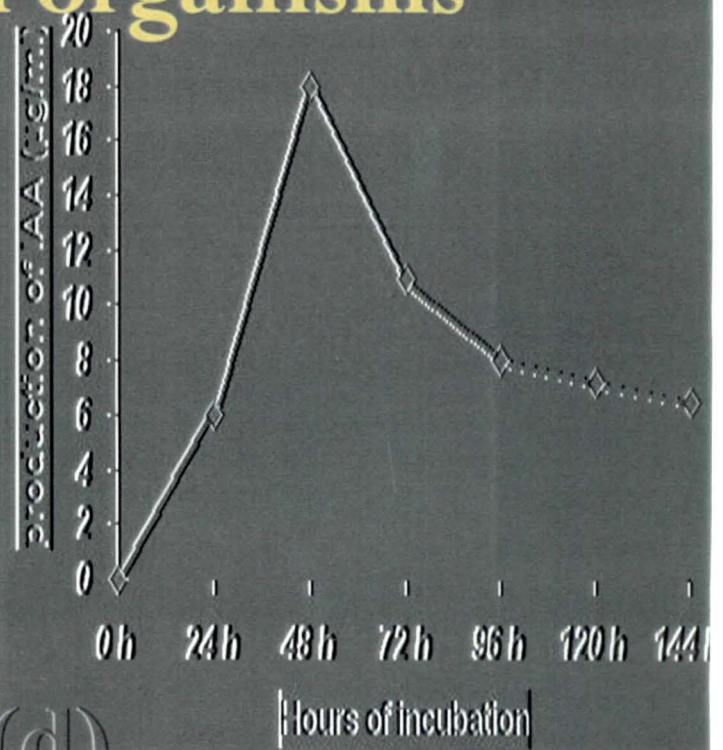
CHAPTER IV

Isolation, characterization and screening of potential antagonists against causal organisms



4.1 Introduction

One of the most interesting aspects of the science of biological control is the study of the mechanisms employed by biocontrol agents to affect disease control (Howell, 2003). The mechanisms of biological control of plant pathogens by antagonistic bacteria and fungi have been a subject of many studies in the past two decades. Most of these studies dealt with antagonists controlling soil borne and to a lesser extent, foliar pathogens. Mechanisms of biological control included antibiosis, parasitism, induced resistance and competition for space and limited resources (Janisiewicz *et al., 2000*). Knowledge of mechanisms involved in biocontrol is important for estimating and predicting its reliability and selection of better strains. Besides other criteria, the choice of an antagonist with its characteristic mechanisms depends on the stage of the life cycle of the pathogen the antagonist is aimed at. Allowable interaction times and niche characteristics determine the suitability of certain modes-of-action during different developmental stages of the pathogen.

4.2. Plant Disease Suppression Mechanisms

4.2.1. Siderophore mediated suppression

The availability of iron for microbial assimilation in environments such as the rhizosphere is extremely limiting. Because almost all living organisms require iron for growth, survival in a heterogenous environment such as the rhizosphere depends largely on the ability to scavenge sufficient iron from a limiting pool. To date, the only known exceptions to this rule are certain lactobacilli, which are devoid of heme proteins and hence have no iron requirement (Archibald, 1983.). The unavailability of iron for growth is surprising, since it is the fourth most abundant element in the Earth's crust (Crichton and Charioteaux-Wauters, 1987). However, it is largely insoluble and thus is unavailable for direct microbial assimilation. In highly oxidized and aerated soils, the predominant form of iron is the ferric form (Matzanke, 1987), which is soluble in water (pH 7.4) at about 10^{-18}M (Nailands *et al.*, 1987). This is too low to support the growth of microorganisms, which

