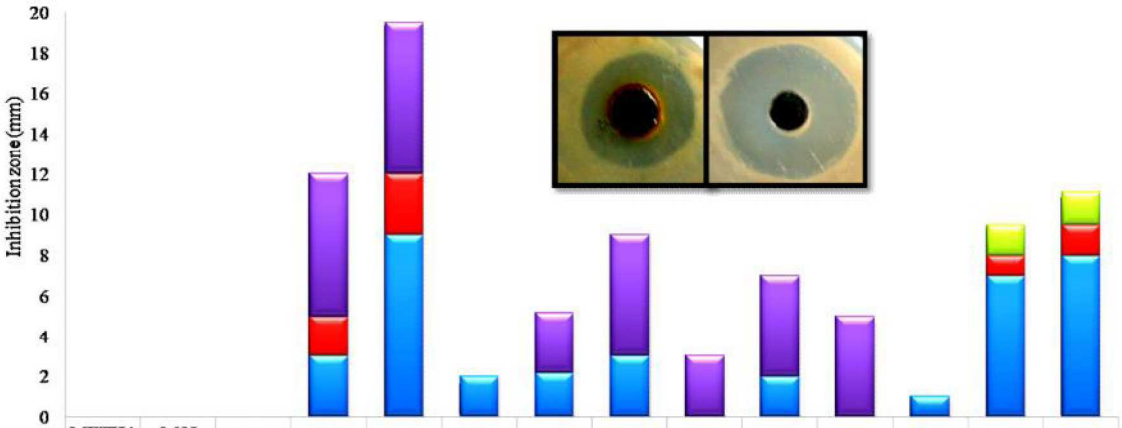
4.2.7 Rhizobial inhibition by ethyl acetate extracts of fluorescent *Pseudomonas* strains grown under different nutritional conditions.

Ethyl acetate extracts of various fluorescent *Pseudomonas* strains grown for 5 days under shaking conditions under different nutritional conditions according to table 3.4 of Chapter 3. The ethyl acetate extracts were tested against the rhizobial cultures.YEMA plates were spread with 0.1 ml of the rhizobial culture using glass spreader. Then using a sterile 1 cm diameter cork borer, wells were bored on the plate periphery and 80 µl of the ethyl acetate extract were loaded in each well. The plates were incubated at 28^0C overnight in upright position and the inhibition of the rhizobial cultures were observed and recorded according to the zone of inhibition obtained along the wells.

4.3 RESULTS & DISCUSSION:

4.3.1 Rhizobial inhibition by antibiotics -2, 4-DAPG, PLT, PRN and PHZ:

Fig. 4.3 shows the effect of pure DAPG, pyrrolnitrin, pyoluteorin and phenazine on rhizobia. Rhizobial strain ST1 was found to be sensitive to DAPG, PRN and PLT followed by *R. leguminosarum* which was inhibited by DAPG and PHZ. Strain IC 3169 is inhibited by DAPG and lesser extent with PHZ while IC 3123 did not get inhibited by 2, 4- DAPG, PRN, PLT (Fig. 4.3). The results show that most of rhizobial strains get inhibition by pure 2, 4- DAPG, PRN, PLT and inhibition was directly proportional to antibiotic concentration.



	METHANOL	0.1N NaOH	1.75	3.5	7	0.7	2.03	4.9	1.75	3.5	7	1.75	3.5	7
	CONTROLS		DAPG(µg)			PRN(µg)			PLT(µg)			PHZ(µg)		
STI	0	0	0	7	7.5	0	3	6	3	5	5	0	0	0
IC 3123	0	0	0	0	0	0	0	0	0	0	0	0	1.5	1.6
IC 3169	0	0	0	2	3	0	0	0	0	0	0	0	1	1.5
R.Leg	0	0	0	3	9	2	2.15	3	0	2	0	1	7	8

Fig. 4.3 Effect of pure antibiotics on *rhizobial spp*

4.3.2 Dual culture tests for rhizobia –fluorescent pseudomonad interaction:

All the isolates which showed antifungal activity better than *Pf C*HA0 or similar to *Pf* CHA0, were selected for the studies by dual culture method. The amounts and type of antifungal metabolites depend on the type of soils and nutrient available (Duffy & Defago 1997). It may be possible that some of these compounds at particular concentrations may be inhibitory to rhizobacteria. Rhizobial strains *R. leg 3841*, IC 3169, IC 3123, ST1, *S.meliloti B. paponicum* were streaked along with *Pf* CHA0, Pf-5, Q2 87, G2 and G20. IC3169 was inhibited by all three model strains viz. Pf-5, Pf CHAO and Q287 (Fig. 4.4, Table 4.5) and also by some isolates. *R. leg 3841* was inhibited by the three model biocontrol strains and by many isolates (Fig. 4.4, Table 4.5). *S. meliloti* was inhibited by *Pf* CHA0, Pf-5 and Q2 87. Rhizobial strain ST1 was inhibited by *P. fluorescens* Pf-5 but showed growth in presence of *Pf* CHAO, Q287 and G20. *R. leg* 3841 was found to be the most sensitive rhizobial strain followed by IC3169. Fluorescent *Pseudomonas* strain G2 and G20 have shown inhibition to all rhizobial strains except IC 3109. *R. leg 3841* and *M. loti* were found to be most sensitive to most of fluorescent *Pseudomonas* strains. *M. loti* was inhibited effectively by *Pf* CHA0, Pf-5, Q287, G2 and G20. *B.japonicum* and IC3123 exhibited growth in presence of all fluorescent *Pseudomonas* strains. Certain combinations of fluorescent *Pseudomonas* strains and particular rhizobial strain increased exopolysaccharide (EPS) production. *Pf* CHAO induced more EPS production by *B. japonicum,* IC3123 and IC 3109 but not in other rhizobial strains.

