CHAPTER 4

Mechanism of Action of Siderophore Producing Rhizobacteria Showing Antagonistic Activity Against Plant Pathogenic Fungi

MECHANISM OF ACTION OF SIDEROPHORE PRODUCING RHIZOBACTERIA SHOWING ANTAGONISTIC ACTIVITY AGAINST PLANT PATHOGENIC FUNGI

4.1 Introduction

Understanding the mechanism of action of a biological control agent may allow the optimum conditions for implementing biocontrol in a given pathosystem to be determined (Mathre et al., 1999; Cabrefiga et al., 2007). However, assessment of the mechanisms of antagonism is a complex and difficult task, starting with prospective studies to reveal the implications of a given process (Cabrefiga et al., 2007). Plant roots release a wide variety of compounds into the surrounding soil, including ethylene, sugars, amino acids, organic acids, vitamins, polysaccharides, and enzymes. These materials create unique environments for the microorganisms living in association with plant roots, in the rhizosphere. On the other hand, rhizosphere bacteria also have a profound effect on plant health. Rhizosphere colonization is important not only as the first step in pathogenesis of soilborne microorganisms, but also is crucial in the application of microorganisms for beneficial purposes (Lugtenberg et al., 2001; Garbeva et al., 2004). Studies on the properties of beneficial bacteria that help them to dominate in the rhizosphere environment and simultaneously exhibit antagonism towards fungal pathogen have attracted a lot of attention of scientists worldwide. Particularly, the mechanism employed by biocontrol organisms in effecting disease control has been the most interesting aspect of biocontrol study (Howell, 2003).

One of the ways in which biocontrol bacteria suppress fungal pathogens is by producing secondary metabolites like antibiotics, siderophore, cell-wall degrading enzymes and hydrogen cyanide (Husen, 2003; Ramirez et al., 2004; Kumar et al., 2005; Ge et al., 2007; Chen et al., 2009). Antibiotics are the most widely studied antifungal metabolites produced by the biocontrol agents to combat the plant pathogens. In several instances, antibiotics have been shown to be particularly effective in suppressing plant pathogens and

the diseases they cause (Ligon et al., 2000; Brodhagen et al., 2005; Kumar et al., 2005; Kumar et al., 2009). Many microorganisms are known to produce multiple antibiotics which can suppress one or more pathogens (Ge et al., 2007). It has been reported that bacterial and fungal biocontrol agents like *Pseudomonas fluorescens, Bacillus subtilis, Serratia* sp., *Burkholderia cepacia* and *Trichoderma virens* produce a wide range of antibiotics involved in plant disease inhibition (Brodhagen et al., 2005; Shen et al., 2007; Kumar et al., 2009). Expression and secretion of lytic enzymes that can hydrolyze a wide variety of polymeric compounds like cell-wall degrading enzymes, protease and DNase by different microbes can also result in direct suppression of plant pathogen activities (Ramirez et al., 2004; Kumar et al., 2005; Chen et al., 2009).

Unlike microbial phytopathogens, plants are not generally harmed by the localized depletion of iron in the soil caused by PGPR. Most plants can grow at much lower (about 1000-fold) iron concentrations than microorganisms (O'Sullivan and O'Gara, 1992). In addition, a number of plants have mechanisms for binding the bacterial iron-siderophore complex, transporting it through the plant, and then reductively releasing the iron from the bacterial siderophore so that it can be used by the plant (Bar-Ness et al., 1991, 1992; Wang et al., 1993). The ability of siderophores to act as effective "disease-suppressive" agents is affected by the particular crop plant, the specific phytopathogen being suppressed, the soil composition, the bacterium that synthesizes the siderophore, and the affinity of the specific siderophore for iron.

This chapter focuses on the characterization of the selected isolates for secretion of antifungal enzymes *in vitro,* for production of HCN and for presence of the PGPR traits like phosphatase activity and IAA production. Additionally the amount of siderophore released by these bacteria *in vitro* has been quantified.

4.2. MATERIALS AND METHOD

4.2.1. Siderophore production assay

Quantification of siderophore was carried out by the CAS shuttle assay method (Payne, 1994). Bacterial isolates were grown overnight under constant shaking in Fiss glucose minimal media (Vellore, 2001). The medium is an iron restricted medium and thus siderophore production is effectively increased. The overnight grown culture was centrifuged at 10,000 rpm for 15 minutes; the cell-free culture supernatant was mixed with an equal volume of CAS reagent (solution 1 in CAS agar media) and incubated for one hour at 37°C. A control was kept where uninoculated broth was mixed with equal volume of CAS reagent. The absorbance was then measured at 630 nm. This assay method determined the amount of siderophore units present in the antagonistic strain culture. Siderophore content was calculated by using the formula:

$$\% \text{ siderophore units} = [(A_r - A_s)/A_r] \times 100,$$

where, A_r = absorbance of reference (uninoculated medium) at 630 nm and A_s = absorbance of the sample at 630 nm.

4.2.2 Antimicrobial metabolite production

The production of antimicrobial metabolites may be detected *in vitro* by specific tests. All the selected isolates were subjected to the tests listed below. Prior to the experiments, each bacterium was subcultured twice from the stock in NA and finally a 24 h NA culture was used for the test. An uninoculated control was included in all tests for comparison. For preparing culture filtrates, bacterial strains were grown overnight in nutrient broth and cultures were centrifuged at 10,000 rpm for 15 min. Culture supernatants were filtered through 0.2µm diameter cellulose acetate filter paper (Sartorius) to obtain the final filtrate which was used in cellulase production and chitinase production tests.