generally need concentrations approaching 10^{-6}M for normal growth. Consequently, to survive in such environments, organisms secrete iron-binding ligands (siderophores), which can bind the ferric iron and make it available to the host microorganism. Most known siderophores can be grouped into hydroxamate and phenolate/catecholate type structures and have different affinities for ferric iron. Soil pseudomonads generally produce fluorescent, yellow-green, water soluble siderophores with both a hydroxamate and phenolate group; these siderophores have been classified as either pyoverdins or pseudobactins. Fluorescent siderophores, which have a very high affinity for ferric iron, are secreted during growth under low-iron conditions. The resulting ferric-siderophore complex is unavailable to other organisms, but the producing strain can utilize this complex via a very specific receptor in its outer cell membrane (Buyer and Leong, 1986). In this way, fluorescent *Pseudomonas* strains may restrict the growth of deleterious bacteria and fungi at the plant root; this has been reviewed by Loper and Buyer in 1991. These iron starvation conditions may also prevent the germination of fungal spores. A direct correlation has been observed *in vitro* between siderophore synthesis in fluorescent pseudomonads and their capacity to inhibit germination of chlamydospores of *Fusarium oxysporum* (Elad and Baker, 1985a; Elad and Baker, 1985b; Sneh *et al.*, 1984). This efficient iron uptake mechanism may also be a significant contributing factor to the ability of these strains to aggressively colonize plant roots, thus aiding the physical displacement of deleterious organisms.

4.2.2. Antibiotic mediated suppression

Biocontrol is often attributed to antibiosis. In many biocontrol systems that have been studied, one or more antibiotics have been shown to play a role in disease suppression. The diversity in the type of antibiotics produced by different strains is only now being fully realized. Compounds such as

phenazines (Thomashow and Weller, 1988.), pyoluteorin (Howell and Stipanovic, 1980), pyrrolnitrin (Howell and Stipanovic, 1979), tropolone (Lindberg, 1981), pyocyanin (Dahiya *et al.*, 1988), and 2,4-diacetylphloroglucinol (DAPG) (Keel *et al.*, 1990) have been isolated from soil fluorescent pseudomonads. The first antibiotics clearly implicated in biocontrol by fluorescent pseudomonads were the phenazine derivatives that contribute to disease suppression by *Pseudomonas fluorescens* strain 2-79 and *P. aureofaciens* strain 30-84, which control take-all of wheat (Weller and Cook, 1983; Brisbane and Rovira, 1988). Genetic analyses have been particularly informative in determining the role of antibiotics in biocontrol, in part because mutants can be screened easily *in vitro* for changes in antibiotic accumulation, providing the means to conduct thorough genetic analyses. Genetic evidence indicated that DAPG could protect sugar beet roots from infection by *Pythium* fungi, since a Tn5-induced DAPG mutant had lost this ability. The same antibiotic compound produced by another fluorescent pseudomonad was found by Keel et al. (1990) to suppress black rot in tobacco and take-all of wheat.

Hydrogen cyanide (HCN) is a broad-spectrum antimicrobial compound involved in biological control of root diseases by many plant-associated fluorescent pseudomonads. Direct inhibition of the fungi by HCN is thought to be the main mechanism of action (Blumer and Haas, 2000), in which case, the effect of the bacterium would be comparable to the HCN-mediated plant defense mechanism (Luckner, 1990). HCN inhibits the terminal cytochrome-c oxidase in the respiratory chain (Knowles, 1976) and binds to metalloenzymes (Blumer and Haas, 2000). However, apart from its role in plant protection, a deleterious effect of microbial HCN on several plants has also been reported (Bakker and Schippers, 1987; Alstrom and Burns, 1989), and indeed, HCN production appears to represent a virulence factor in *P. aeruginosa* (Gallagher and Manoil, 2001). Deleterious HCN-producing strains have even been studied for biological control of weeds (Kremer and Souissi, 2001).

4.2.3. Parasitism

If fungal cells are lysed and cell walls are degraded then it is generally assumed that cell wall-degrading enzymes produced by the bacteria are responsible, even though antibiotics may be produced at the same time. Considerable effort has gone into identifying cell wall-degrading enzymes produced by biocontrol strains of bacteria even though relatively little direct evidence for their presence and activity in the rhizosphere has been obtained. For example protease and chitinase activity has also been reported among fluorescent pseudomonads (Nielsen *et al.*, 1998; Nielsen, *et al.*, 2002).

4.2.4. Colonization

It seems logical that a biocontrol agent should grow and persist, or "colonize," the surface of the plant it protects, and colonization is widely believed to be essential for biocontrol (Weller, 1983; de Weger *et al.*, 1987; Parke, 1991). However, colonization, or even the initial population size of the biocontrol agent, has been shown to be significantly correlated with disease suppression in only a few instances (Paulitz and Baker, 1987; Parke, 1990; Bull *et al.*, 1991). Suppression of take-all of wheat is correlated with colonization of roots by *P. fluorescens* strain 2-79 (Bull *et al.*, 1991). However, even in interactions that require colonization for disease suppression, the biocontrol agent may not be required at high population density.