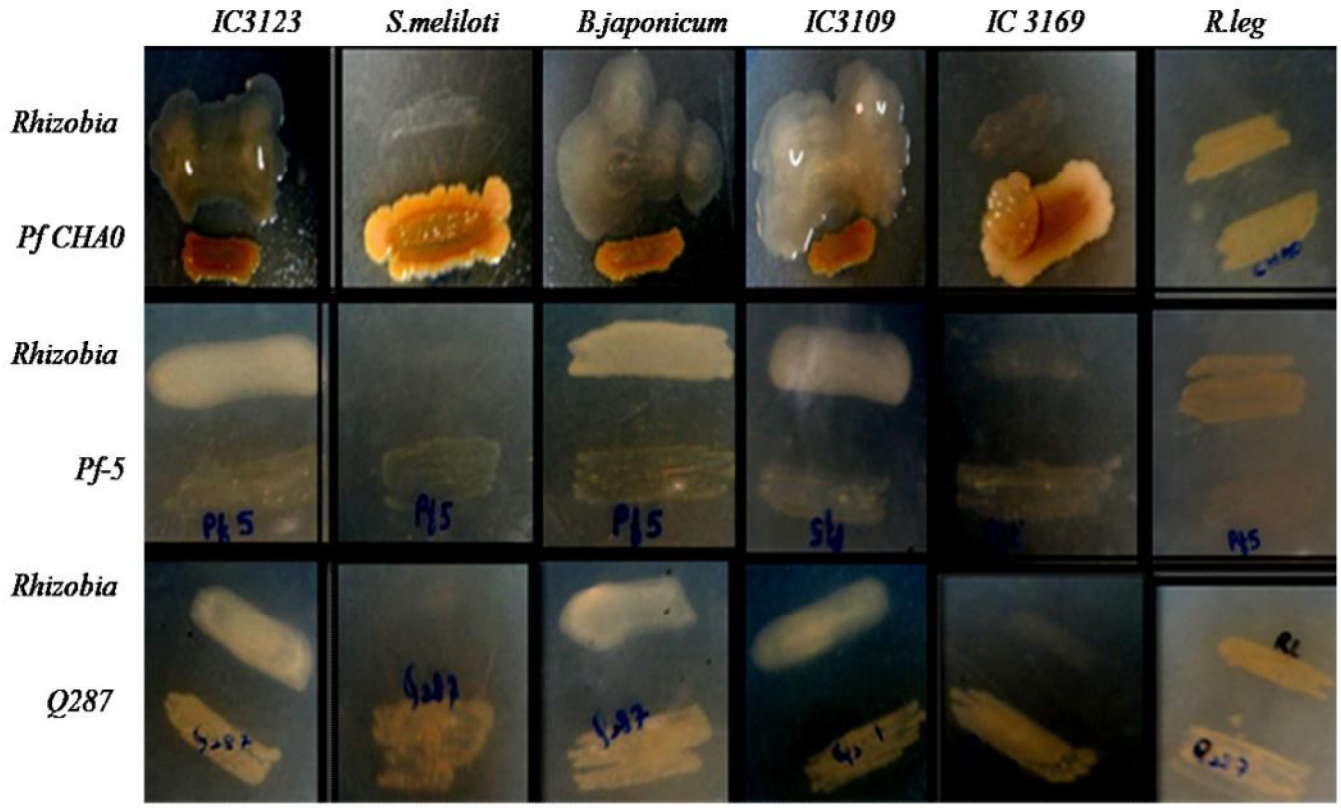


Fig. 4.4 Dual culture interaction of model fluorescent *Pseudomonas* and rhizobia

Table 4.5 Inhibition of rhizobial growth by fluorescent *Pseudomonas* strains

Pseudomonas Strains	*R.leg 3841*	*S.meliloti*	*M.loti*	*IC 3123*	*IC 3169*	*IC 3109*
Pf-5	++	-	+++	-	+	-
Pf CHA0	+	+	+++	-	+	-
Q 2 87	++	-	+++	-	++	-
G2	+++	+	+++	+	+	-
G20	+++	++	+++	+	+	-
G45	++	+	-	+	+	+
C7	+	-	+	+	+	+
G16	++	-	+	-	+	+
G13	+	-	+	-	+	-
G44	+	-	-	+	-	-