4.2.2.1 Cellulase production

To test for cellulolytic activity, M9 agar medium was amended with 10 g/L cellulose and 1.2 g/L yeast extract. After sterilization of media, it was poured onto petriplates (90 mm diameter) and allowed to solidify. Wells were cut on cellulose agar plates and 100µl bacterial culture filtrates were loaded in the wells. For preparing the culture filtrates, bacterial strains were grown overnight in nutrient broth and cultures were centrifuged at 10,000 rpm for 15 min. Culture supernatants were filtered through 0.2µm diameter cellulose acetate filter paper (Sartorius). The plates were incubated at 37°C for 8 days and subsequently stained with 0.1% Congo red solution overnight and destained four times with 1M NaCl at 1 h interval. Plates were checked for the formation of clear halo around the wells that indicated positive result for cellulase production (Cattelan et al., 1999; Kumar et al., 2005).

4.2.2.2. Pectinase activity

For detection of pectinase activity, M9 agar medium was used which was supplemented with 1% pectin (Cattelan et al., 1999; Kumar et al., 2005). Plates were prepared after autoclaving the medium and pouring it onto petriplates (90 mm diameter). Solidified plates were used for streak inoculation of bacterial strains. The inoculated plates were incubated for 48 hours at 30°C and subsequently flooded with 2M HCl. Clear halos around the colonies were considered as positive for pectinase production.

4.2.2.3. Lipase activity

Tween 80 agar plates were used to determine lipase activity. Plates were inoculated by streaking individual bacterial strains on the surface of the agar and incubated over night. An opaque halo of precipitation around the growth indicated hydrolysis of Tween 80 (Barrow and Feltham, 1993).

4.2.2.4. Chitinase activity

Chitinolytic activity was observed by the method of Bargabus et al. (2002). M9 media was supplemented with 0.1% glycol chitosan and 1% agarose.

Bacterial cultures (24 h) in NB were centrifuged at 10,000 rpm for 15 min and the supernatants were filtered through 0.2µm diameter cellulose acetate filter paper (Sartorius). Wells were made in the plates where 100µl of culture filtrate was added for the detection of chitinase activity. Plates were incubated at 30°C for 48 hours, stained with fluorescent brightener 28 and finally observed under UV light. Positive result was indicated by the presence non-fluorescent lytic zones around wells.

4.2.2.5. Amylase activity

Starch agar plates were used for testing amylase activity (Aneja, 2003). The plates were inoculated with the bacterial antagonists by single streak and incubated at 30°C for 48 hours in an inverted position. The plates were then flooded with iodine solution and after holding for 30 seconds the solution was poured off. Development of clear zone around the bacterial line of streaking in a dark blue or purple background was considered as positive result.

4.2.2.6. Protease activity

Skimmed milk agar plates were used to check for protease activity. Plates were inoculated with bacterial isolates as a single streak and incubated for 24-48 h at 30°C in an inverted position. Formation of a clear zone around the bacterial line of inoculation in the background of a turbid medium indicated positive result (Aneja, 2003).

4.2.3. Evaluation of PGPR traits

All the selected bacterial isolates were tested for two major PGPR traits. Each bacterium was subcultured twice from the stock in NA and finally a 24 h NA culture was used for the test. An uninoculated control was included in all tests for comparison.

4.2.3.1. Indole-3-acetic acid production

Production of IAA was determined according to Patten and Glick (2002). Twenty-four-hour-old bacterial isolates were cultured on Luria-Bertani (LB)

broth supplemented with 5 mM of L-tryptophan and were centrifuged to obtain culture supernatants. The supernatants (1 ml) were mixed vigorously with 4 ml of Salkowski's reagent (Gordon and Weber, 1951; Ahmad et al., 2005) and the absorbance was measured after 20 min at 535 nm. The concentration of IAA was determined by comparison with the standard curve. IAA standard curve was prepared from a series of known IAA concentrations which were similarly assayed.

4.2.3.2. Phosphatase activity

Phosphatase activity was determined in Pikovskaya's agar medium (Pikovskaya, 1948). The medium was inoculated with bacterial strains and incubated at 30°C for at least 5 days. Development of a clear zone around bacterial growth was considered as positive for phosphatase production.

4.2.4. Detection of HCN Production.

Test for HCN production was carried out by the method of Bakker and Schippers (1987). Bacteria were heavily inoculated in nutrient agar plates supplemented with 4.4 g/L glycine and incubated in an inverted position at 30°C with filter paper strips dipped in picric acid solution (0.5% picric acid in 2% Na_2CO_3 aqueous solution) placed inside the lids. Change of colour of the indicator strip from yellow to brown was considered as a positive result.

4.2.5. Antagonism by crude extracellular products

Crude extracellular products were extracted from the culture of the bacterial isolate *Pseudomonas putida* strain AS04 which showed the highest antifungal activity. The strain was allowed to grow on semi-solid nutrient agar media containing 0.6 % agar at 30°C for 48 h.

4.2.5.1. Preparation of crude extract

The entire semi solid agar containing bacterial culture (300 ml) was crushed and homogenized with 150 ml of 80% aqueous acetone. The mixture was centrifuged and the supernatant containing the antifungal metabolite was collected and condensed to 2 ml under vacuum in a rotary vacuum

evaporator (Eyela A-1000S, Japan) and used for bioassay (Shanahan et al., 1992).

4.2.5.2. *In vitro* antifungal activity of the crude extract

The crude culture extract was tested *in vitro* for antifungal activity against the two fungal pathogens *Fusarium solani* and *F. equiseti*. Fungal inoculum was prepared in sterile distilled water by gently brushing the surface of 10 day old PDA cultures of the fungus with inoculation needle. The suspension of mycelial fragment and spores were collected from each fungus by filtering aseptically through cheese cloth and mixed (1 ml) with 19 ml molten PDA (45°C) and allowed to solidify. For testing the antifungal activity of the crude extract, two wells were cut at a distance of approximately 3cm from each other in the PDA plates seeded with the fungal pathogen and the crude extract (100 µl) was loaded in one of the wells. The other well was loaded with acetone only which served as the control. The plates were incubated at 30°C for 3 days and observed for zone of clearing around the well.