4.2.5. Induced Resistance

Some biocontrol agents induce a sustained change in the plant, increasing its tolerance to infection by a pathogen, a phenomenon known as induced resistance. In some cases, it is clear that induced resistance by biocontrol agents involves the same suite of genes and gene products involved in the well documented plant response known as systemic acquired resistance (SAR), but this is not always the case. SAR is typically a response to a localized infection or an attenuated pathogen, which is manifested in subsequent resistance to a broad range of other pathogens (Ross, 1961; Uknes *et al.*, 1992; Ryals *et al.*, 1996) in

this issue. It has been shown that the biocontrol agent *P. fluorescens* strain CHAO (Maurhofer *et al.*, 1994) induces SAR-associated proteins, confers systemic resistance to a viral pathogen, and induces accumulation of salicylic acid, which plays a role in signal transduction in SAR (Gaffney *et al.*, 1993; Ryals *et al.*, 1996). Mutants of CHAO that do not produce the siderophore pyoverdin do not induce SAR, suggesting a novel role for bacterial metabolites in disease suppression (Maurhofer *et al.*, 1994). Another fluorescent pseudomonad, *P. putida*, induces expression of the gene encoding PRIa, which is associated with the classical SAR response (Zdor and Anderson, 1992). Other strains of *P. fluorescens* do not induce expression of the gene products associated with the classic SAR response but appear instead of inducing a functionally analogous response (Hoffland *et al.*, 1995).

This chapter will focus on the mechanism of action of fluorescent *Pseudomonas* BRL-1 against the pathogens responsible for *Cymbidium* rot.

4.3. Materials and Methods

4.3.1. Microscopic study

To study the morphological changes of the mycelium of *Fusarium oxysporum* and *Mucor hiemalis* f. *hiemalis* a small amount of fungal culture from the interacting zone of the dual plate culture as well as a drop of fungal culture from the dual liquid culture (discussed in the preceding chapter) were taken in a clean glass slide and stained with lactophenol and cotton blue and observed under Leica DMLS Research microscope. Mycelial morphology was compared with those of the control sets.

4.3.2. Effect of non-volatile substances

The production of non-volatile compounds by fluorescent *Pseudomonas* BRL-1 was tested by an 'agar layer technique' (Dennis and Webster, 1971a). A single sterile cellophane paper was placed aseptically on the solidified Peptone Glucose Agar (PGA) plates. At the centre of the plate 0.5 ml of fluorescent *Pseudomonas* BRL-1 was inoculated at a concentration of 10^7 cfu /ml and these plates were incubated at 30^0C for 48 hours. Then cellophane paper along with the grown bacterial isolate was removed carefully. An agar block (5 mm diameter) of four days old culture of *F. oxysporum* and *M. hiemalis* f. *hiemalis* were placed separately on the centre of these plate whereas point inoculation at the center of the plate was done for *E. carotovora* on nutrient agar (NA) at an concentration of 10^6 cfu /ml. Plates with cellophane paper inoculated with sterile distilled water served as control. Each treatment was replicated thrice. After three days of incubation at 30^0C colony diameter of the test fungi were recorded as well as morphological changes of the bacterial pathogen was documented by comparing with the control sets.

4.3.3. Effect of volatile substance

To investigate the inhibitory effect on fluorescent *Pseudomonas* BRL-1 for the production of volatile compound 'inverted plate technique' was followed (Dennis and Webster, 1971b). An agar block (5 mm diameter) of four days old culture of

F. oxysporum and *M. hiemalis* f. *hiemalis* were placed separately on the centre of the petri plate containing 20 ml of PGA media and for bacterial pathogen NA plates were streaked with 24 hours old culture of *E. carotovora* containing 10^6 cfu /ml. A loopful of 24 hours old fluorescent *Pseudomonas* BRL-1 culture (10^7 cfu /ml) was streaked on petriplate having 20 ml of PGA as well as NA then inverted over the plates inoculated with the fungal pathogens and bacterial pathogens. Two plates were sealed together (mouth to mouth) with parafilm, control plates consist of only fungal and bacterial pathogen inverted over uninoculated PGA and NA plate respectively. These plates were then incubated at 30^0C and three replicates were maintained for each treatment. After 5 days of incubation colony diameter of the test fungal pathogens was measured, as well as morphological changes of the colonies of the bacterial pathogen was compared with the control.

