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Antagonistic Effect of *Pseudomonas spp* on *Pythium aphanidermatum* and on Plant Growth of Chilli. (*Capsicum annuum* L.)

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A B S T R A C T

Pre and post-emergence damping-off disease caused by *Pythium* spp which is a facultative parasite and lives saprophytically on the moist humus in soil and attacks seedlings at the soil level. "Damping off", "Soft rot", "Wheat rot", or the "foot rot" of the seedlings is commo diseases caused by the fungus. *P. aphanidermatum* and also causes "damping off" disease of tobacco, mustard, chillies and cress seedlings. Rapid germination of sporangia of *Pythium* after exposure to exudates or volatiles from seeds or roots followed by immediate infection make management of Pythium very difficult. It is estimated that diseases caused by *Pythium* species in different crops are responsible for losses worldwide. Besides this, *P. aphanidermatum* became resistant to the common fungicides used against it. So, using a biocontrol agent against the phytopathogens was always significant because Pseudomonads possess many traits that make them well suited as bio control and growth-promoting agents. In addition, pseudomonads are responsible for the natural suppressiveness of some soils to soil borne pathogens and can provide biological control of soil borne pathogens on a wide range of crops, and they have a key role in the suppressiveness of some soils to plant pathogens. *Pseudomonas* isolates were isolated from the rhizosphere of vegetable and ornamental crops to use as biocontrol agents. A total of six isolates were isolated and identified through bio-chemical tests and characterization of *Pseudomonas*. Then the isolates were screened for antagonism *in vitro* against *Pythium aphanidermatum* in laboratory conditions through dual culture assay. Among six isolates, all were inhibiting the *Pythium aphanidermatum* at various levels. Overall, the isolate 2 was inhibiting maximum percent over reduction at 48 hrs with 58.25% and isolates 6 was inhibiting the minimum percent over reduction at with 54.69%. Whereas, in field trails, after 55DAT the isolate 1 was most effective and excelled among all the isolates with 34.46% by following all the isolates participated certainly in increasing the growth parameters of chilli.

Introduction

The *Pseudomonas* (subclass-Proteobacteria, order-Pseudmonadales, family- Pseudomonaceae) posses a strictly respiratory metabolism using oxygen, they are motile (one or several polar flagella), nonsporulating rods giving Gram negative reaction and 58–

69% GC (Guanine & Cytosine) content, chemo-organotrophic, and in some cases nitrate as terminal electron acceptor. The fluorescent pseudomonads include all *Pseudomonas* species with the ability to produce fluorescent pyoverdine siderophores, noticeably *P. aeruginosa, P. syringae, P. putida* and *P. fluorescens* (Bossis *et al.*, 2000). *Pseudomonas fluorescens* is adapted to survival in soil and colonization of plant roots, and this applies also to the particular case of bio control agents from this species. Pseudomonads possess many traits that make them well suited as bio control and growth-promoting agents. These include the ability to grow rapidly in vitro and to be mass produced, rapidly utilize seed and root exudates, colonize and multiply in the rhizosphere and spermosphere environments and in the interior of the plant, produce a wide spectrum of bioactive metabolites (i.e., antibiotics, siderophores, volatiles, and growth-promoting substances, compete aggressively with other microorganisms, and adapt to environmental stresses. In addition, pseudomonads are responsible for the natural suppressiveness of some soils to soil borne pathogens and it produce 2,4 diacetylphloroglucinol (2,4-DAPG) can provide biological control of soil borne pathogens on a wide range of crops, and they have a key role in the suppressiveness of some soils to plant pathogens (Mavrodi *et al.*, 2013). In this investigation the attempts were made to identify the pseudomonads and testing their activity against soil borne pathogen, *P. aphanidermatum*. The *Pythium* species are fungal-like organisms, commonly referred to as water molds, (kingdom Straminopila, phylum oomycot, class Oomycetes, subclass Peronosporomycetidae, order Pythiales and family Pythiaceae) are worldwide in distribution and associated with a wide variety of habitats ranging from terrestrial or aquatic environments, in cultivated or fallow soils, in plants or animals, in saline or fresh water. The genus *Pythium* is one of the largest oomycete genus and consists of more than 130 recognized species which are isolated from different regions of the world.