G5	+++	-	-	-	-	+
H9	++	-	-	-	-	+
P1	++	-	-	-	-	+
C2	-	-	+	-	-	+++
Un -inoculated	-	-	-	-	-	-

(+ Inhibition - No Inhibition)

4.3.3 Rhizobial inhibition by ethyl acetate extraction of antibiotics from fluorescent *Pseudomonas* strains:

The effect of partially purified metabolites on rhizobial strains was studied using ethyl acetate extracts of fluorescent *Pseudomonas* strains (Fig. 4.5). *R. leg* 3841 was found to be the most sensitive rhizobial strain in these experiments also and was inhibited by extracts of all fluorescent *Pseudomonas* strains except strain C2. IC3169 was inhibited by extracts of *Pf* CHAO, Pf-5, Q287, C7, G2, G13, G16 and G45. *M. loti* was inhibited by *Pf* CHA0, Pf-5, Q287, G2 and G20 extracts. IC 3123 was inhibited moderately by extracts of C7,G2, G13, G20, G44 and G45. Strains G2 and G20 have shown inhibition to all rhizobial strains except IC 3109. Strains G13, G44, H9 and P1 showed inhibition to few rhizobial strains only.

Fluorescent pseudomonads could be categorized based on their compatibility with rhizobial strains (Table 4.6) and both *Pseudomonas* as well as rhizobial strains were clustered on the dendrogram based on their pattern of inhibition (Figs. 4.6 & 4.7). In some cases it was observed that the rhizobial strains inhibited by extracts of fluorescent *Pseudomonas* strains may not necessarily get inhibited by live culture of the same fluorescent *Pseudomonas* strain. The inhibition shown by the extracts may be due the fact that antibiotics produced by fluorescent *Pseudomonas* strains get concentrated in ethyl acetate extracts; the effect of which either gets diluted out in live cultures or is produced in very low concentrations which are ineffective on rhizobium strains. Also there are cases where a particular fluorescent *Pseudomonas* strain inhibits growth inhibition of rhizobial strains in dual culture assay while the extract of the same fluorescent *Pseudomonas* strain is unable to cause inhibition. This may happen because the inhibition caused may be due to some metabolite which could not get extracted during ethyl acetate extraction. Strain IC 3123 did not get inhibited even on treatment with varying concentrations of pure antibacterial compounds while it got moderately inhibited by extracts of fluorescent *Pseudomonas* strains G2, G13, G20, G44, G45 and C7 suggesting the role of some other

component of the ethyl acetate extract other than the antibiotics. Similar was the case with rhizobial strain IC 3169 which got inhibited only slightly by DAPG that too at a higher concentration, but exhibited inhibition by extracts of strains namely *Pf* CHAO, Pf5, Q287, G2, G13, G16, G45 and C7 in the plate assay.

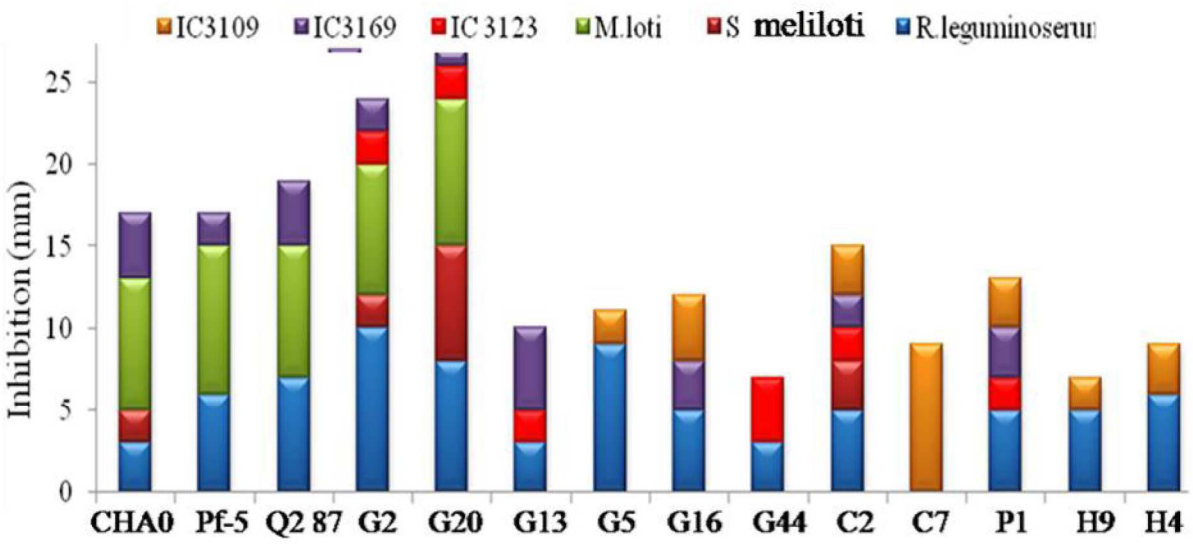
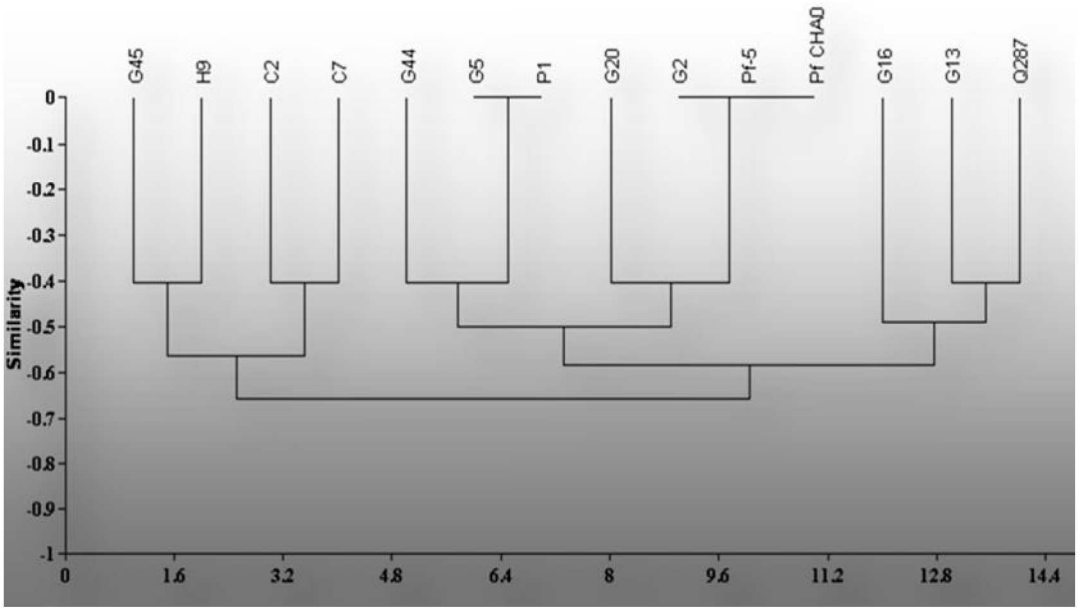


