# Role of Siderophore of *Pseudomonas Fluorescens* in Biocontrol of Plant Pathogenic Fungi *Rhizoctonia Solani*

BY

Jannet Vennila J.

A THESIS SUBMITTED TO THE AVINASHILINGAM INSTITUTE FOR HOME SCIENCE AND HIGHER EDUCATION FOR WOMEN (DEEMED UNIVERSITY) COIMBATORE-641 043, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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# I INTRODUCTION

The control of pathogens is very essential in realising the full potential of any crop plant. The pathogens generally reduce the crop yield by destroying the plant's growth by utilising the plant's nutrients, minerals etc, and causing disease to the plant. Especially seedling disease complex of cotton, caused by pathogens like **Rhizoctonia solani, Macrophomia phaseolina** and **Pythium** is a major problem around the world. (Verma 1986; Singh and Verma, 1988).

Many of these pathogens can live in soil for long periods, particularly in presence of host debris and accordingly difficult to control by chemical means or resistancen breeding. Moreover chemical control of pathogens has many disadvantageous like toxicity, resistance development, cost effect and so on. Hence now biological control using antagonistic bacteria have come into practice which is more preferable than chemical control in the sense, more active, no toxic effect and no resistance development in the plants.

In the recent years, there has been more success in biological control of soil -borne diseases with the use of antagonist fluorescent Pseudomonads through seed bacterization (Weller and Cook, 1983; Colyer and Mount, 1984).

The fluorescent Pseudomonads antagonize pathogens by producing one or more metabolites that include, antibiotics (Fravel, 1988), siderophores (Leong 1986) and cyanide (Voisard **et al.,** 1989). Some of the fluorescent Pseudomonad strains also behave as plant growth promoting Rhizobacteria (Suslow and Schroth, 1982 b).

Siderophores are produced by many bacteria. They are low molecular weight ferric iron transport agents. The function of siderophores is to supply iron to the bacterial cell. Siderophores selectively complex with iron with very high affinity and make it unavailable to other micro organisms including pathogens.

**Pseudomonas fluorescens** produce siderophore which control many diseases caused by **Rhizoctonia solani.** The siderophores of Pseudomonas efficiently sequester ferric iron in plant root zone, making it unavailable to other rhizoplane micro organisms including plant pathogen **R. solani.** Hence the populations of the **R. solani** is reduced and the roots appear healthier (Vidhyasekaran, 1993).

The present study carried out on the "Role of siderophore of **Pseudomonas fluorescens** in biocontrol of **Rhizoctonia solani**" has following objectives.

1. To extract the siderophore from **P. fluorescens** cells which has been taken from cotton grown fields.

2. To find the fungistatic activity of the purified siderophore against **R. solani.**

3. To assess the effect of **P. fluorescens** on **R. solani** growth.

4. To identify the type of siderophore in **P. fluorescens** by thin layer chromatography.

5. To quantify the siderophore present in **P. fluorescens** isolates.

# Review of Literature

## II REVIEW OF LITERATURE

The review of literature pertaining to the study " Role of siderophore of **Pseudomonas fluorescens** in biocontrol of plant pathogenic fungi **Rhizoctonia solani**," are discussed under the following headings.

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## 2.1. RHIZOCIONIA SOLANI

**Rhizoctonia solani** is a species complex of wide host range. Adams (1990) found that **R.solani** is the asexual form of fungal species, **R.solani** is cosmopolitan in soils, but the sexual form is rarely seen except on stems of **Solanum tuberosum.**

### 2.1.1. OCCURENCE

It is a destructive plant pathogen with an almost unlimited host range. The fungus inhabits soils and causes damping off seedlings according to **Laha et al.** (1992). It causes damping off of seedlings mainly in cotton and cucumber (Cubeta **et al.**, 1991). It causes black scurf in potato (Tanii **et al.**, 1990). It also includes the formation of root, crown and stem rots and occasionally foliar blights (Baker, 1970). **R.solani** is an important symbiont with the saprophyte seedlings stage (Protocorn) of many orchids (Warcup, 1985).

### 2.1.2 PROPERTIES

**Rhizoctonia solani** is both a pathogen and a saprophyte, it aggressively colonizes organic debris and is one of the fastest growing fungi. The cells of the **R.solani** are multinucleate (plurinucleate) and the cytoplasm is interconnected through a septal pore (dolipore) that is characteristic of the Basidiomycotina. No asexual spores are found.

Only sclerotia form as soil - borne propagules. The sclerotia are knots of undifferentiated, intertwined, pigmented monilioid cells. Kloepper (1993) has developed and evaluated two **invivo** assays for possible use as prescreens in selecting plant growth promoting rhizobacteria (PGPR) with biological control effects of pre and post emergence damping off disease on cotton seedlings caused by **R.solani.**

## 2.2. PSEUDOMONAS FLUORESCENS

### 2.2.1. OCCURENCE

**Pseudomonas** species have been successively used in laboratory and green house experiments by Howell and Stipanovic, 1979; Nair and Fahy, (1972); and Kawamoto and Lorbeer, 1976. Leyns **et al.**, (1990) have found that among the several antifungal bacteria isolated from different crops, **Pseudomonas fluorescens** was the common one.

## 2.2.2. ROLE OF P. FLUORESCENS IN RHIZOSPHERE

### 2.2.2.a. GROWTH PROMOTING ACTIVITY

Burr **et al.,** (1978) have reported significant increases of potato yields following treatment of tubers with specific strains of **Pseudomonas fluorescens** and **P.putida.** Kloepper and Schroth, (1978) have reported similar response on radish and Suslow and Suchroth, (1982 a) on sugar beets.Buyer and Leong, (1985) have showed that both plant growth promoting **Pseudomonas B - 10** and its yellow green fluorescent iron transport agent (Siderophore) Pseudobactin enhance potato growth and biologically control certain soil - borne fungal diseases in part by depriving specific root colonizing endemic micro - organisms including phytopathogens of iron(III) thus inhibiting their growth.

The growth inhibition of certain beneficial fluorescent **pseudomonads** is due in part to the inability of susceptable strains to utilise Siderophores from beneficial strains to transport iron (lll).

## 2.2.2. b. BIOCONTROL OF PLANT PATHOGENS

**Pseudomonads** have been shown to suppress various pathogens in soil including **Gaeumannomyces graminis** var **tritici** by Sivasithamparam and Parker, (1978); Weller and Cook, (1983). Weger **et al.**, (1986) have made an approach to understand the molecular basis of the reduction in plant yield depression by root colonizing **Pseudomonas** species. They characterised 30 plant root colonizing **Pseudomonas** species with respect to siderophore production, antagonistic activity, plasmid content and sodium dodecyl sulphate polyacrylamide gel electrophoresis pattterns of their cell envelope proteins. O'Sullivan and O'Gara,(1990), made an effort to study the regulation of ferric iron uptake in fluorescent **Pseudomonads** and cloning of the regulatory gene, Thomashow and Weller, (1992) made a study to find the role of antibiotics and siderophores of **Pseudomonas fluorescens** in biocontrol of "take all" disease of wheat caused by **G. graminis**. A positive relationship between root colonization by **P. fluorescens** Str 2 -79 RN and biological control of "take all" of wheat caused control of scab, black scurf and soft rot of potato caused by **G.graminis** was demonstrated by Bull **et al.**, (1992).Tanii **et al.**,(1990) have suggested the biological control of scab, black scurf and soft rot of potato caused by **R.solani** and **Erwinia** species, using seed tuber bacterization with **Pseudomonas fluorescens**.