Pythium spp cause Pre and post-emergence damping-off. The fungus is facultative parasite and lives saprophytically on the moist humus in soil and attacks seedlings at the soil level. It also grows readily on floating vegetable matter and decaying animals. "Damping off", "Soft rot",

"Wheat rot", or the "foot rot" of the seedlings are common diseases caused by the fungus. *P. aphanidermatum* causes "damping off" disease of tobacco, mustard, chillies and cress seedlings. Besides this, *P. aphanidermatum* became resistant to the common fungicides used against it. Keeping in view of this above role of *Pseudomonas* spp and *Pythium* the isolation and characterization of *Pseudomonas* spp and *Pythium* spp and their invitro screening for antagonism and field evaluation were undertaken.

Materials and Methods

The present study was carried out in Laboratory, Dept. of Plant Pathology, Sam Higginbottom University of Agriculture, Technology and Sciences (SHUATS), Naini, Prayagraj during 2019-20, "Antagonistic effect of *Pseudomonas spp* on *Pythium aphanidermatum* and on plant growth of chilli". The experiment was laid out in Completely Randomized Design (CRD) and Randomized block design in 3 replications.

Collection of soil sample

Samples were collected from the rhizospheric soils of college farm, Allahabad. Soil samples were collected up to the depth of 10 to 15 cm from the rhizosphere of plants i.e. Lady's

finger, spinach, cabbage, onion, tomato, mustard, drumstick, turnip, rose. Sampling procedures were performed as described by Gomes *et al.,* (2001). Briefly, eight composite rhizosphere samples of each crop were taken. Rhizosphere samples were collected which are intact to the roots, transported to the laboratory in sterile plastic bag and processed for isolation of *Pseudomonas* spp. and characterization.

Isolation of *Pseudomonas* spp.

Isolation of *Pseudomonas* spp was made from fresh rhizosphere soil samples of different crops such as Lady's finger, Spinach, Cabbage, Onion, Tomato, Mustard, Drumstick, Turnip, Rose crops from central research field of Naini agricultural Institute, Allahabad. The soil samples which are intact the root system was dug out, carefully taken in plastic bags and labelled well and stored at 4°C (Gomes *et al.,* 2001). *Pseudomonas* species were isolated using dilution method with Nutrient Agar medium and King's Medium B. Rhizosphere soil samples of 1 g were suspended in 9 mL of normal saline water (pH 7.0) and shaken vigorously.

The resulting solution was serially diluted and appropriate dilution (10-4 to 10-6) of this suspension (0.1 mL) was spread plated on Nutrient Agar medium. Cultures were incubated in the bacterial incubator at 37°C±2 for 2 d. For experimental use, isolates were transferred when needed to nutrient agar medium that was stored at 4°C. Each colony was assayed further for morphological and physiological characteristics including Gram reaction, Motility, Oxidase and Catalase enzyme, KOH soluble activity.

Morphological characterization

The *Pseudomonas* spp isolated and streaked for colony development on Kings B media agar plates separately. The individual colonies were examined for shape, size, margin, pigmentation, and structure of colonies (Belkar and Gade 2012).

Biochemical characterization

After phenotypic identification of the isolates they were further proceeded to various classical biochemical tests in order to obtain the accurate identification of isolated bacteria. Biochemical methods (Gram Staining, KOH test, Catalase, Growth in 7%, 14% of NaCl, Gelatin Hydrolysis, Nitrate to Nitrite Reduction, Casein Hydrolysis, Starch Hydrolysis, Growth at 4˚C, 10˚C, 30˚C and 45˚c). Biochemical tests for identification were done following the Bergey's manual of determinative bacteriology (1994). Isolated bacteria were sub cultured on to Kings B medium in 24-36 hrs.