Fig.4.5 Inhibition of rhizobial strains by the ethyl acetate extracts of fluorescent *Pseudomonas* strains

Table 4.6 Categorization of fluorescent pseudomonad based on their compatibility with rhizobial strains

Rhizobium spp	Compatible fluorescent pseudomonad	Non compatible fluorescent pseudomonad
R.leg	C2, G25, G29, G31, H4	CHAO,Q287,Pf-5, C7,G2, G5, G13, G16, G20,G44, G45, H9,P1
S.melliloti	Q287,Pf-5, C2,C7,G5, G13, G16, G25,G31, G44,H4,H9,P1	CHAO, C7,G2,G20,G45
M.loti	C2, G5, G25, G29,G31,G45, H4,H9,P1	CHAO,Q287,Pf-5,C2,C7, G13, G16,G44,G20,G2
IC 3123	CHAO,Q287,Pf-5, C2,G5,G13, G16,G25,G29, G31, H4, P1,H9	C7, G2, G20,G44,G45
IC 3169	C2, G5, G25, G29, G31, H4, P1, G44, H9	CHAO,Q287,Pf-5, , C7, G2, G13, G16,G20, G45
IC 3109	Pf CHAO,Q287,Pf-5, C2,G2, G13,G20,G25, G29, G31, G44, H4	C2,C7,G5,G16, ,G45, H9,P1



Based on inhibition effect to rhizobium strains

Fig 4.6 Clustering of fluorescent *Pseudomonas* strains based on their inhibitory effect on rhizobial strains (Cut-Off value ≥2 mm)