4.3.4. HCN production

Production of hydrogen cyanide was tested qualitatively according the method of Wei et al. (1991). The fluorescent *Pseudomonas* BRL-1 was inoculated in Tryptone Soya Agar (TSA) medium supplemented with amino acid glycine (4.4 gm/lit. of medium). A strip of sterilized filter paper saturated with a solution containing picric acid 0.5% (yellow) and sodium carbonate (2%) was placed in the upper lid of the petridish. The petridishes were then sealed with parafilm and incubated at 30^0C for 4 days. A change of colour of the filter paper strip from yellow to light brown, brown or reddish brown was recorded.

4.3.5. IAA production

To test the production of IAA, active culture of fluorescent *Pseudomonas* BRL-1 was inoculated in 10 ml of minimal salt media (5X: $(NH_4)_2SO_4$ –5 gm; KH_2PO_4-22.5 gm; K_2HPO_4-52.5 gm; Na-citrate-2.5 gm; distilled water-1 lit.) supplemented with 100 µg/ml of tryptophan, and incubated at 30^0C under shaking for 48 hours. Culture broth was centrifuged at 7500 rpm for 10 min. To 1 ml of culture supernatant, 2 ml of Salkovsky reagent was added and contents

incubated at 30°C for 25 min. Absorption was read at 530 nm and levels quantified from standard curve of IAA (Gaur *et al.*, 2004).

4.3.6. Chitinase production

To test the chitinolytic property of fluorescent *Pseudomonas* BRL-1 it was inoculated on Luria Bertani (LB) medium (Gunasekaran, 1995) supplemented with 0.5% colloidal chitin as principal source of carbon. Plates were incubated at 30°C for three days. Formation of a clear halo region around the colonies indicates chitinase activity of the strain (Basha and Ulagnathan, 2002).

4.3.7. Production of hydrolytic enzyme

Production of hydrolytic enzyme was qualitatively assayed in 5X minimal media containing gelatin (0.4%), starch (1%), pectin (1%), Carboxy methyl cellulose (1%), for protease, amylase, pectinase, cellulase respectively. Plates were incubated for 72 hours at 30°C and formation of clear zone around bacterial colony was read as positive. (Gaur *et al.*, 2004).

4.3.8. Siderophore production

Chrome Azurol S (CAS) agar medium was prepared as described by Schwyn and Neilands (1987) to detect the siderophore production. CAS agar (blue agar) was inoculated with 24 hours old fluorescent *Pseudomonas* BRL-1 at the center of the plate and kept for incubation at 30°C for 72 hours. The change of the blue colour of the medium to orange or presence of yellow to light orange halo surrounding the bacterial colony indicates the production of siderophore.

Chemical assay was performed to detect the nature of siderophore-

Tetrazolium test (Snow, 1954) was performed to detect the hydroxamate nature. Triphenyltrtrazolium chloride (about 1mg) was dissolved in a drop of culture supernatant of fluorescent *Pseudomonas* BRL-1, on a tile and a drop of 2 N of sodium hydroxide (NaOH) added. Immediate development of deep red colour was taken as a positive reaction.

Arnow's test (Arnow, 1937) was performed to detect catecholate siderophore. The cell free filtrate of fluorescent *Pseudomonas* BRL-1 was used as a source of

catechol. Catechol (Sigma Co.) was used as standard. Culture filtrate and standard catechol each in a volume of 1 ml were taken separately in two test tubes graduated at 5 ml. To each test tube was added 1 ml of 0.5 N hydrochloric acid, 1 ml of nitrate molybdate reagent (10 gm of sodium nitrate and 10 gm of sodium molybdate in 100 ml of distilled water), 1 ml of 1 N NaOH and enough distilled water to make the volume of 5 ml. Development of yellow colour of solution following the addition of nitrate molybdate reagent and its subsequent intensification of deep orange colour upon addition to NaOH indicated the presence of catechol.