Gram's reaction

This test is essential to differentiate bacteria into gram positive and gram negative bacteria. A loopful of bacterial suspension was smeared on to a glass slide. It is air- dried and heat fixed by passing the slide rapidly two to three times on Bunsen burner. The smear was flooded with crystal violet solution for 1 min. The slide was washed with a gentle stream of tap water blot dried and flooded with lugol's iodine for 1 min. Again the slide was washed with water and blot dried, and decolorized by washing in a gentle stream of 95% ethyl alcohol for 30 sec to remove excess stain that will easily wash away, counter staining was done by flooding with safranin for 20 sec. The slide was again washed with tap water and blot dried. The preparation was observed under compound microscope at different magnifications for pink-red or blue-violet stained bacteria representing gram-negative or gram-positive nature respectively.

Catalase test

In this test 24hrs old slant culture of *Pseudomonas* isolates was flooded individually with one ml of 3% hydrogen peroxide (H_2O_2) and observed the isolate for the production of gas bubbles. The effervescence indicated the positive catalase activity (Clarke *et al.,* 1953).

KOH solubility test

The main principle behind this test is that, the lipopolysaccharides present in the bacterial cell wall gets dissolved in 3% KOH and forms a mucoid thread. A loopful of *Pseudomonas* isolate from a well grown colony was mixed in a drop of 3% aqueous KOH solution for not more than 10 seconds with the help of a toothpick. Tooth pick was raised a few centimetres from the slide and was observed for the formation of a mucoid thread. The gram negative *Pseudomonas* isolates produce strands even on repeated strokes of the toothpick, whereas gram positive bacteria don't produce any mucoid threads (Dhankar *et al.,* 2019).

Starch hydrolysis

Starch is an insoluble polymer of glucose. Some bacteria possess the ability to produce amylase that breaks starch into glucose units. Starch agar was prepared (1g of potato starch is suspended into 10ml of cold distilled water and mixed with 100ml of nutrient agar , Autoclave at 121℃ for 20min. cool to 45℃.), sterilized and dispensed into sterile petri plates. The plates were inoculated with the *Pseudomonas* isolates.

After incubation at 37˚C for three days, the culture plates were flooded with Lugol's Iodine and the plates were observed for the formation of a clear zone around the bacterial growth (Clarke 1953).

Gelatin hydrolysis

This test is used to determine whether an organism can hydrolyze gelatin by the action of gelatinase enzyme. Test tubes containing gelatin medium was stabbed with loop containing *Pseudomonas* isolate is inoculated. Uninoculated control and inoculated tubes were incubated at 37˚C for 2-3 days (Clarke 1953).

Physiological characterization

Rhizobacterial growth at different temperatures

Cultures of *Pseudomonas* isolates are incubated at different temperatures 4˚c, 10˚c, 30˚c & 45˚c The little growth of bacteria was observed at 4˚c and 10˚c and better growth at 30˚c and no growth at 45˚c. All the isolates grew well at 37˚c. Therefore optimum temperature for the growth of all the three isolates was 28˚C - 37˚C (Seleen & Stark 1943).

Effect of different NaCl concentrations on the growth of rhizobacterial isolates

The growth of *Pseudomonas* bacteria was tested under different concentrations of NaCl. The growth of bacterial isolates was observed at 70,140 mM NaCl. Less growth was observed at 70mM NaCl concentration. Concentrations of 140mMNaCl had better growth. (Seleen & Stark 1943)

Characterization of rhizobacteria for PGPR traits

Production of extra-cellular i.e. antifungal metabolites with antagonistic properties Siderophore production

Siderophore production was tested qualitatively using chrome azurol S (CAS)

agar. The *Pseudomonas* culture was spotted on the CAS agar plates with two replications. Yellow-Orange pigment (pyoveridin/ pyocyanin) around the colonies after 1-5 days of incubation indicated siderophore production (Schwyn and Neilands, 1987).