The incidence of **P. fluorescens** in rhizosphere of rice and their antagonism towards **Sclerotium oryzae** was reported by Elangovan and Gnanamanickam (1992); Gnanamanickam and Mew, (1990) have showed that these bacteria are particularly useful in relation to the microbial suppression of "take all" fungus . **G.graminis** following wheat monoculture and for the management of rice diseases, **Pseudomonas. fluorescens** Biotype G, now referred to as Biovar G , was the dominant fluorescent group in south Australian soils and wheat rhizospheres.

## 2.3. SIDEROPHORES :

Campell (1989) has defined siderophore as "a chemical produced by an organism which binds cations especially $Fe^{3+}$ and helps to transport it into the organism in iron limited conditions". According to Neilands(1981) "Siderophores are low molecular weight compounds that are produced under iron limiting conditions, chelate the ferric ion with high specific activity, and serve as vehicles for the transport of Fe(iii) into microbial cell.

It was shown by Kim and Misaghi(1992) that siderophore production by fluorescent Pseudomonads is sensitive to changes in atmospheric oxygen and carbondioxide concentrations. Considerable amount of research is being

carried out implicating siderophores produced by PGPR which are partly responsible for enhanced plant growth and biocontrol (Schroth and Hancock 1982;Mew and Rosales 1986). Leong(1986) has discussed the siderophores, their biochemistry, and their role in the biocontrol of plant pathogens. They have also showed that the beneficial strains **P. fluorescens** and **P. putida** enhance plant growth, by effective biocontrol mechanisms against soil borne fungal pathogens through siderophore production.

Neilands (1981) in his "Microbial iron compounds" has discussed various aspects of siderophores like chemical and physical properties, classification, their importance, their distribution in different species of bacteria and so on. Meyer and Abdallah(1978) have shown, that the biosynthesis of a yellow green fluorescent water soluble pigment produced by **P. fluorescens** occured when the bacteria was iron deficient.

The specificity of siderophore receptors and biocontrol by **Pseudomonas** species was analysed by Bakker **et al**(1990). Colin and Maraite(1990) did a special experiment on spectrophotometric characterization **and biological** activity of siderophores of fluorescent Pseudomonads antagonists of phytopathogenic bacteria. The importance of microbial siderophores in plant pathology, as determinants and factors

influencing the iron nutrition to plants, virulence factors or ecological determinants is the subject of many reviews (Leong, 1986; Leong and Expert, 1990; Neilands and Leong, 1986; Schippers **et al.**, 1987).

Waid(1975) produced diverse groups of siderophores by soil microbes in culture media. Azegame **et al** (1988); and Gill and Warren, (1988) have showed that iron regulated antagonism cannot be attributed solely to pyoveridine (siderophore) production, because **Pseudomonas** species are known to produce several antimicrobial activity. Elsherif and Grossmann(1992) developed different methods of application of fluorescent of Pseudomonads to biologically control plant pathogenic fungi.

### 2.3.1 CLASSIFICATION OF SIDEROPHORES

Although siderophores vary greatly in chemical structure, most have either hydroxamate or catechol groups that are involved in iron (iii) chelation. Neilands(1981) has reported that transport of iron into the cell is mediated by specific membrane receptor and transport systems that recognize the iron - siderophore complex.

### 2.3.2. STRUCTURE

The structural diversity among the different siderophores is quiet considerable and depends on the microorganism which produce them as per O'Sullivan and O'Gara, (1992). However a common feature of all siderophors is that they form six coordinate octahedral complexes with ferric iron (Raymond **et al.,** 1984). Siderophores with phenolate Catecholate type structure have higher formation constants with ferric iron, but the stability of these iron complexes is highly pH dependent (Matzanke 1987). The hydroxamate complex is much more stable (Neilands, 1981) and hence potentially more significant in the rhizosphere.

### 2.3.3 INHIBITORY EFFECT OF SIDEROPHORES

**Elad and Baker(1985) and Sneh et al** (1984) found a direct correlation in **invitro** siderophore synthesis in fluorescent **Pseudomonads** and their capacity to inhibit germinatin of chlamydospores of **Fusarium oxysporum.** There are a number of reports suggesting that plant species are capable of obtaining iron from some microbial siderophores (Orlande and Neilands, 1982; Powell **et al.,** 1982);

Siderophores from fluorescent **Pseudomonads** have been implicated in iron uptake by tomato plants as per Duss **et al.,**(1986), and the remedy of lime induced chlorosis by

peanut according to Jurkevitch **et al.,** (1988). Carnations and barley may utilise the fluorescent siderophore pseudobactin - 358 for uptake of iron (Duijff **et al.,** 1991).

### 2.3.4 FACTORS INFLUENCING SIDEROPHORE PRODUCTION

Siderophore production by fluorescent **Pseudomonads** is influenced by a great variety of factors, eg concentration of iron (Kloepper **et al.,** 1980); nature and concentration of carbon and nitrogen sources (Gouda and Greppin, 1965; Park **et al.,** 1988), level of phosphates (Barbhaiya and Rao, 1988), pH and light (Greppin and Gouda, 1965), degree of aeration (Lenhoff, 1963), presence of trace elements such as magnesium, zinc (Chakrabarty and Roy, 1964) and temperature (Weisbeek **et al.,** 1986).

### 2.3.5 BACTERIA PRODUCING SIDEROPHORES

Bacteria that produce siderophores include **Escherichia coli** (Braun, 1981), **Pseudomonas** species (Cox and Graham 1979; Meyer and Abdallah, 1980) **Bacillus megaterium** (Byers **et al.,** 1967) **Actinomyces species** (Bickel **et al.,** 1960) **Mycobacterium** species (Snow, 1970) and plant pathogen **Agrobacterium tumefaciens** (Leong and Neilands, 1981).

## 2.3.6 SIDEROPHORE OF PSEUDOMONAS SPECIES

### 2.3.6.a. OCCURENCE:-

Neilands(1981) has showed pseudobactin is the fluorescent siderophore of **Pseudomonas** B-10. It increases the potato growth as per Kloepper **et al.**, (1980):. Meyer and Abdallah, (1978) have found that pyoveridine, the yellow green fluorescent pigment of **Pseudomonas fluorescens** is synthesized and excreted by iron - deficient cells and has a high specific affinity for Fe(iii).

### 2.3.6.b. STRUCTURE

Partial determination of structure of pyoverdine has shown that an unusual aminoacid $\delta$ - N - hydroxy ornithine is present in a cyclic peptide chain This aminoacid is also a constituent of several hydroxamate iron binding compounds.