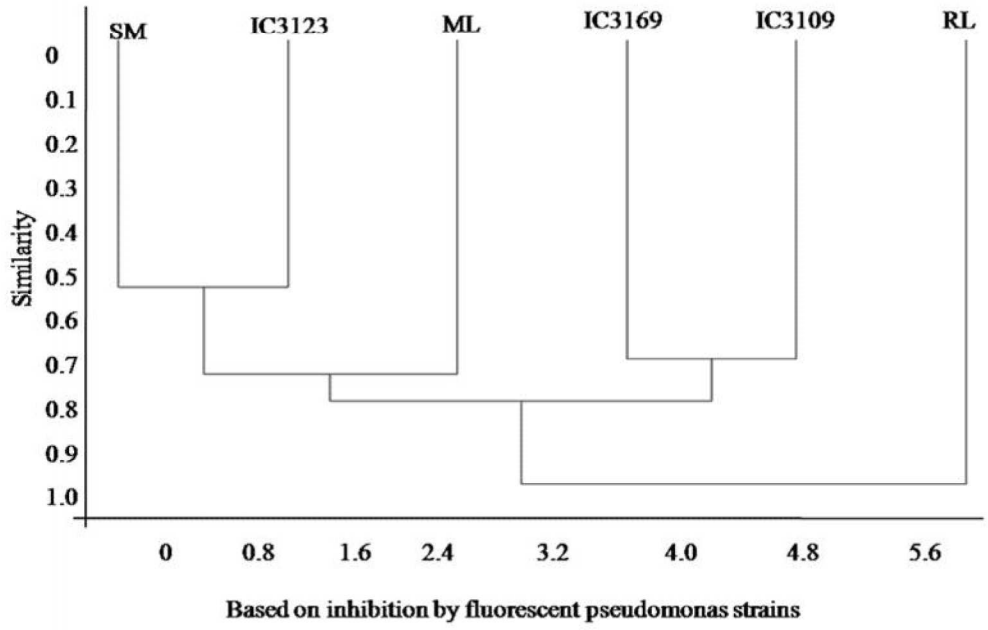


Fig.4.7 Clustering of rhizobial strains based on their inhibition pattern by fluorescent *Pseudomonas* strains (Cut-Off value ≥2 mm)

(ML-*M.loti*, SM-*Sinorhizobium meliloti*)

4.3.4 Rhizobial inhibition by fluorescent *Pseudomonas* strains under various nutritional conditions

Certain media compositions found to to be very significant for the effective biocontrol by *Pf* CHA0 as the metabolites were produced in high amount. The percentage inhibition of fungal culture by fluorescent *Pseudomonas* strains was significantly high under certain nutritional amendments as described in Table 3.2 of Chapter 3 as compared to control without any nutritional amendments. Such media and nutrient combinations were used for studying the antagonistic effect of fluorescent pseudomonads on rhizobia.

4.3.4.1 Effect of DAPG production supportive media on rhizobial inhibition by fluorescent pseudomonads:

Pf CHA0 was grown on KMB and MALT medium at 28^0 C for 4 d and DAPG extraction was done. 50µl of the extract was loaded and checked for antibacterial activity. As seen in Fig 4.8 both KMB and MALT media extracts were showing inhibitory affects on the rhizobial strains tested.

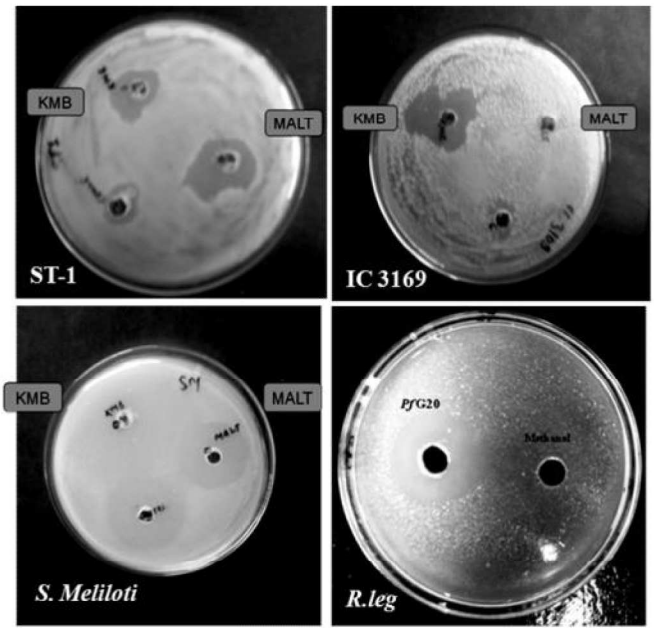
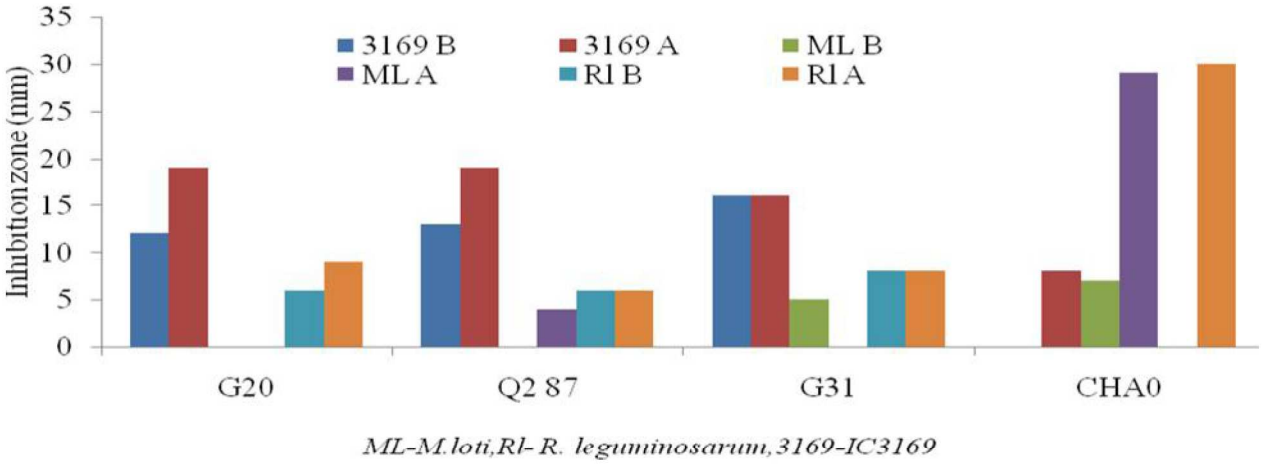