4.2.6. Scanning Electron Microscopy

Scanning electron microscopy was used as a tool to study the interaction between the antagonistic bacteria and the fungal pathogen. Two most potent antagonistic bacterial isolates, *Pseudomonas putida* strains AS01 and AS04 were selected for the study against the pathogens *Fusarium equiseti* and *F. solani*. A dual culture plate was set up as described earlier (Section 3.2.4.1). A mycelial disc (4 mm diameter) of the fungus was placed centrally in sterile PDA plates while the bacteria were streaked circularly at a distance of 20 mm from the centre. One sterile cover glass was placed carefully between the fungus and bacterial line of inoculation (closer to bacterial inoculation line) for obtaining the bacteria-fungus interaction zone over the cover glass. Another plate inoculated by the fungal pathogen was treated as control. Both the plates were incubated at 28°C until the mycelia in the control plate reached the edge of the petriplate. The cover slip was removed after fungal mycelia grew over the cover glass and finally subjected to series of treatment prior to observation under SEM. The treatments were

done following the process of Samaranayake et al. (2005) and Masaphy et al. (1987). Fungal mycelia were fixed with 2.5% glutaraldehyde solution for 1 hour. Glutaraldehyde was removed by slight decanting. This step was followed by dehydration through an ascending series of ethanol: 50%, 70% and 90% (vol/vol) ethanol for 5 min each; two changes with 90% ethanol for 15 min each and with 100% ethanol for 20 min each. All ethanol dilutions were made with distilled, deionized water. After dehydration, the samples were coated with gold (IB2-ion coater, Japan) and observed under scanning electron microscope [Model: Hitachi S-530 (Japan) 1986].

4.3. RESULTS

4.3.1. Siderophore production

In the quantitative assay of siderophore production, it was found that the isolate *Pseudomonas putida* strain AS04 was the highest producer recording 62.33 % units of siderophore followed by MD01 which produced 54.80% units. The lowest amount of siderophore was produced by CR07 with 8.30% (Table 12). BB05, MB01, MB02, NG05 and AS01 showed moderate siderophore production that ranged from 41.7% to 45.8%. A comparative chart is given in fig. 23b, which shows the amount of siderophore produced by different bacterial isolates.

4.3.2. Antimicrobial Metabolite Production

Antagonistic bacterial isolates were found to be capable of producing several hydrolytic enzymes which are considered to contribute towards their antagonistic activity (Table 13). Clear halo under UV light confirmed chitinase production by only three isolates namely AS04 and MD01 and CB02 strains (Fig. 21a) whereas rest showed negative result. Test for cellulase and pectinase production was found to be negative for all twenty isolates. Protease production or casein hydrolysis test was also performed where clear zone around opaque media confirmed positive result; the cultures showing positive result were BB05, BB07, AS01, AS04, CR07, CR10, CR12, CR14, CB02, MD01, MB01, MB02, NG07 and KT05 (Fig. 21b). The strains tested positive for lipid hydrolysis were BB05, AS01, AS04,

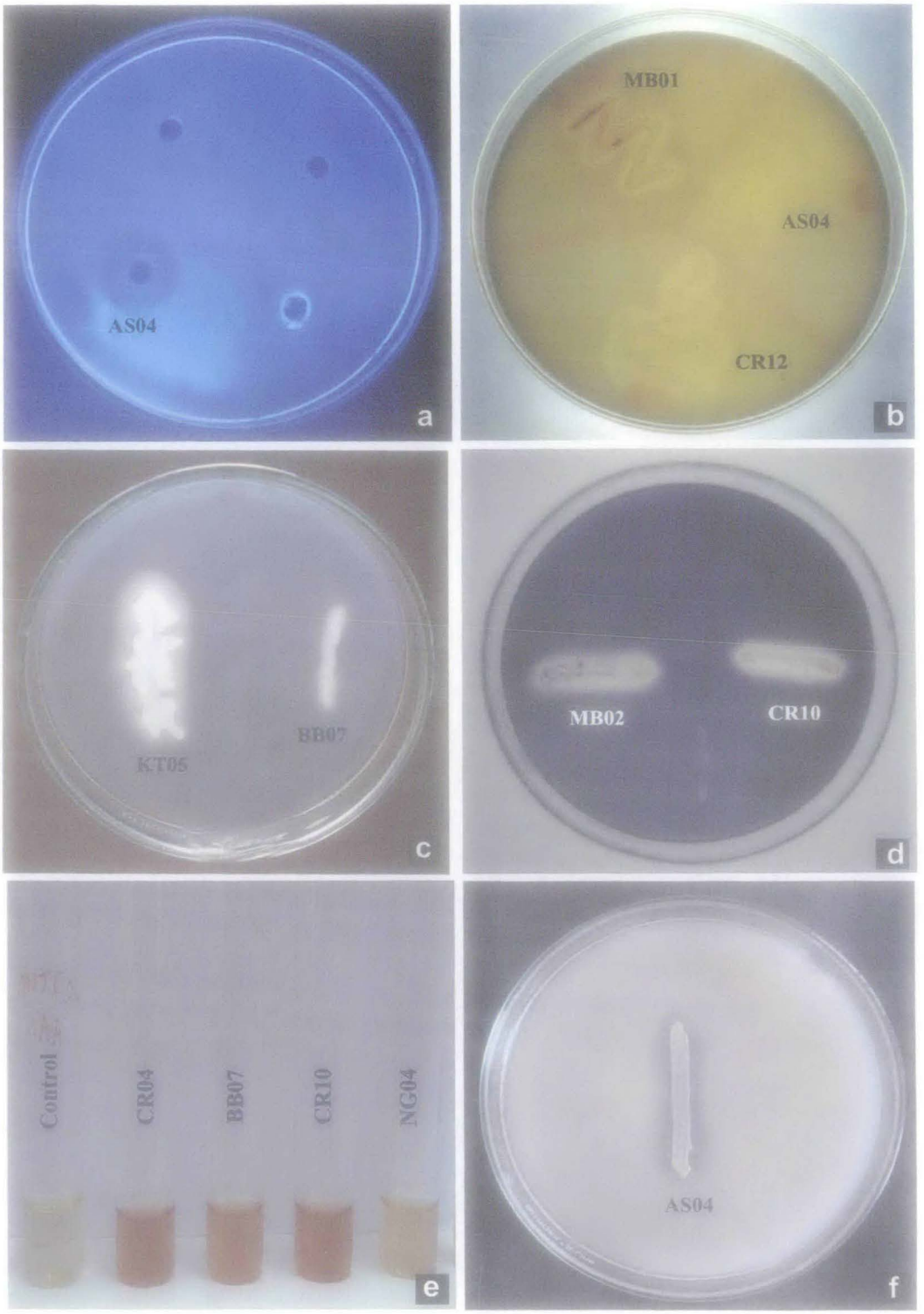


Fig.21: Production of extracellular lytic enzymes by selected antagonistic isolates:

(a) Non-fluorescent zone around the well showing chitinase production by strain AS04; (b) Clearing zone in SMA plate shown by AS04, CR12 and MB01;(c) Turbid zone around bacterial culture showing lipase activity by KT05 and negative result by BB07 showing no turbid zone; (d) Amylase activity evident by clearing zone around purple background by MB02 and CR10; (e) Amount of IAA shown by varying intensity of brown colouration in culture supernatant on addition of Salkowski's reagent by bacterial isolates CR04, BB07, CR10 and NG04. Extreme left tube represents control (f) Phosphatase activity shown by clearing zone in Pikovskaya agar by AS04.

CR07, CR10, CR12, CR13, CR14, CB02, MB02, NG04, NG07 and KT05 (Fig. 21c). The isolates that hydrolyzed starch indicating amylase production were CR04, CR07, CR10, CR14, JL11, MB02, MB05, NG04, NG07 and KT05 (Fig. 21d).

Table12: Amount of siderophore produced by antagonistic bacterial isolates

Bacterial Strains	Absorbance at 630 nm (A_s)	% Siderophore units[a]
BB05	0.14	41.70±0.79
BB07	0.15	37.50±0.29
AS01	0.13	45.80±0.46
AS04	0.06	62.33±0.95
MB01	0.14	41.66±0.34
MB02	0.14	41.66±0.20
MB05	0.17	29.20±0.59
CB02	0.17	29.20±0.49
MD01	0.07	54.80±0.65
CR04	0.16	33.33±0.52
CR07	0.22	08.30±0.40
CR10	0.16	33.33±0.43
CR12	0.20	16.70±0.35
CR13	0.16	33.33±0.46
CR14	0.17	29.20±0.36
JL11	0.19	20.80±0.42
NG04	0.17	29.20±0.53
NG05	0.14	41.70±0.39
NG07	0.16	33.33±0.34
KT05	0.20	16.70±0.35

[a]: % siderophore units = $[(A_r - A_s)/A_r] \times 100$; A_r =0.240 (A_r is the absorbance of uninoculated medium)

Table 13: Production of hydrolytic enzymes related to antagonistic activity by the isolated bacterial strains[a]

Bacterial Isolates	Chitinase	Cellulase	Pectinase	Protease	Lipase	Amylase
BB05	-	-	-	+	+	-
BB07	-	-	-	+	-	-
AS01	-	-	-	+	+	-
AS04	+	-	-	+	+	-
CR04	-	-	-	-	-	+
CR07	-	-	-	+	+	+
CR10	-	-	-	+	+	+
CR12	-	-	-	+	+	-
CR13	-	-	-	-	+	-
CR14	-	-	-	+	+	+
CB02	+	-	-	+	+	-
MD01	+	-	-	+	-	-
JL11	-	-	-	-	-	+
MB01	-	-	-	+	-	-
MB02	-	-	-	+	+	+
MB05	-	-	-	-	-	+
NG04	-	-	-	-	+	-
NG05	-	-	-	-	-	+
NG07	-	-	-	+	+	+
KT05	-	-	-	+	+	+

[a]:'+' tested positive; '-'tested negative.

4.3.3. Detection of HCN Production.

All the twenty anatagonistic isolates showed a negative test for HCN production. This was evident when the colour of the filter paper did not record the expected color shift from yellow to brown.

4.3.4. Antagonism by crude extracellular products

The crude extracellular product of the *Pseudomonas putida* strain AS04 extracted from semi solid culture medium was found to exhibit antifungal activity under *in vitro* condition against *Fusarium solani* (Fig. 22a) and *F. equiseti* (Fig. 22b). This was evident by a clear zone of inhibition around the well containing the culture extracts of the strain AS04. Inhibition zones were not noticeable around the control wells.

4.3.5. Evaluation of PGPR traits

For evaluating PGPR traits, all the antagonistic isolates were tested for IAA production and phosphatase activity. Results showed that all 20 antagonistic isolates produced IAA (Fig. 23a). CR10 produced highest amount of IAA, i.e., 68 µg/ml followed by CR04, BB07 (Table 14). The concentration of IAA was estimated by an IAA standard curve. Streaking of bacterial isolates on the Pikovskaya's agar plate and incubation for 2-5 days at 28°C led to the development of a clear zone indicating positive phosphatase activity. Of the twenty isolates only AS04 produced phosphatase (Fig.17c) on Pikovskaya's agar.

4.3.6. Scanning Electron Microscopy

Scanning electron microscopic studies of the interacting zones of the antagonistic bacteria and plant pathogenic fungi revealed severe mycelial deformities of the pathogens. The results of the study were recorded in photographs (fig. 24a-h). It is clear from the figures that bacterial cells were attached to the hyphal surface of *F. equiseti* (fig 24a). This was accompanied by clearing of the hyphal fluid in some areas (fig 24c). Such events may occur due to secretion of secondary metabolites and diffusible lytic enzymes by this bacterium. Mycelial deformities like hyphal bulging and bursting of the mycelia were also observed in *F. equiseti* in presence of the strain AS04 (Fig. 24b). The strain AS01 also caused degeneration of cell wall, lysis of mycelia and subsequent release of cell contents of *F. solani* (Fig 24e and 24f). Disruption of mycelia of *F. solani* was also observed evidently due to

the action of the strain AS04 (Fig. 24g). The results were compared with respective controls (Fig 24d and 24h).