## 2.3.7. SIDEROPHORE OF PSEUDOMONAS FLUORESCENS

**Pseudomonas fluorescens** generally produce fluorescent, yellow green, water - soluble siderophores with both a hydroxamate and phenolate group; these siderophores have been classified as either pyoverdines or pseudobactins. Analysis of pseudobactin and pyoveridine type siderophores from different fluorescent pseudomonad strains showed that

the principal difference is the composition, number and configuration of the amino acids in the peptide backbone (Neilands, and Leong, 1986). The production of the siderophores has been linked to the disease suppression ability of certain **fluorescent Pseudomonads** (Loper and Buyer, 1991).

## 2.4.SIDEROPHORE MEDIATED PATHOGEN SUPPRESSION BY PSEUDOMONAS FLUORESCENS

### 2.4.1. MECHANISM OF ACTION

Neilands and Leong (1986) gave a short review on the relationship between siderophores and plant growth and disease. The differnt types of siderophores and how they are involved in biocontrol of disease was well explained by them. The proposed mechanism for siderophores mediated disease suppression by fluorescent **Pseudomonas** is illustrated by O' Sullivan and O'Gara (1992). Fluorescent siderophores which have a very high affinity for ferric 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 (Loper and Buyer, 1991). The essentiality of iron to micro organisms has been reviewed by Neilands (1977).

### 2.4.2. IMPORTANCE OF SIDEROPHORES IN PLANT GROWTH

Bakker **et al.,** (1988) made a study on **Pseudomonas putida** strain WCS358, **P.fluorescens** strain WCS374, and siderophore negative mutants of these strains obtained by Tn5 transposon mutagenesis; were used in experiments to assess the role of siderophores in potato growth stimulation by WCS358, Vandenbergh **et al.,**(1983), has discussed about the iron chelating compounds produced by soil **Pseudomonas** like **P.putida, P.fluorescens** and **P.aeruginosa** and their correlation with fungal growth inhibition. Meyer and Hornsperger (1978) have found that experiments with Fe(iii) showed that **pseudomonas fluorescens** had an active system for iron transport. When the purified iron binding pigment synthesized by this bacterium was added to the external medium, the rate of iron uptake, by the cells increased significantly.

### 2.4.3. SUPPRESSION OF PLANT PATHOGENIC FUNGI

#### 2.4.3.a. RHIZOCTONIA SOLANI

It was shown by Laha **et al..,** (1992) that isolates of fluorescent **Pseudomonads** have the ability to suppress the root rot and damping off of cotton caused by **Rhizoctonia solani.** These fluorescent **Pseudomonads** antagonize pathogens by producing one or more metabolites that

includes antibiotics, HCN and siderophores (Leong, 1986). Mathot (1990) analysed the usefulness of specific strains of **P.fluorescens** to improvee the growth of beans **(Phaseclus vulgaries)** and to give protection against **R.solani.**

A field evaluation of bacterial inoculants of **Pseudomonas** was done to control seedling diseases pathogens like **R. solani,** on cotton by Hagedorn **et al.,**(1993). Gnanamanickam and Mew (1993) have demonstrated the biological control of rice diseases caused by **R. solani** (sheath blights) with bacterial antagonists like **P. fluorescens.** Similarly Rath and Wolf (1993) have develcped a method of biological control of **R. solani** which causes damping off of sugar beet with **P.fluorescens. Ryu et al.,** (1992) selected and identified antagonistic bacteria like **Pseudomonas** species for controlling soil borne diseases of vegetables caused by **R. solani, F.oxysporum, Phytophthora Capsici.** Cubeta and Echandi (1991) demonstrated the biological control of **R. solani** and **Pythium** damping off of cucumber. Podile **et al.,**(1992) showed that antibiotic produced by **P. fluorescens** can act as an antagonists against **R. solani, F. solani, and S. rolfsi.** Bacterization with **P. fluorescens** can act as an antagonists and resulted in the effective biological control of sheath rot of rice and stem rot of ground - nut caused by **R. solani** according to Sakthivel **et al.,**(1992).

Kloepper (1993) developed **in vivo** assays for selecting potential rhizobacterial biological control agents against **R. solani** on cotton. Tu (1993) in his management of root rot diseases of peas, beans and tomatoes has discussed the control of **R. solani** with **P. fluorescens.** The two fluorescent compounds produced by **P. fluorescens** are pyocyanin and pyrrolnitrin (Suslow, 1982) which are effective inhibitors of fungal pathogens (Howell and Stipanovic, 1979; Suslow and Schroth, 1982 b). The antibiotic pyrrolnitrin increased emergence and survival of cotton seedlings when planted in **R. solani** infested soil. Weller and Cook (1986) have showed that **pseudomonas** can control "take all" by both antibiotic production and by the use of siderophores. It is thought that the siderophores may be important in early colonisation when the take all fungus competes for iron, but the antibiotic as a secondary metabolite may be produced later and then inhibit the growth in the lesions and the stele.

### 2.4.3.b. ERWINIA CARATOVORA

Siderophore production by **P. fluorescens** and **P. putida** and the effective control of soft rot of potato caused by **E. caratovora** was discussed by Vidhyasekaran (1993).

## 2.4.3.c. PYTHIUM, GAEUMANNOMYCES, F. SOLANI AND OTHER PATHOGENIC FUNGI

Fluorescent **Pseudomonads** for plant disease control was well established by Ray **et al.**, (1993). The pathogens include **Aspergillus niger, F. Oxysporum, Macrophomina Phaseolina** and **R. solani.** Mishagi **et al.**,(1982) found that water soluble fluorescent pigments from 156 **Pseudomonas** strains inhibited growth of **Geotrichum, candidum,** and pigments from some strains studied more thoroughly also inhibited several fungi **invitro.** Yield depression of plant growth in higher frequency cropping soil caused by an increase of deleterious micro organisms or their products can be reduced by seed inoculation with **P. fluorescens** was proposed by Geels and Schippers(1983); Schippers **et al.**, (1985). Furthermore, a significant reduction of the fungal and bacterial population in the rhizosphere was observed by kloepper **et al.**,(1980); Suslow and Schroth (1982 b). The factors influencing siderophore production by **Pseudomonas** species were characterised by Loper and Ishimaru (1992). The role of antibiotics and siderophores in biocontrol of "take all" disease of wheat caused by **G.graminis** was demonstrated by Thomashow and Weller (1990). Tanii **et al.**, (1990) explained the biological control of scab ,black scurf and soft rot of potato by seed tuber

bacterization. Heungens **et al.,** (1993) have analysed the role of siderophores in biological control of **Pythium** species by **Pseudomonas** strain 7NSK2. Gagne **et al.,** (1991) have showed that volatile compounds produced by some PGPR strains inhibit **Pythium, Phytophthora** and **R. solani.** A special study was done on the biological control of**R. solani** by Roy (1990) and Kloepper **et al.,** (1980) have shown that fluorescent **Pseudomonads** produce siderophores to suppress the **Fusarium** wilt pathogens. Fungistatic activity of water soluble fluorescent pigments of fluorescent **Pseudomonads** especially **P. fluorescens** against **R. solani, Pythium,** was explained by Misaghi **et al.,** (1981). Pseudobactin the pyoveridine produced by **Pseudomonas** B - 10 inhibits grwoth of **Fusarium Oxysporum** and **G. graminis** as per Kloepper **et al.,** (1980). Compound S, the Pyoverdine produced by the foliar epiphyte **P. fluorescens** UV - 3, enhances germination of iron - replete, but not iron deplete conidia of **C. acutatum** according to Mc Cracken and Swinburne (1979).