Fig.4.8 Inhibition of rhizobial strains by antibiotic extracted from different DAPG-supporitve media by *Pf* CHA0 and G20

4.3.4.2 Rhizobial inhibition by ethyl acetate extracts of fluorescent *Pseudomonas* grown under different nutritional conditions:

Presence of various nutritional amendments in the growth media increases the antifungal property of the fluorescent *Pseudomonas* strain CHAO by the production of antibiotics by fluorescent *Pseudomonas* strains. It was hypothesized that the pattern of inhibition of rhizobial strains by the extracts of fluorescent *Pseudomonas* strains and differ from the live culture interactions. Therefore experiment was performed to observe the inhibition of rhizobial strains by the fluorescent *Pseudomonas* strains in presence of different nutritional amendments which are said to increase the biocontrol trait of fluorescent *Pseudomonas* strains. The results show that *R.leguminosarum* which most sensitive strain and got inhibited by extracts of all fluorescent *Pseudomonas* strains, also did not shown growth in presence of most fluorescent *Pseudomonas* strains in media A & B (Figs. 4.9 & 4.10). It shows growth in presence of Pf-5 and G2 and when *P.fluorescens* CHAO grown in media A & B. *M. loti* also does not show any growth when present along with any fluorescent *Pseudomonas* strain. *B.japonicum* does not get inhibited significantly by most fluorescent *Pseudomonas* strains in presence of any nutritional combination but is inhibited by G2 in media A& B. To conclude the results suggest that the various nutritional combinations increase the antifungal activity of fluorescent *Pseudomonas* strains In presence of these nutritional combinations the fluorescent *Pseudomonas* strains inhibit *R.leguminosarum and M.loti.*Hence *R.leguminosarum and M.loti* can be considered as sensitive strains which upon coinoculation with fluorescent *Pseudomonas* strains get inhibited and hence should not be used together as bioinoculants. Strains G20, G38 and G45 have shown strong antifungal activity but not a growth inhibition of any of rhizobial strains which could be due to a metabolite/concentrations which is inhibitory to fungal growth but not to rhizobial strains.G2 has shown both the antifungal activity as well as inhibition of rhizobial growth so it could be conclude that inhibitory molecule could be same for both inhibition.Rhizobial cultures inhibition assay in the presence of extracellular metabolites released by biocontrol strains.



(mM)	Citrate	Glucose	Pi	KNO3	Fe2+	Zn2+	Mo2+
A-	100	100	0	0	0	0.35	0.5
B -	100	100	10	10	0.5	0.35	0.5

Fig. 4.9 Inhibition of rhizobia by *Pf* CHA0 antibiotic extracts from culture supernatants from the biocontrol supportive media (A&B)