4.4. Results and Discussion

Microscopic observation of the mycelium from the interacting zone showed hyphal shriveling, mycelial deformities, swelling, fragmentation, short branching and granulation of cytoplasm ultimately resulting into lysis (Figure 4.1). Morphological abnormalities in hyphae of fungal pathogen, was clearly observed under microscope. Such abnormalities occurred due to secondary metabolites and diffusible lytic substances produced by the antagonist. Further study was made to screen for the production of different secondary metabolites including siderophore, hydrogencyanide, non-volatile and volatile compound, hydrolytic enzymes, IAA investigated for their effect on fungal as well as bacterial pathogens (Table 4.1).

Table 4.1: Showing different secondary metabolites production and enzymatic activity of fluorescent Pseudomonas BRL-1

Different Metabolites	Rate of Production
Siderophore production	+++
Antibiotic Substances	-
HCN production	-
Volatile substances	-
Chitinase activity	+
Protease activity	++
IAA production	+++

'+++' Stronger production; '++' Moderate production; '+' Low production; '-' No production.

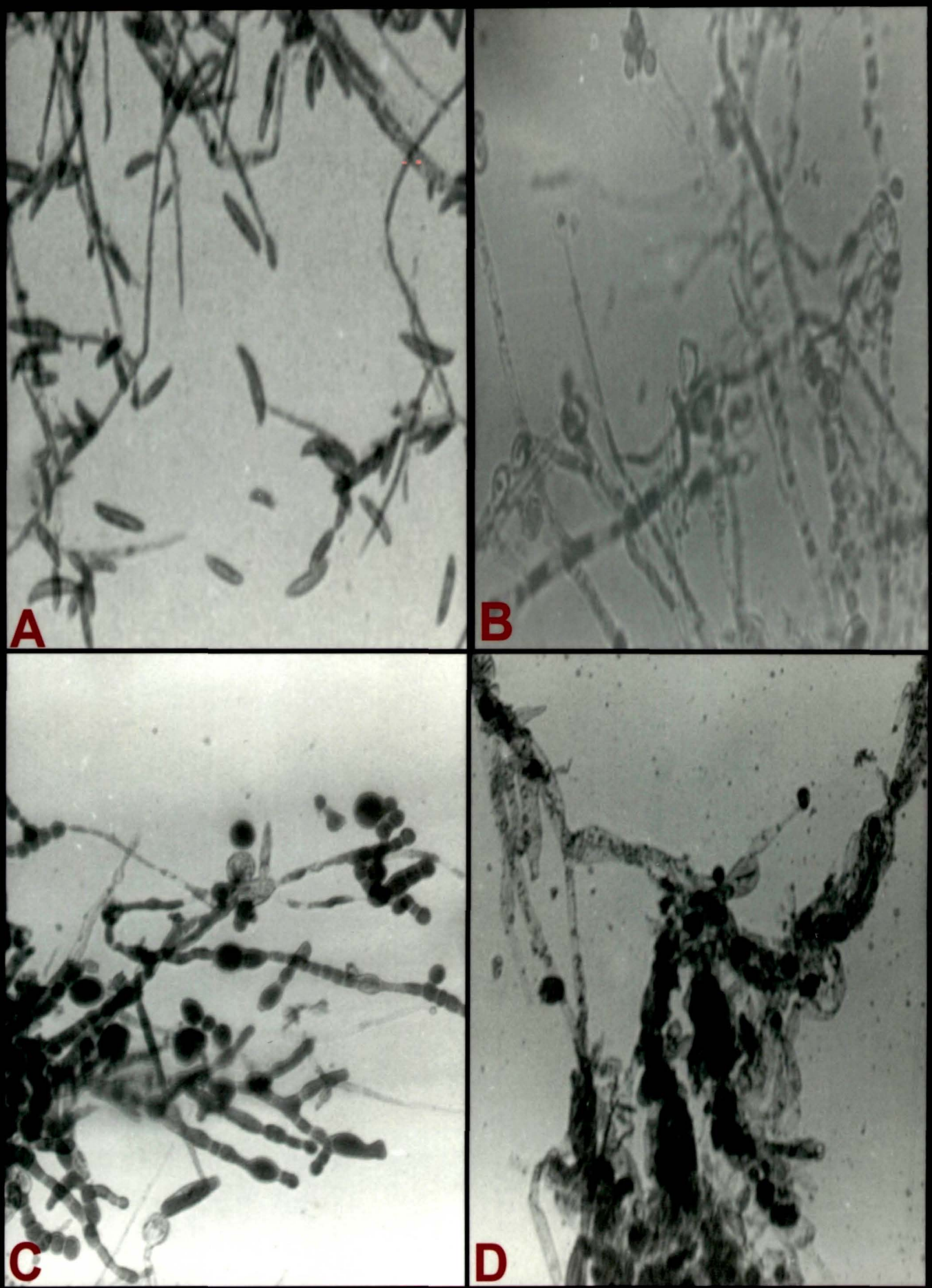


Figure 4.1. Microscopic observations of mycelium inhibited by fluorescent Pseudomonas BRL-1 strain: (A) Mycelium of F. oxysporum grown in PGA media (Control); (B) Present in the inhibition zone, when grown with fluorescent Pseudomonas BRL-1 on PGA; (C) Mycelium of M. hiemalis f. hiemalis grown on PGA media (Control); and (D) Present in the inhibition zone when grown with fluorescent Pseudomonas BRL-1 on PGA.

The effect of non-volatile and volatile substances production by antagonist fluorescent *Pseudomonas* BRL-1 was tested against *Fusarium oxysporum, Mucor hiemalis* f. *hiemalis* and *Erwinia carotovora* as described in materials and methods. Even after 96 hours of incubation there were no significant reductions in colony diameter of the fungal pathogens as well as no morphological changes of the colony of *Erwinia carotovora* was observed.