The ferric pyoveridine of **P.fluorescens** CHAO inhibits endoconidial germination of soil borne fungus **T. basicola**, Siderophore production by a fluorescent **Pseudomonad** strain RBT - 13 isolated from the rhizoplane of tomato plant which showed **in vitro** antagonism against several fungal and bacterial plant pathogens and simultaneously increased the plant growth and disease suppression in four crop plants (Kumar and Dube,1991 and 1992).

# Experimental Procedure

# III EXPERIMENTAL PROCEDURE

The experimenatal design for this project namely " Role of siderophores of **Pseudomonas fluorescens** in biocontrol of plant pathogenic fungi **Rhizoctonia solani** " is presented under the following headings.

3.1. Source of **Rhizoctonia solani.**

3.2. Source, isolation and maintainence of **Pseudomonas fluorescens.**

3.3. Extraction and purification of siderophores fron **Pseudomonas fluorescens** isolates.

3.4. Fungistatic activity of purified siderophores.

3.5. Inhibition of **Rhizoctonia solani** by **Pseudomonas fluorescens** isolates.

3.6. Identification of siderophore type by Thin layers chromatography.

3.7. Quantification of siderophores present in the **Pseudomonas fluorescens** isolates.

## 3.1. SOURCE OF RHIZOCTONIA SOLANI

Cotton seedlings infected with **R. solani** was used for isolation of the pathogen. Cotton seedlings was collected from cotton breeding station, TNAU, Coimbatore. The pathogen was isolated using selective medium.

**MEDIUM COMPOSITION**

$K_2HPO_4$ - I O g.

$MgSO_4$ $7H_2O$ - 0.5g

KCl - 0.5g

Fe $SO_4$ - 0.1g

Sodium nitrate - 0.2g

Chloromycetin - 0.05g

Streptomycin Sulphate - 0.05g

Demoson - 30 ppm.

Agar agar - 15g

Water - 1000 ml.

## 3.2. SOURCE, ISOLATION AND MAINTAINENCE OF PSEUDOMONAS FLUORESCENS

Soil samples were collected from the cotton grown field where **R. solani** incidence was observed. For the isolation of **P. fluorescens**, Kings' B selective medium was used (Kings', 1954).

**MEDIUM COMPOSITION**

Peptone - 20g

Glycerol - 10 ml

$K_2HPO_4$ - 1.5g

$MgSO_4$ - 1.5g

Cycloheximide - 100 g/ml.

Chloramphenicol - 12.5 g/ml

Ampicillin - 50 g/ ml

Agar - 15 g/l

Water - 1000 ml

The **Pseudomonas fluorescens** was identified with staining and standard biochemical tests(Palleroni, 1984). The identified **P. fluorescens** isolates were transferred to Kings' B medium slants in duplication for maintainence. One set of isolates were used for further studies.

### 3.3. EXTRACTION AND PURIFICATION OF SIDEROPHORES FROM P. FLUORESCENS ISOLATES. (MISAGHI ET AL., 1982).

Succinate medium ($K_2HPO_4$ - 0.6% ; $KH_2PO_4$ - 0.3% ; $(NH_4)_2\ SO_4$ - 0.1% ; $MgSO_4\ 7H_2O$ - 0.02% ; Succinic acid-0.4%) was used for the extraction of siderophore. Medium was adjusted to $P^H$ 7.2 by addition of 2N NaOH prior to sterilization. Flasks (250 ml) containing 50 ml of the medium were inoculated with the bacterium and incubated at 24 - 26$^o$c for 38 hours without shaking. Bacterial cells were removed by centrifugation at 10,000 g for 20 minutes. Three hundred milli litres of cell free extract was centrifuged at 10,000 g for 20 minutes after addition of 2 volumes of acetone. The fluorescent pigment was precipitated from the extract by adding two additional volumes of acetone followed by centrifigation at 10,000g for 20 minutes. The pigment was washed three times with reagent grade acetone and dissolved in 12ml of 0.03M acetic acid buffer, $P^H$ 5.2. Two tenths milli liter of pigment solution was placed at the top of the column (1.5 x 25 cms) of Sephadex G - 25.

The column was eluted with water at a rate of 1.0 ml/minite at 24 - 25°c. Absorbtion spectra of each 1ml fraction was determined by a recording spectrophotometer.

### 3.4. FUNGISTATIC ACTIVITY OF THE PURIFIED SIDEROPHORE

The purified siderophore was obtained according to the procedure described above. In sterile petri dishes, molten potato dextrose agar medium was transferred and allowed to solidify. At the centre of the plate, 8mm diameter **R.solani** disc was placed, Wells were formed with cork borer on the potato dextrose agar medium and in each well, 5 micro litre of purified siderophore was placed for testing their fungistatic activity against **R. solani.**

### 3.5. INHIBITION OF R. SOLANI BY PSEUDOMONAS FLUORESCENS ISOLATES

The molten Kings' B agar medium was transferred to sterile petri dishes. At one end of the plate, the **P.fluorescens** isolates was streaked with the help of inoculation needle. On the opposite side, a 8mm diameter disc of R. solani was placed. For control, **R. solani** disc alone was placed, and the plates were incubated for 48 hours, and inhibition zone was measured.

### 3.6.IDENTIFICATION OF SIDEROPHORE TYPE BY THIN LAYER CHROMATOGRAPHY (MODI et al, 1985)

TLC plates were prepared with silica gel G. The siderophore of **P. fluorescens** were spotted along with standards 3,4, DHBA and sodium salicylate. The solvent system used was Butanol : Acetic acid : Water (4 : 1 : 1). The chromatogram was observed under ultra violet light for fluorescence and developed by Hathway's reagent, prepared as per the procedure described by Reeves **et al.**, (1983). The Rf values were measured as follows :

$$Rf = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent front.}}$$

Based on the Rf values, the compound present in the siderophore was identified.

### 3.7. QUANTIFICATION OF SIDEROPHORES PRESENT IN P. FLUORESCENS ISOLATES:

One micromole of standard 3,4 DHBA was prepared (0.154 mg/litre) and absorbance was determined at 700nm. For the assay, one volume of the sample (20ml) and one volume of Hathway's reagent (20ml) was added and and absorbance was measured at 700 nm. Based on the optical density values, the quantity of siderophores present in the **P. fluorescens** isolates was calculated as given below and expressed as mg/litre.

CALCULATION:

$$\text{Quantity of siderophore present in 20 ml of the sample} = \frac{0.154 \times \text{Absorbance of sample}}{\text{Absorbance of standard}} \text{ mg/litre.}$$

$$\text{Quantity of siderophore present in one litre of the culture filtrate} = \frac{0.154 \times \text{Absorbance of sample} \times 1000}{\text{Absorbance of standard} \times 20} \text{ mg/litre.}$$

# Results and Discussion

## III RESULTS AND DISCUSSION

The results and discussion of the project namely "Role of the siderophores of **Pseudomonas fluorescens** in the biocontrol of plant pathogenic fungi **Rhizoctonia solani**" is discussed under following headings.