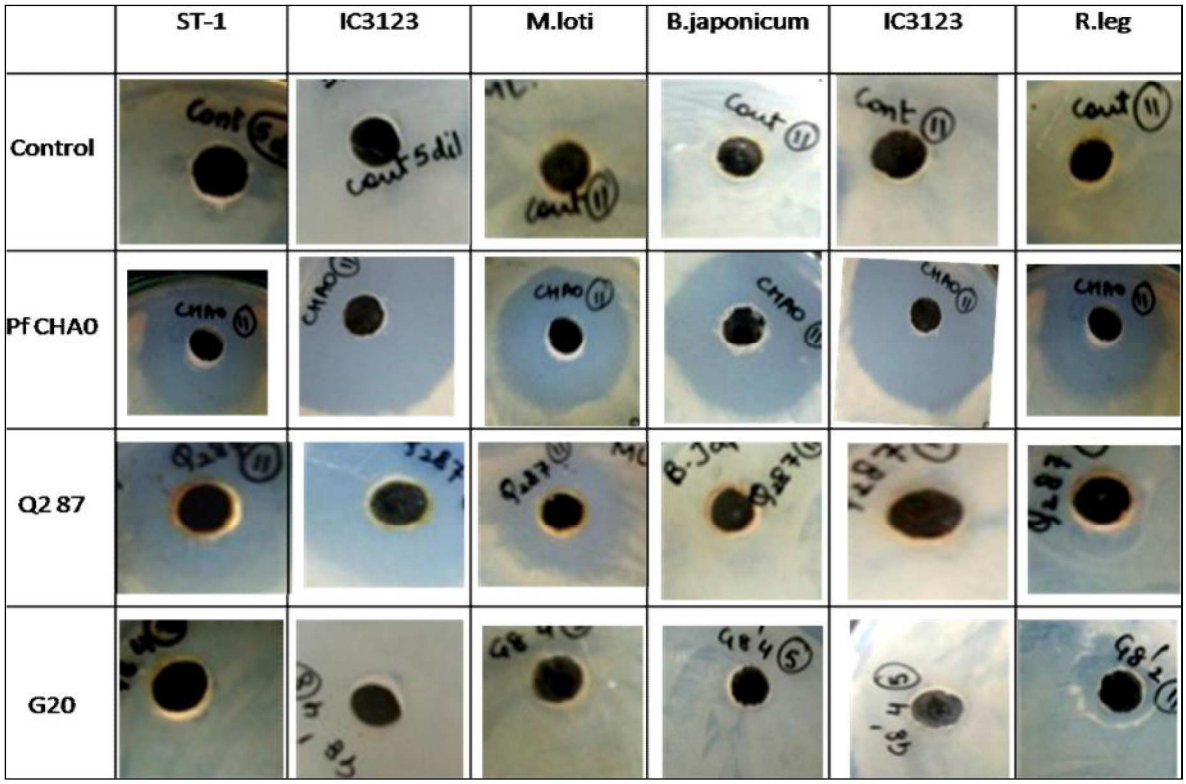


Fig.4.10 Inhibition of rhizobia by antibiotic extracts of fluorescent *Pseudomonas* strains from cultures grown under biocontrol supportive nutrient combinations

4.3.5 Triple culture technique to study the interaction of rhizobia, fluorescent *Pseudomonas and R. bataticola*

These experiments deal with the effect of rhizobial growth by the extracellular metabolites produced by bio-control strains of fluorescent *Pseudomonas* in presence of fungal phytopathogens. This experiment was done based on hypothesis that fluorescent pseudomonads are known to be induced for the antifungal metabolites gene expression in the presence of the fungal pathogen or its extracellular metabolites e.g. stimulation od DAPG swynthesis by fusaric acid etc. The strategy of the experiment was planned such that the growth of the rhizobial cultures could be tested in presence of both fungal inoculum and fluorescent *Pseudomonas* strains (Fig. 4.11). As can be inferred from the Table 4.7, strains *Pf* CHAO, Q287 and Pf-5 show consistently high antifungal activity in presence of rhizobium cultures. Fluorescent *Pseudomonas* strain G20 shows a lower anti fungal activity as compared to strains *Pf* CHAO, Q287 and Pf 5. Strain G2 and G1 has shown 35.5% and 22.22% fungal inhibition and also a strong inhibition of *R.leguminosarum*, while strain G38, G45 and G20 have shown fungal inhibition of 55.5%, 46.66% and 42% respectively but not the inhibition of rhizobial strains.