Table 14: Production of IAA, phosphatase and HCN by antagonistic bacterial isolates[a]

Bacterial strains	Conc. of IAA produced (µg/ml)	Phosphatase production	HCN production
BB05	52.0±0.50	-	-
BB07	60.0±0.87	-	-
JL11	36.0±0.58	-	-
MD01	34.0±0.37	-	-
CB02	47.0±0.34	-	-
AS01	39.0±0.51	-	-
AS04	35.0±0.56	+	-
CR04	60.0±0.68	-	-
CR07	35.0±0.71	-	-
CR10	68.0±0.53	-	-
CR12	26.0±0.73	-	-
CR13	36.0±0.42	-	-
CR14	41.0±0.42	-	-
MB01	36.0±0.55	-	-
MB02	45.0±0.56	-	-
MB05	29.0±0.66	-	-
NG04	19.0±0.68	-	-
NG05	33.0±0.52	-	-
NG07	40.0±0.30	-	-
KT05	55.0±0.68	-	-

[a]:'+' tested positive; '-'tested negative

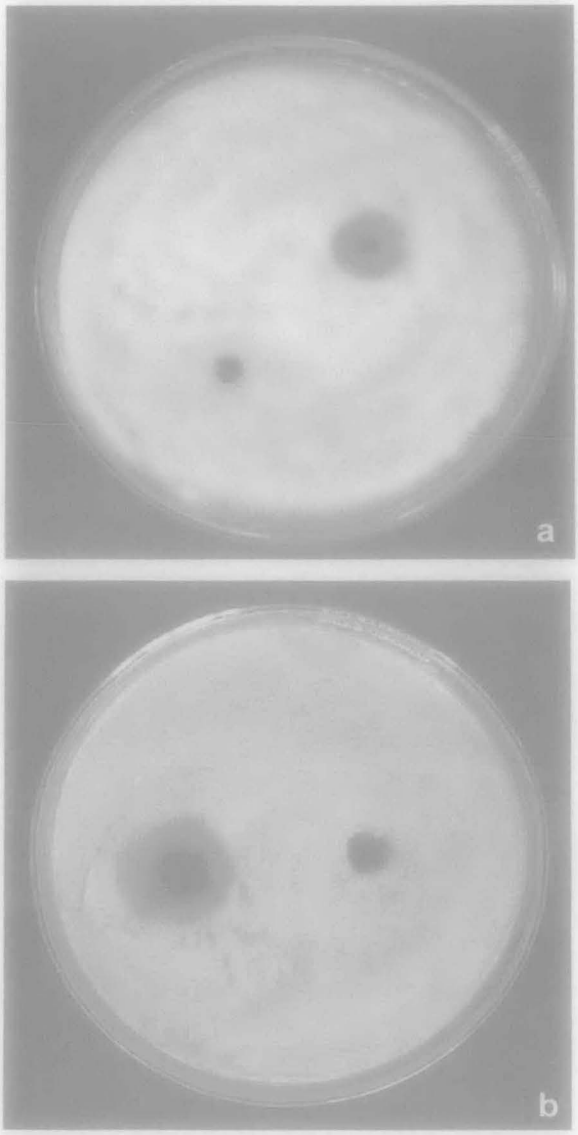
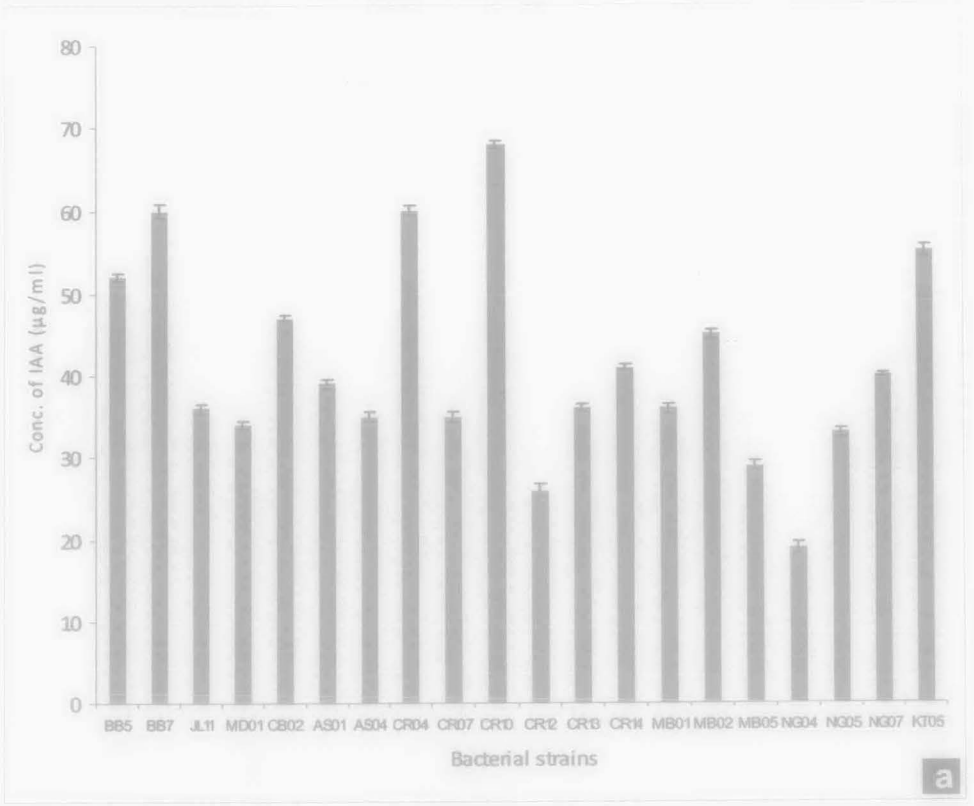