4.1. Isolation of **Rhizoctonia solani.**

4.2. Isolation of **Pseudomonas fluorescens.**

4.3. Extraction and purification of siderophores from **Pseudomonas fluorescens** isolates.

4.4. Fungistatic activity of the purified pigment.

4.5. Inhibition of **Rhizoctonia solani** by **Pseudomonas fluorescens** isolates.

4.6. Identification of siderophore type in **Pseudomonas fluorescens** isolates.

4.7. Quantification of siderophore present in **Pseudomonas fluorescens** isolates.

## 4.1. ISOLATION OF RHIZOCTONIA SOLANI

One virulent isolate of **Rhizoctonia solani** was obtained from the cotton seedlings affected by seedling blight. This isolate was used for further studies .

## 4.2. ISOLATION OF PSEUDOMONAS FLUORESCENS

Five bacterial isolates were obtained from the cotton grown field soil sample.Among these five bacterial isolates , three isolates were found to be **Pseudomonas fluorescens** based on staining and standard biochemical tests(Palleroni, 1984). So the other two isolates were rejected. The three isolates were numbered Pf-1,Pf-2 & Pf-3 respectively and these were maintained in kings' B agar slants.These were used for further studies.

In this attempt of isolation of **Pseudomonas fluorescens** from cotton rhizosphere, it was found that the presense of ampicillin, chloramphenicol and cycloheximide in Kings' B agar medium suppressed the growth of most of the fungi and bacteria normally encountered in the rhizosphere, but they were not inhibitory to the **Pseudomonas fluorescens** .Laha **et al**, (1992)also obtained **Pseudomonas fluorescens** isolates from cotton rhizosphere using Kings' B agar medium supplemented with the above antibiotics and reported similar results. A strain of **Pseudomonas fluorescens** was isolated from cotton rhizosphere where **Rhizoctonia solani** incidence was more by Howell and Stipanovic,(1979) and they reported similar results.

## 4.3. EXTRACTION AND PURIFICATION OF SIDEROPHORES FROM PSEUDOMONAS FLUORESCENS ISOLATES.

Siderophores were isolated from acetone extract of **Pseudomonas fluorescens** Pf-1,Pf-2 & Pf-3 culture filtrates. The crude pigment thus obtained from three isolates, was purified by Sephadex gel G-25 column chromatography. Out of three **Pseudomonas fluorescens** isolates, two isolates (Pf-2 and Pf-3) yielded peak at 405nm. Pf-1 did not yield any peak.The absorbance of purified fractions of Pf-3 and Pf-2 was illustrated in figures I and II.

The results of this aspect of the present study, is in confirmation with the results of Misaghi **et al**(1982) who also recorded peak at 403nm for the purified pigment of **Pseudomonas fluorescens** in their study related to isolation and purification of siderophore from **Pseudomonas fluorescens** .

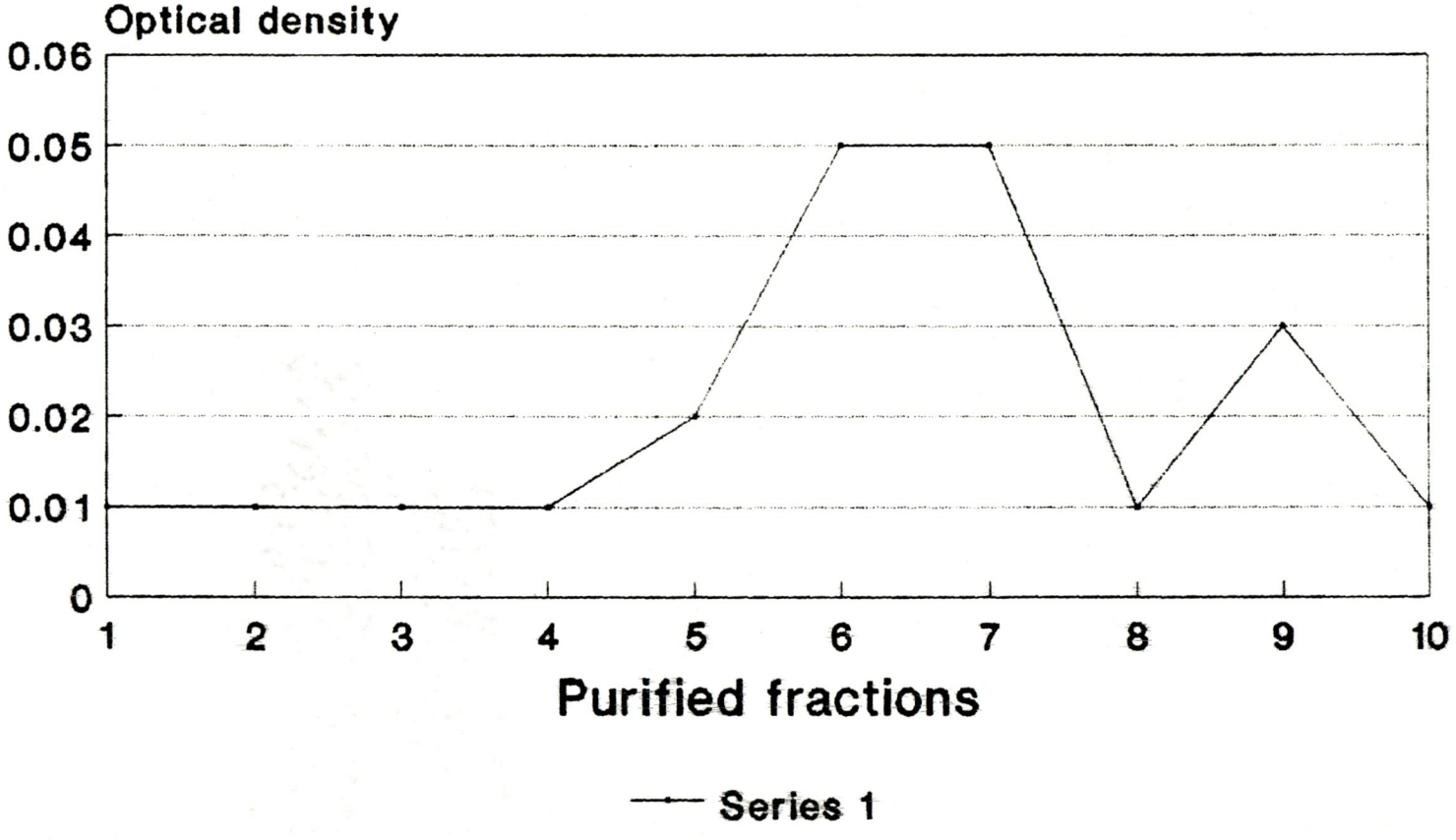
Isolation and characterization of the fluorescent pigment produced by **Pseudomonas** spp has been reported by many workers. Demange **et al** (1986) characterised the siderophore produced by **Pseudomonas aeruginosa** and Hofstad **et al**(1986) characterized a siderophore from **Pseudomonas putida.**