HCN production by fluorescent *Pseudomonas* BRL-1 was tested and found that the there was no change in colour of the picric acid saturated filter paper strip and results was recorded as negative. In fact it was reported that production of HCN is deleterious to plant (Bakker and Schippers, 1987; Alstrom and Burns, 1989).

In general, Indole acetic acid is universally accepted as a plant growth promoter. Other than that, it has been reported by Chirst and Mosinger (1989) that this phytohormone could also induce resistance through PR protein production. Again in the year 2004, Sharaf and Farrag reported that IAA reduce spore germination, mycelial dry weight and protein content of the pathogenic fungi and thus prevent significantly any chance for disease induction by soil pathogens. The present study complements their findings by demonstrating significant production of IAA by fluorescent *Pseudomonas* BRL-1 after 48 hours of incubation (Figure 4.2A).

Lorito et al. (1994) and Dunne et al. (1997) reported that the exposure of selected phytopathogenic fungi to lytic enzymes such as chitinase, protease could result in the degradation of the structural matrix of the fungal cell wall. Fluorescent *Pseudomonas* BRL-1 has been found to produce chitinase and protease, when they were grown in chitin and gelatin media respectively, as a sole carbon source (Figure 4.2B and 4.2C). As the fungal cell wall contains chitin, as the major component and chitinase are well known to lyse the cell walls of both live and dead fungi (Ueno *et al.*, 1990), the reduction in dry weight of the fungal pathogen may be due to the proteolytic and chitinolytic activity of fluorescent *Pseudomonas* BRL-1.

CAS agar (blue agar) plate assay indicate siderophore production by fluorescent *Pseudomonas* BRL-1. The strain forms colonies with an orange halo. This colour change is based on the principle that the blue colour of the CAS medium is due to the Fe-dye