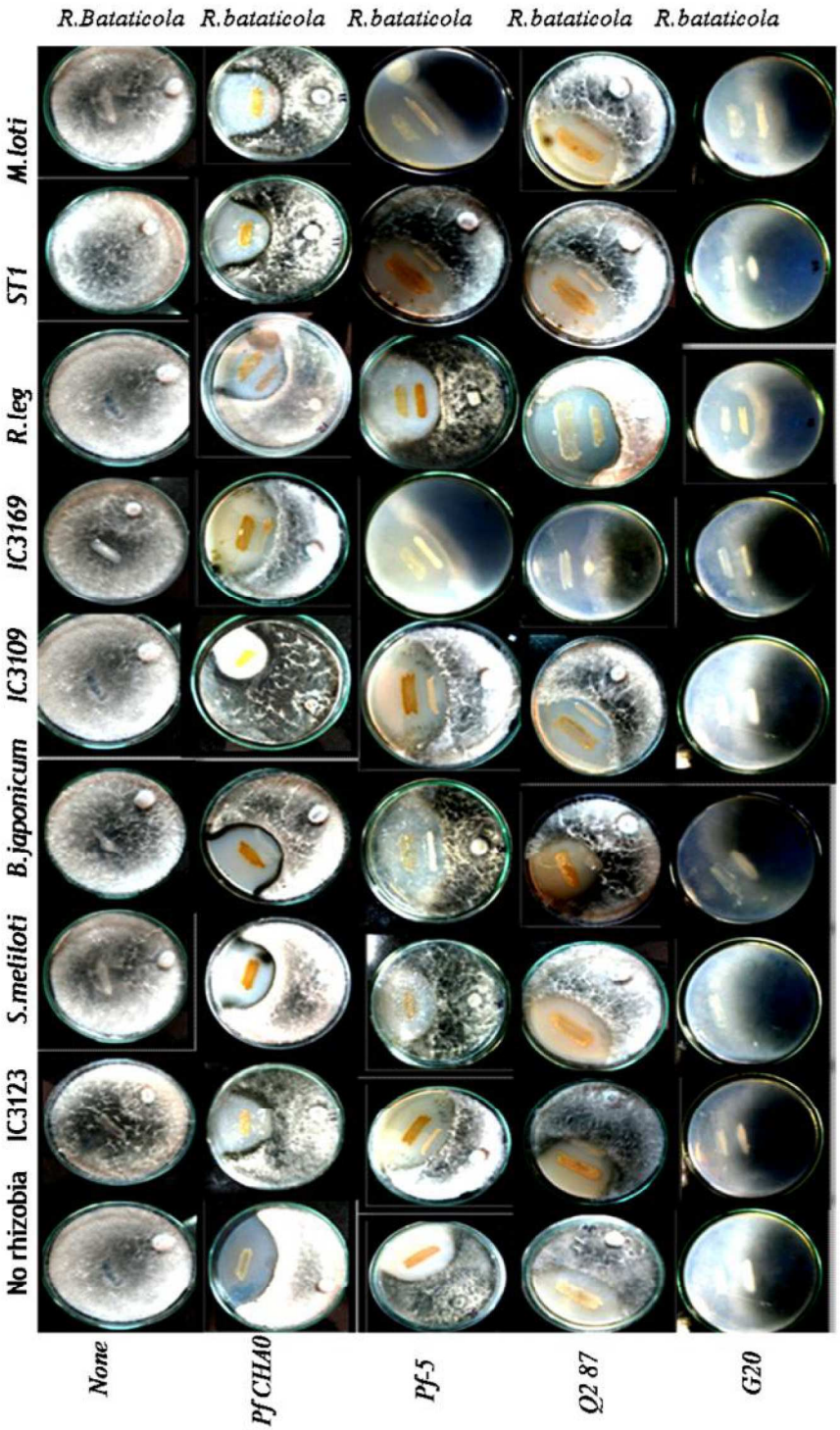


Fig.4.11 Interaction of fluorescent *Pseudomonas* and rhizobia in presence of *R.bataticola*

Table 4.7: Interaction of biocontrol strains of fluorescent pseudomonad and standard rhizobial strains in presence of *R.bataticola* (+ : Growth - : No growth)

Strains	Un-inoculated	Fungus	*R.leg*	*S.meliloliti*	*M.loti*	*B.japonicum*	ST1	IC3123	IC3169	IC3109
G20 + F	-(26.6)	NA	-(53.3)	-(33.3)	-(35.5)	-(26.6)	-(28.8)	-(26.6)	-(26.6)	-(26.6)
G20	+	-(26.6)	-	No growth	-	-	-	-	No growth	-
Q2 87 + F	-(33.3)	NA	-(55.5)	-(40)	-(48.8)	(33.3)	-(40)	-(33.3)	-(33.3)	-(33.3)
Q2 87	+	-(33.3)	-	No growth	-	-	-	-	-	-
Pf-5 + F	-(33.3)	NA	-(48.8)	-(42.2)	-(46.6)	-(33.3)	-(44.4)	-(33.3)	-(33.3)	-(33.3)
Pf-5	+	-(33.33)	-	No growth	-	-	-	-	No growth	-
CHA0 + F	-(33.33)	NA	-(40)	-(33.3)	-(44.4)	-(31.1)	-(42.2)	-(31.1)	-(31.1)	-(31.1)
CHA0	+	-(33.33)	-	-	-	-	-	-	-	-
Fungus	+	NA	+	+	+	+	+	+	+	+
Un-inoculated	NA	+	+	+	+	+	+	+	+	+

4.4 CONCLUSION:

Fourteen fluorescent *Pseudomonas* isolates with good antifungal activity were tested for their inhibition against seven rhizobial cultures using ethyl acetate extracts as well as by dual plate method. Effect of pure antibacterial/antifungal metabolites produced by fluorescent *Pseudomonas* strains showed that rhizobial strain ST1 was sensitive for DAPG, PRN and PLT , followed by *R.leguminosarum* which was inhibited by DAPG and PHZ while IC 3123 did not get inhibited by any of the pure antibacterial compounds. Certain rhizobial strains like *B.japonicum,* IC3123 and IC3109 exhibited increased EPS production in presence of *Pf* CHAO. The growth of fluorescent *Pseudomonas* strains and fungus in different nutritional combinations was observed. The fungal growth was indifferent both with and without nutrient supplements.Extracts of *Pf* CHAO and *Pf* Q287 prepared in media without any supplement significantly inhibited most rhizobial strains. The extracts prepared in media A & B exhibited very less or almost no inhibition to rhizobial cultures. *R. leguminosarum* 3841 was found to be the most sensitive rhizobial strain followed by *M. loti* which were was inhibited effectively by extracts from several *Pseudomonas* biocontrol strains. *B.japonicum* does not get inhibited significantly by most fluorescent *Pseudomonas* strains.

Although ideally plant growth promoting rhizobacteria such as fluorescent *Pseudomonas* strains should interact synergistically with root nodulating rhizobia and hence positively effect plant growth, but the results obtained here suggest that a few combinations of fluorescent *Pseudomonas* strains and *Rhizobium* spp. may prove hazardorous for the plant as the fluorescent *Pseudomonas* strains inhibit the growth of rhizobial cultures. The significance of work would be thus that the compatibility of the claimed biocontrol strains should be thoroughly checked with other rhizospheric resident PGPRs before their application in the fields for maximum economic gains. This work signifies that certain bio-control strain may have adverse effect on the rhizobial populations present in rhizosphere and hence these criteria should be checked during application of bio-control strains. An understanding of ecological and mutual interactions between putative inoculants and resident rhizosphere microorganisms is required to be thoroughly studied for maximum benefits to the host plant.

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