## 4.4 FUNGISTATIC ACTIVITY OF THE PURIFIED PIGMENT

Fluorescent **Pseudomonads** antagonize pathogens by producing one or more metabolites that include antibiotics(Fravel, 1988) siderphores (Leong, 1986) and cyanide (Voisard **et al**, 1989). The involvement of siderophores produced by fluorescent Pseudomonas in biocontrol of many phytopathogenic fungi has been proved by

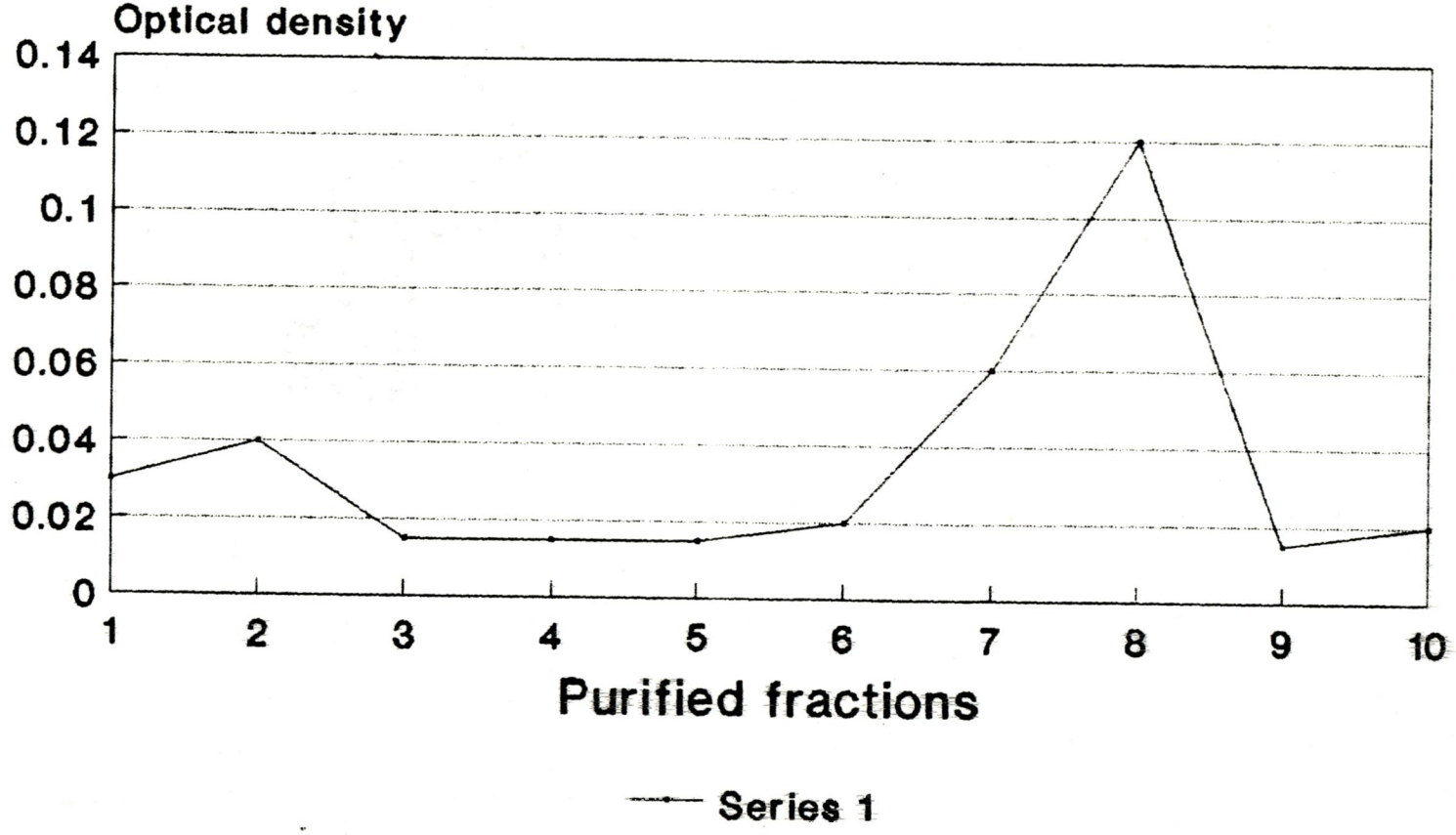
# FIGURE - I

## ABSORBANCE OF SIDEROPHORE OF PSEUDOMONAS FLUORESCENS ISOLATES - (Pf - 3)



# FIGURE - II

## ABSORBANCE OF SIDEROPHORE OF PSEUDOMONAS FLUORESCENS ISOLATES - (Pf - 2)



Leong, (1986).

Table I shows the fungistatic activity of fluorescent pigment of **P.fluorescens** isolates.

**TABLE 1**

**FUNGISTATIC ACTIVITY OF PURIFIED PIGMENT OF PSEUDOMONAS FLUORESCENS ISOLATES:**

| S. No | ISOLATE | INHIBITION ZONE (cms) |
|---|---|---|
| 1. | Pf - 1 | 0.6 |
| 2. | Pf - 2 | 1.5 |
| 3. | Pf - 3 | 1.0 |

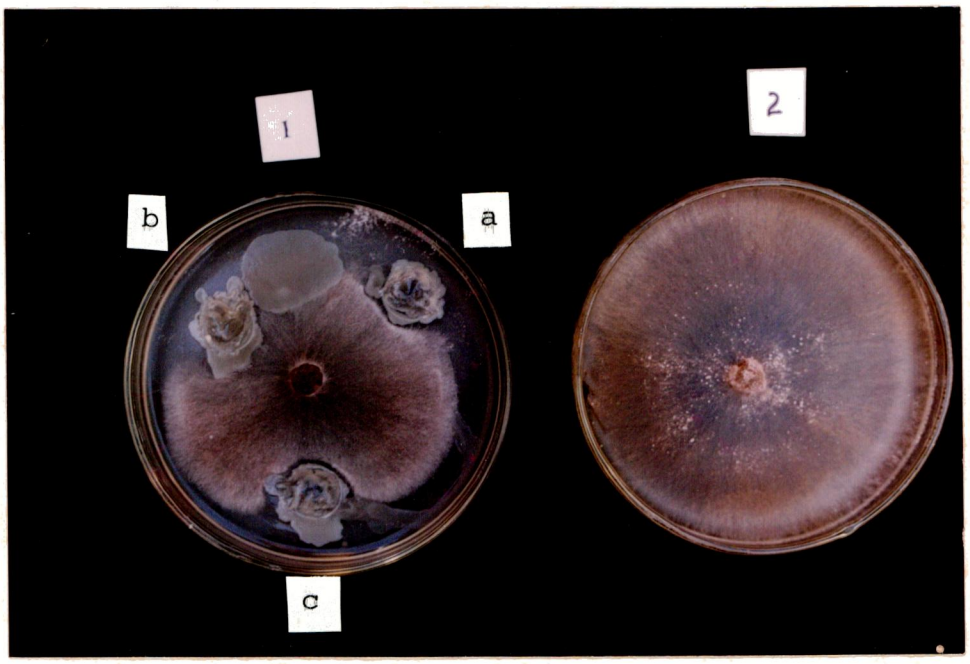
Among the three pigment samples obtained, So far , Pf - 2 and Pf - 3 were found to inhibit **Rhizoctonia solani**, to a greater extent than the pigment obtained from Pf - 1. The inhibition zone was 0.6 cms, 1.5 cm, and 1.0 in pigments of Pf - 1, Pf - 2 and Pf - 3 respectively.