Fig. 22: Inhibition of mycelial growth by crude acetone extract obtained from strain AS04 culture supernatant of; (a) *F. solani* (b) *F. equiseti*



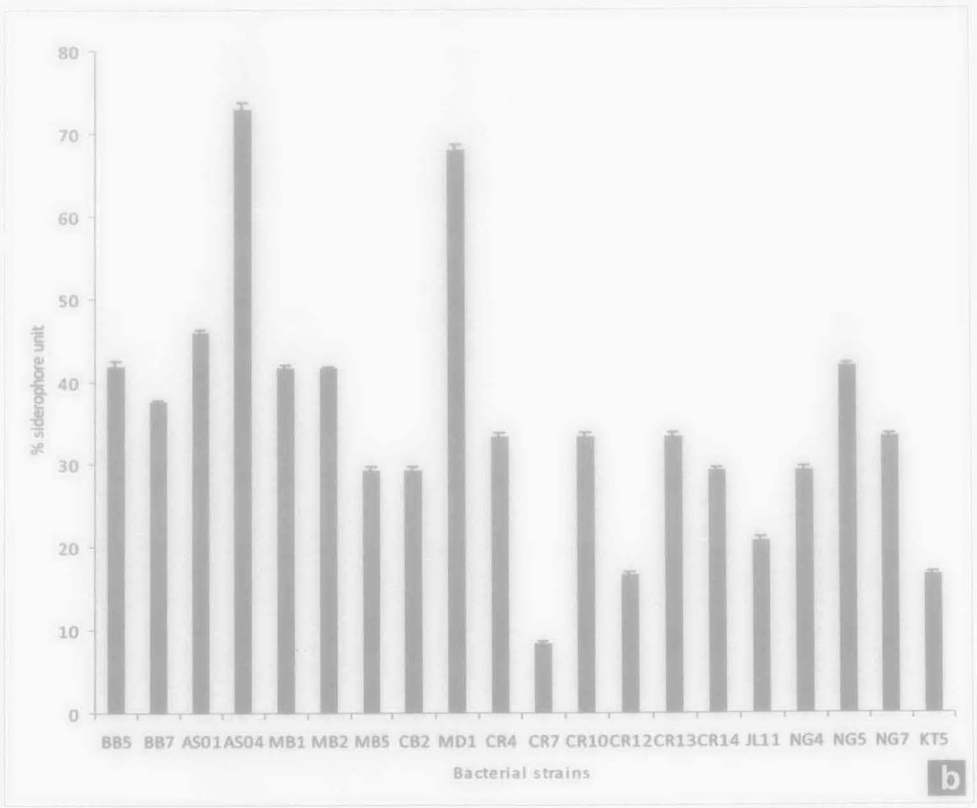


Fig. 23: (a) Amount of IAA produced by the selected antagonistic isolates;