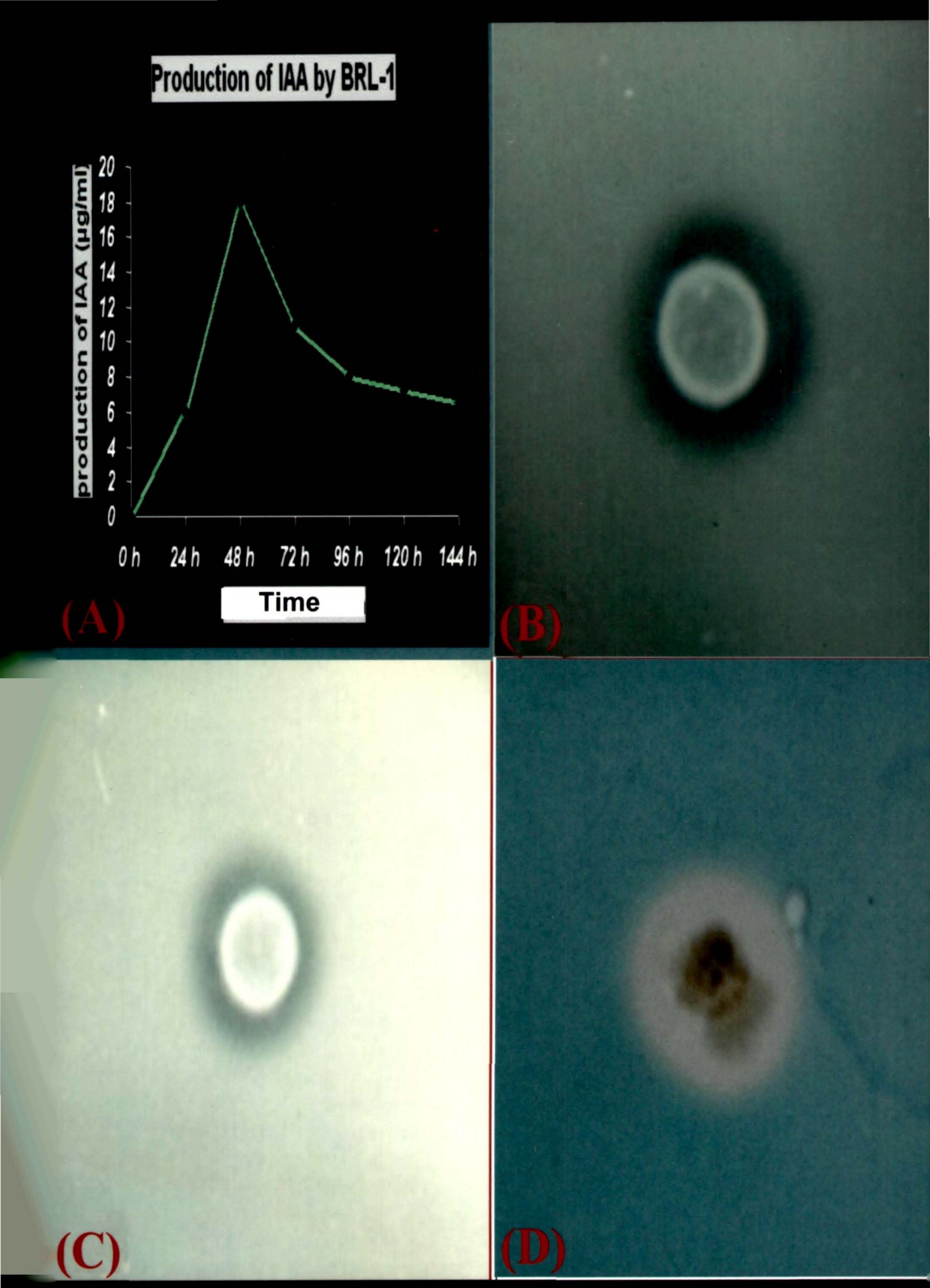


Figure 4.2: (A) Production of IAA by the isolate on minimal media supplemented with tryptophan; (B) Zone around the colony indicate chitinolytic property of the strain in LB media; (C) Proteolytic activity of the strain in gelatin agar media; and (D) Orange halo indicates the production of siderophore in CAS agar plate

complex and when siderophore is produced by the fluorescent *Pseudomonas* BRL-1 the iron is released from the Fe-dye complex resulting in the change in colour to orange which indicate the siderophore production. The isolate produced hydroxamate siderophore as evidenced by positive tetrazolium test and negative in case of Arnow test (Figure 4.2D).

On the basis of these studies it was well understood that the fluorescent *Pseudomonas* BRL-1 isolate was showing significant antagonistic property through combined and/ or individual effect of siderophore, production of proteolytic enzyme, IAA and chitinolytic activity. Considerable attention has been paid to plant growth promoting rhizobacteria (PGPR), as the best alternative to chemicals to facilitate eco-friendly biological control of soil and seed borne pathogen (Remeshkumar *et al.*, 2002). These observations might help in developing this PGPR (fluorescent *Pseudomonas* BRL-1) as a potential rhizospheric biocontrol agent against *Cymbidium* rot pathogens.

The next chapter deals with (a) development of a cost effective media for large-scale biomass production of the potential antagonistic fluorescent *Pseudomonas* BRL-1, (b) powder formulation of fluorescent *Pseudomonas* BRL-1 and lastly (c) field evaluation of the powdered formulation of the antagonist to check its efficacy.

4.5. Reference

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