Plate - 1 indicates the fungistatic activity of the fluorescent pigment of **P.fluorscens** isolates.

Misaghi **et al.,** (1982) also have reported the similar fungistatic activity of the crude pigment of **Pseudomonas fluorescens** against **R. solani.** But they haven't mentioned the length of inhibition zone. This observation is also supported by Kloepper **et al.,** (1980) who partially purified fluorescent siderophore produced by **P. fluorescens** to analyse the fungistatic activity of the siderophore against a number of important soilborne pathogenic fungi and has reported similar results.

## PLATE-1

FUNGISTATIC ACTIVITY OF THE FLUORESCENT PIGMENT OF **Pseudomonas fluorescens** ISOLATES




1. a - **Pf-1** b - **Pf-2** c - **Pf-3**
2. **CONTROL**

Likewise, Burr and Caesar, (1984) has shown that fluorescent pigment of 156 Pseudomonas strains inhibited growth of several fungi in **in vitro** conditions. Researchers like Scher and Baker (1980) have now implicated **Pseudomonas** sp. ' as a major factor in inhibition of several pathogens in suppressive soils, through production of siderophores.

**4.5. INHIBITION OF RHIZOCTONIA SOLANI BY PSEUDOMONAS FLUORESCENS ISOLATES :**

Antagonistic fluorescent Pseudomonads are important natural antagonists of plant pathogens like **Rhizoctonia solani**, **Fusarium solani** and **Pythium** sp. These fluorescent Pseudomonads have now been implicated in control of many plant diseases caused by these fungi.

Table II shows the effect of **Psuedomonas fluorescens** isolates on **Rhizoctonia solani.**

**TABLE II.**

**EFFECT OF PSEUDOMONAS FLUORESCENS ISOLATES ON RHIZOCTONIA SOLANI :**

| S.No | ISOLATES | GROWTH OF RHIZOCTONIA SOLANI (mm) | REDUCTION OF RHIZOCTONIA SOLANI GROWTH (%) |
|---|---|---|---|
| 1. | Pf - 2 | 62 | 30 |
| 2. | Pf - 3 | 74 | 16 |
| 3. | **Rhizoctonia solani** (control) | 88 | - |

Out of the three isolates, Pf - 2 and Pf - 3 were found to be superior than Pf - 1. These two isolates were tested for antagonistic activity against **Rhizoctonia solani**. Pf - 2 was found to be more inhibitory, reduced the **R. solani** growth by 30% than Pf-3 which has reduced the growth by 16%.

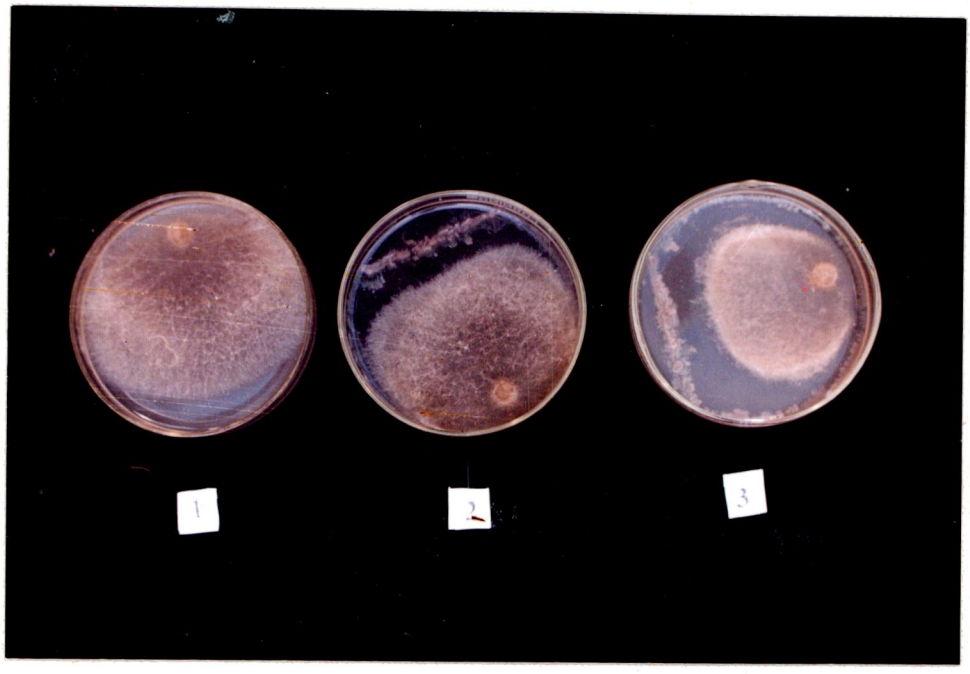
Plate - 2 illustrates the inhibition of **Rhizoctonia solani** by **Pseudomonas fluorescens** isolates.

Similar inhibitory effect was noticed by Laha **et al.**, (1982),when they used fluorescent Pseudomonads to biocontrol **Rhizoctonia solani** in cotton rhizosphere. In this study, 85% maximum growth suppression was noticed. The difference in the percent of inhibition may be due to different **Pseudomonas** sp. **(Pseudomonas putida)** used.

PLATE-2

INHIBITION OF **Rhizoctonia solani** BY

**Pseudomonas fluorescens** ISOLATES




1 - CONTROL 2 - Pf-3 3 - Pf-2

## 4.6. IDENTIFICATION OF SIDEROPHORE TYPE IN PSEUDOMONAS FLUORESCENS ISOLATES :

Microorganisms are known to priduce two types of siderophores, the secondary bydroxamic acids and the phenolics (derivatives of 2,3 dihydroxy benzoic acids or catechol). Miles and Khimji (1975) have reported that fluorescent Pseudomonads produce a catechol - type siderophore.

The identification of 3,4 dihydroxybenzoic acid in the siderophore produced by **Pseudomonas fluorescens** is predicted in table III.

**TABLE III**

**IDENTIFICATION OF 3,4 DIHYDROXY BENZOIC ACID IN SIDEROPHORE OF PSEUDOMONAS FLUORESCENS ISOLATES.**

| S.No. | SAMPLE | DISTANCE TRAVELLED BY SOLUTE (cms) | SOLVENT (cms) | Rf |
|---|---|---|---|---|
| 1. | Pf 1 | 11.4 | 15 | 0.76 |
| 2. | Pf 2 | 11.8 | 15 | 0.79 |
| 3. | Pf 3 | 11.7 | 15 | 0.78 |
| 4. | Standard 1 (3,4 DHB4) | 11.7 | 15 | 0.78 |
| 5. | Standard 2 (Sodium salicylate) | 10.5 | 15 | 0.70 |

By the thin layer chromatography technique it was noticed that the Rf value of the pigments obtained from **Pseudomonas fluorescens** isolates Pf - 1, Pf - 2 and Pf - 3 was in accordance with the Rf value of standard -1 (3,4 DHBA). So 3,4 DHBA was detected as a component of the siderophore of **P. fluorescens** isolates. Hence the siderophore of **P.fluorescens** was found to be catechol - type.