(b)Amount of siderophore produced by selected antagonistic isolates

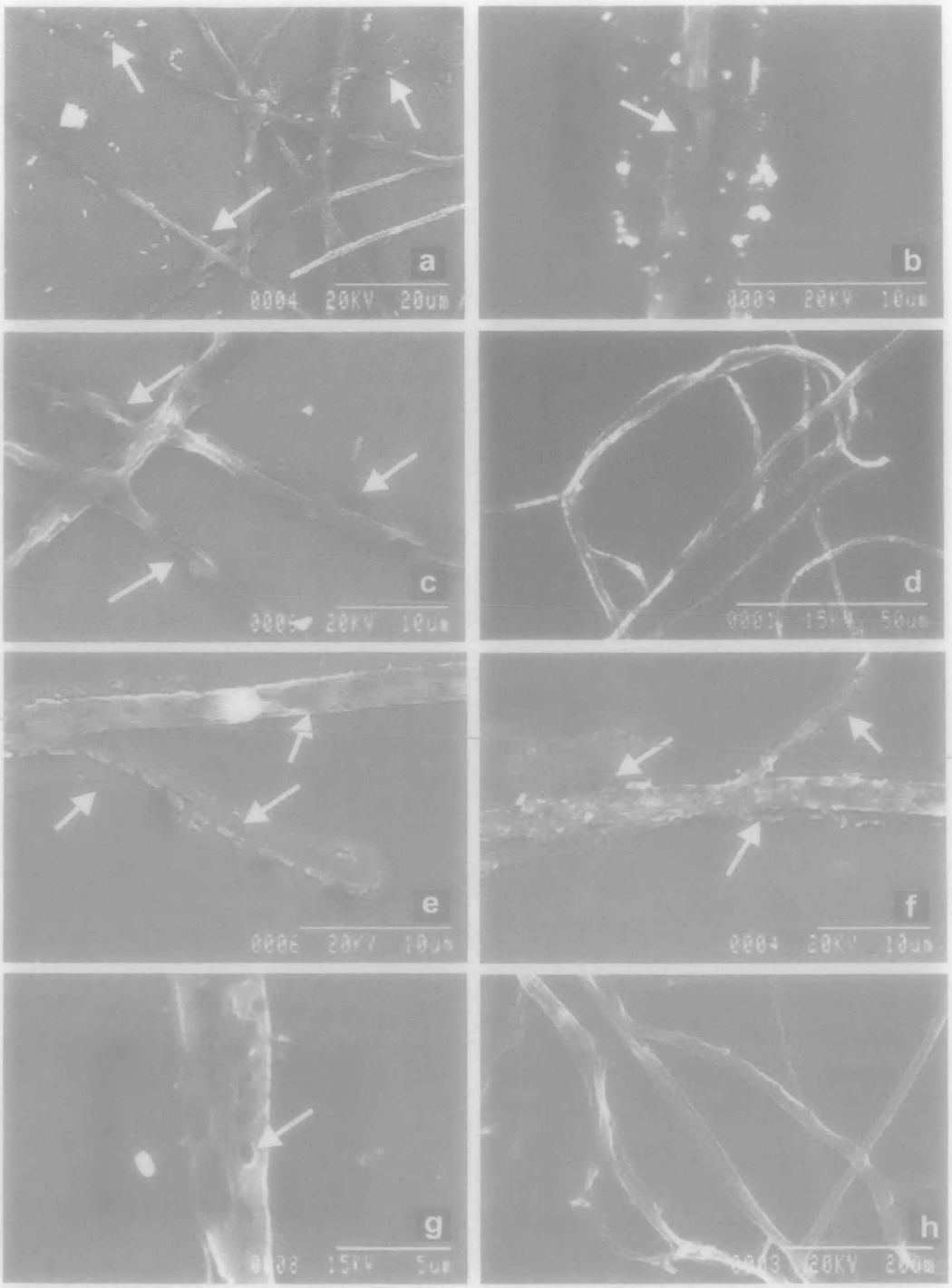


Fig.24: Scanning Electron Microscopic observation of the interaction zone between fungus and antagonistic bacteria grown in PDA plates in dual cultures. The sites of changes are indicated by arrows:

(a) Bacterial cells of *P.putida* strain AS01 attached to the hyphal surface of *F.equiseti* (1500x); (b) Mycelial deformities and lysis of the hyphae of *F.equiseti* by *P.putida* strain AS04 (5000x); (c) Clearing of *F.equiseti* hyphal fluid by *P.putida* strain AS01 (3000x); (d) *F.equiseti* control (1000x); (e) Clearance of mycelia of *F.solani* caused by *P.putida* strain AS01 (4000x);(f)Lysis of mycelia and release of cell contents of *F.solani* by *P.putida* strain AS01 (3000x); (g)Disruption of mycelia of *F.solani* by *P.putida* strain AS04 (6000x); (h) *F. Solani* control (2000x).

4.4. DISCUSSION

Microbes that demonstrate the ability to antagonize plant pathogens are taxonomically diverse. The characteristics that they share that are important determinants of this ability include the aggressive colonization of the plant rhizosphere and the production of antifungal metabolites. Many active biocontrol microbes especially those belonging to the genera *Pseudomonas* and *Bacillus* are known to produce a diverse array of antifungal compounds and in many cases the production of these compounds has been directly correlated with biocontrol activity (Ligon et al., 2000). The biological role of metabolite production by these bacteria appears to be in providing a competitive advantage in the colonization of the rhizosphere, an environment that is rich in plant exuded nutrients. Offensive bacterial colonization by biocontrol strains and defensive retention of rhizosphere niches are enabled by production of bacterial allelochemicals, including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes, and detoxification enzymes (Ligon et al., 2000; Compant et al., 2005).

In the present study, the antagonistic isolate, *Pseudomonas putida* strain AS04 recorded maximum siderophore production among all the twenty selected isolates. Several other strains noticeably, MD01, BB05, MB01 and AS01 also produced high amount of siderophores. All these strains were fluorescent pseudomonads. The group comprising of fluorescent pseudomonads derive their name from the yellow green fluorescent pigment pyoverdin which they produce under iron limiting conditions (O'Sullivan and O'Gara, 1992). Several researchers (Gupta et al., 2002; Omidvari et al., 2010) have assayed the amount of siderophore produced by fluorescent pseudomonads. The CAS shuttle assay used in the present study was also used by Sayyed et al. (2005) for determining the amount of siderophore produced by *P. fluorescens* and *P. putida* strains. The authors observed a high amount (87 and 83% units) of siderophore production by these strains. Siderophores have been demonstrated to play a major role in plant disease suppression by some bacterial biocontrol agents (O'Sullivan and O'Gara,

1992). Although various bacterial siderophores differ in their abilities to sequester iron, in general, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (Loper and Henkels, 1999). It has been reported that apart from pseudomonads, many gram-positive bacteria including *Bacillus subtilis* synthesize siderophores both of hydroxamate and catecholate type (Temirov et al., 2003).

In the present study, the *P. putida* strain AS04 with maximum antifungal activity was found to exhibit chitinase activity on chitin supplemented plates. Besides, two other strains *Serratia* sp. CB02 and *P. fluorescens* MD01 also produced chitinases. Extracellular lipase activity was evident in most of the tested strains. Extracellular protease can contribute to the ability of bacteria to suppress fungal diseases (Ahmadzadeh et al., 2006) and here, 14 of the 20 displayed proteolytic activity in SMA medium. The extracellular product extracted from the spent culture medium during the present study was found to inhibit the growth of *F. equiseti* and *F. solani* in PDA. Several biocontrol PGPR strains have been found to produce enzymes including chitinase, protease and lipase that can lyse fungal cells (Chet and Inbar, 1994). For example, the role of chitinase produced by antagonistic microorganisms like *Pseudomonas*, *Enterobacter*, *Bacillus*, *Serratia* and *Trichoderma* in inhibition of phytopathogens has been demonstrated by several workers (Nielson et al., 1998; Manjula and Podile, 2005; Jaiganesh et al., 2007). Chitin, an unbranched homopolymer of 1,4-β-linked *N*-acetyl-d-glucosamine, is a major cell-wall component of most phytopathogenic fungi which does not occur in plants or other microbes. Although the physiological function of microbial chitinases has yet to be clarified, there is strong correlative evidence that they are proteins with antifungal activity (Schickler and Chet, 1997). Chitinases, along with proteases and 1,3-β-glucanases, degrade fungal cell walls, inhibit fungal growth at the hyphal tips and have been shown to associate with hyphal walls *in planta* (Schickler and Chet, 1997). Lim et al. (1991) isolated a strain of *Pseudomonas stutzeri* that produced extracellular chitinase and laminarinase, and found that these enzymes could digest and lyse *Fusarium solani* mycelia thereby preventing the fungus from causing crop loss due to