Plate - 3 shows the thin layer chromatography of the siderophore of three **Pseudomonas fluorescens** isolates which identifies the 3,4, DHBA in the siderophore.

Modi **et al.,**(1990) also have reported catechol type of siderophore in cowpea **Rhizobium** using TLC technique. Here 2,3 DHBA was identified as a component part in the siderophore of **Rhizobium**. The difference in derivatives of the component may be due to the different organism used. Chakraborty **et al.,** (1990) have also reported that **Pseudomonas stutzeri** produces a Catechol type siderophore. But this **P. stutzeri** is a nonfluorescent Pseudomonad.

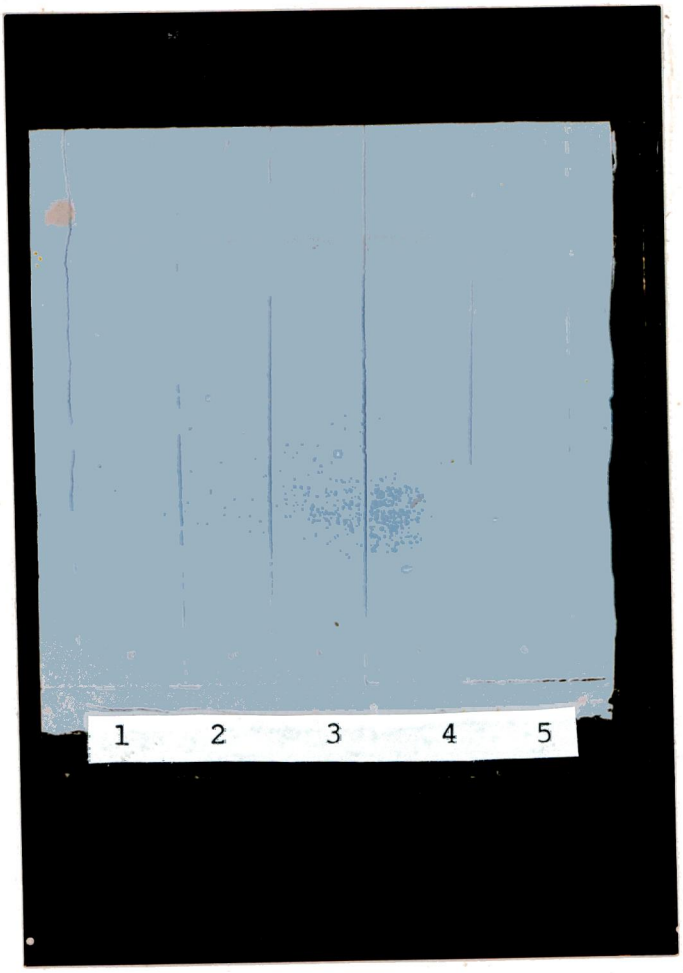
## 4.7. QUANTIFICATION OF SIDEROPHORE PRESENT IN PSEUDOMONAS FLUORESCENS ISOLATES :

The quantity of siderophores produced by micro organisms may vary in accordance to their inhibitory effect on pathogens.

Table IV gives the quantity of siderophore present in **Pseudomonas fluorescens** isolates :

## PLATE-3

THIN LAYER CHROMATOGRAPHY OF SIDEROPHORE OF **Pseudomonas fluorescens** ISOLATES.




1 - Pf-1 2 - Pf-2 3 - Pf-3

4 - STANDARD - 1 (3,4 DHBA)

5 - STANDARD - 2 (SODIUM SALICYLATE)

## TABLE - IV

**QUANTIFICATION OF SIDEROPHORE PRESENT IN PSEUDOMONAS FLUORESCENS ISOLATES :**

| S.No | ISOLATE | OPTICAL DENSITY | QUANTITY (mgs) | QUANTITY/LITRE OF CULTURE FILTRATE (mgs) |
|---|---|---|---|---|
| 1. | Standard (3,4 DHBA) | 0.75 | 0.154 | - |
| 2. | Pf - 1 | 0.10 | 0.020 | 1.0 |
| 3. | Pf - 2 | 0.38 | 0.078 | 3.9 |
| 4. | Pf - 3 | 0.35 | 0.072 | 3.6 |

One litre culture filtrate of Pf 1, Pf-2 and Pf - 3 yielded 1.0, 3.9 and 3.6 mg of Catechol type siderophore respectively. No absorbance was observed at 560 nm indicating the absence of Salicylate type siderophore.

Modi **et al.,** (1985) has reported one litre culture supernatant of Rhizobium gave 6.2 mg of 2,3 DHBA siderophore. The variation in the quantity may be due to the different organism involved in the study.

Higher production of siderophore was also reported by Alstrom (1987) by **Pseudomonas fluorescens** which antagonizes **Gaeumannomyces graminis.**

# Summary and Conclusion

# V SUMMARY AND CONCLUSION

The possibility of replacing chemical control of plant root diseases with a biological alternative remains an exciting and challenging objective. Some fluorescent **Pseudomonas**.spp. do appear to be prime candidates for this role through production of siderophore.

In the present study, the siderophore of **Pseudomonas fluorescens** isolates Pf - 1, Pf - 2 and Pf - 3 were extracted from the culture filtrates. The purified pigments of these isolates were tested for the activity against **Rhizoctonia solani.** The summary of the results obtained by this study is given below.

1. The purified siderophore pigment obtained from three isolates of **Pseudomonas fluorescens** isolates Pf - 1, Pf - 2 and Pf - 3 was found to inhibit **Rhizoctonia solani** growth with the inhibition zone of 0.6, 1.0 and 1.5 cms respectively.

2. **Pseudomonas fluorescens** isolates Pf -2 and Pf - 3 were found to reduce the growth of **Rhizoctoria solani** growth by 30% and 16% respectively. Pf-1 was found to be inferior than Pf-2 and Pf-3.

3. Microrganisms are known to produce two types of siderophores, the secondary hydroxamic acids and the phenolic acids (derivatives of 2,3 dihydroxy benzoic acid or Catechol).3,4 dihydroxy benzoic acid was detected

as a component of siderophore of **Pseudomonas fluorescens** isolates Pf-1, Pf-2 and Pf-3.

4. One litre culture of **Pseudomonas fluorescens** isolates of Pf - 1, Pf - 2 and Pf - 3 yielded 1.0, 3.9 and 3.6 mg of siderophore respectively.

Siderophores of **Pseudomonas** spp has been proved to be an excellent challenge against many soil pathogens. Progress is now being made at understanding some of the traits of fluorescent **Pseudomonas** which are necessary for biological control of pathogens. The integration of these traits with the ecological fitness of strains will be prerequisite for designing biocontrol agents for specific purpose. A better understanding of root colonization and disease suppression mechanisms is needed before suitable strains can be directly selected and/or engineered by genetic means into commercially liable products. The wild type strains containing desired attributes can be recognized. More siderophore producing **Pseudomonas** spp can be selected to biocontrol pathogens. Research along these, will increase the impact of fluorescent Pseudomonads on the biocontrol of plant root diseases in the commercial world.

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