root rot. Similarly, Fridlender et al. (1993) were able to reduce the incidence of plant disease caused by the phytopathogenic fungi *Rhizoctonia solani, Sclerotium rolfsii* and *Pythium ultimum* by using a glucanase-producing strain of *Pseudomonas cepacia* which was able to damage fungal mycelia. Nielsen et al. (1998) reported that in the sugar beet rhizosphere fluorescent pseudomonads inhibit plant pathogenic fungi *Rhizoctonia solani* by production of cell wall-degrading endochitinase. Chernin et al. (1995) observed that three different strains of the biocontrol bacteria *Enterobacter agglomerans* that are antagonistic to fungal pathogens including *Rhizoctonia solani,* possess a complex of four separate enzymes that is responsible for the chitinolytic activity of the bacteria. These bacteria significantly decreased the damage to cotton plants following infection with *Rhizoctonia solani.* Moreover, Tn5 mutants of one of these biocontrol strains that were deficient in chitinase activity were unable to protect the plant against damage caused by the fungal pathogen. Trivedi et al. (2008) observed that the soil bacterium *Pseudomonas corrugata* which showed antagonism against *Alternaria alternata* and *Fusarium oxysporum* recorded lipase and chitinase production in growth medium and they were considered as contributory factors to the antagonistic activity of the strain. Arora et al. (2008) observed that fluorescent *Pseudomonas* isolates PGC1 and PGC2 with antifungal potential against *R. solani* and *P. capsici* produced the enzymes chitinase and beta-1,3-glucanase. Their results indicated the role of chitinase and beta-1,3-glucanase in the inhibition of *R. solani.* Samavat et al. (2011) reported that *Pseudomonas fluorescens* UTPF 68 and UTPF 109 showing antagonism against *R. solani* produced chitinase which was thought to play a role in antagonism. Other authors (Diby et al., 2005; Srividya et al., 2012; Wahyudi et al., 2011; Tabarraei et al., 2011; Viswanathan and Samiyappan, 2001) also reported production of multiple lytic enzymes like protease, lipase and chtinase by *Pseudomonas* strains.

In the present study, all the antagonistic isolates were able to produce the plant growth hormone IAA. Additionally, the most antagonistic *P. putida* strain AS04 was able to produce phosphatase on Pikovskaya's agar plates. Low levels of soluble phosphate can limit the growth of plants. Some plant-

growth promoting bacteria solubilize phosphate from either organic or inorganic bound phosphates, thereby facilitating plant growth (Lugtenberg and Kamilova, 2009). Additionally, it has been documented that IAA production by bacteria associated with plants enhances the development of host plant root system, thereby favouring the growth of crop plants (Patten and Glick, 2002). Plant growth promoting rhizobacteria (PGPR) primarily fluorescent pseudomonads which are aggressive root colonizers and possess ability to solubilise phosphates and produce IAA, play an important role in the biological control of plant diseases caused by soil-borne fungal pathogens (Gupta et al., 2002). Yasmin et al. (2009) isolated 15 rhizobacterial strains from sweet potato rhizoshpere including *Pseudomonas*, *Serratia* and *Klebsiella* which produced phosphatase, IAA and siderophores. These strains were antagonistic against *Rhizoctonia* sp. and *Pythium* sp. Ramyasmruthi et al. (2012) isolated 18 bacterial strains from the rhizophere of brinjal, capsicum and chilli out of which *Pseudomonas fluoresecens* isolate R which controlled *Colletotrichum gloeosporioides* causing anthracnose in chilli was found to produce both phosphatase and IAA. Gupta et al. (2002) isolated 12 fluorescent *Pseudomonas* strains from the rhizosphere which showed a strong antagonistic effect against *Macrophomina phaseolina*, a charcoal rot pathogen of peanut. Of the 12 strains, 11 produced high amount of IAA. Due to its IAA-producing ability, it was found to be effective for promoting the growth of peanut plants. Other microbial by-products like hydrogen cyanide may also contribute to pathogen suppression by blocking the cytochrome oxidase pathway and is toxic to all aerobic microorganisms at picomolar concentrations (Ramette et al., 2003; Kumar et al., 2005; Senthilkumar et al., 2009).

Scanning electron microscopic studies of the interacting zones of the antagonistic bacteria (strains AS01 and AS04) and plant pathogenic fungi (*F. solani* and *F. equiseti*) revealed severe mycelial deformities of the pathogens during the present study. Bacterial cells were found attached to the hyphal surface. Degeneration of cell wall, lysis of mycelia and subsequent release of cell contents was observed. Lim et al. (1991) reported

abnormal hyphal swelling and lysis of hyphae in *F. solani* when co-cultured *in vitro* with the biocontrol strain YPL-1 of *Pseudomonas stutzeri*. Severe mycelial deformations of *Curvularia lunata* caused by *Bacillus* sp. strain BC121 was observed by Basha and Ulaganathan (2002). Senthilkumar et al. (2007) have also reported several structural deformities like hyphal lysis and bulging of the mycelium of *Rhizoctonia bataticola* caused by *Paenibacillus* sp. HKA-15. Arora et al. (2008) observed lysis, distortion, swelling in hyphae of the pathogen *P. capsici* taken from the inhibition zone in microscopic studies on the antagonism of the chitinase producing fluorescent *Pseudomonas* isolates PGC1 and PGC2.

The present siderophore producing antagonistic isolates were found to produce a number of lytic enzymes including chitinase, protease and lipase. Cellulase and pectinase activity was not observed in any of our isolates which can be regarded as a desirable trait because the production of cellulase and pectinase is considered an undesirable characteristic of plant beneficial bacteria (Cattelan et al., 1999). Lack of hydrogen cyanide production is another beneficial trait present in our isolates for HCN is considered to inhibit plant growth and yield due to the interference with cytochrome oxidation (Bakker and Schippers, 1987). The other experimental evidences such as the inhibitory activity of the extracellular crude products as well as the cell wall degradations observed through electron microscope indicate that exoenzymes produced by the present isolated strains can play a crucial role in limiting the growth of the phytopathogens.