Cyanide production

Cyanide production was determined in petriplates of NA amended with Glycine (4.4 g/l) and FeCl3.6H2O (0.3mM) A strip of sterilized filter paper saturated with a solution containing picric acid, 0.5 % and Sodium carbonate (2.0%) was placed inside the plates which were already inoculated with *Pseudomonas* were sealed air tightly (Bakker and Schippers, 1987) with parafilm and incubated for four days. Hydrogen cyanide (HCN) production was assessed by the presence of a colored zone around the bacteria and the yellow colour of the filter paper turning brown to reddish brown. Reactions were scored as weak yellow to light brown, moderate brown and strong reddish brown. (Castric 1977).

Production of cell wall degrading enzymes Casein hydrolysis

This test is used to determine whether the bacteria can hydrolyze casein by the action of enzyme casein hydrolase . Inoculate plates of milk agar (Suspend Skim milk powder , 5g in 50ml of distilled water ; agar, 1g in 50ml of distilled water . Autoclave separately at 121℃ for 20min, cool to 45℃, mix together and pour into Petri dishes). The plates were streaked with *Pseudomonas* the test isolate and incubated at 37C for 48h. Bacterial cultures were recorded positive if zone of hydrolysis was seen around the colonies**.**

Primarily, all the bacterial isolates are checked for fluorescence indicating for presence of pigmentation under UV light. Based on visual pigmentation by presence of pigment(pyocyanin/ pyoverdin), if there is no visible pigmentation then need to perform motility test.

Then presence of any pigment identifies the isolate as P.aeruginosa and if it is negative then test of growth at 41C should be performed. Later positive motility test subjected to flagella test and if it is a negative for motility test then it is not a pseudomonad. After growth at 41c if it is positive then the isolate is probably P.aeruginosa and if negative then gelatin hydrolysis test need to be done.(Bergey's *manual of Determinative bacteriology*, fourth edition,1934).

Identification of *Pythium* spp

Identification of the fungal pathogen *Pythium* characteristics were done through microscopic examination. Small pieces of tissues about 3mm from infected collar region with some healthy tissue where cut with sterile scalpel. The sample is placed on a glass slide containing lactophenol and chopped into small pieces using a sterile blade. It is stained using cotton blue. Then, the microscope is used for the examination of mo morphology and culture characteristics of fungal structures.

1- Vesicle 2 -Empty sporangium 3–Vegetative hyphae

In vitro screening for antagonistic activity

The pseudomonas isolates were screened for antifungal activity which was checked against *Pythium* through dual-culture technique (Jones 2008). The *Pseudomonas* isolates were initially streaked on the periphery of the

Potato dextrose agar plates as a single line and fresh mycelia of phytopathogenic fungi *Pythium* spp. of 5mm disc were spot inoculated on the opposite sides of the streak line of the isolate.

All the isolates were surveyed on the individual fungi. The plates were then incubated at 30°C for 3–5 days and were observed for the inhibition of the fungal mycelium. Plates streaked with sterilized water in place of bacterial isolates were kept as control. (Ramanathan *et al.*, 2002).

Antagonists were tested for the production of extra cellular secondary metabolites. The clear zones in skimmed milk agar were measured and used as indicators of protease activity/casein degradation displays the antagonism (Nielsen and Sorensen, 1997).

Inhibition percent of growth was calculated using the following equation.

$$Inhibition(\%) = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

Results and Discussion

Phenotypic identification of fluorescent *Pseudomonas* spp.

A Total of six isolates of *Pseudomonas* spp were isolated with small to medium, smooth, glistening colonies, convex elevation and these isolates were gram negative, rods without sporulation when observed under microscope in table No.1 Out of the total six isolates two showed yellowish pigmentation and two showed orange pigmentation and remaining were off white to cream pigmentation.

Biochemical characterization of *Pseudomonas* spp

All the isolates of *Pseudomonas* spp were negative for Starch and Casein hydrolysis and all are positive for catalase test, KOH test and HCN production. Whereas four isolates were positive for Gelatin test and two were negative. And for NaCl @14% four isolates were positive and remaining two isolates were negative to the test. For the test Growth at 41°C only two isolates were positive and remaining were negative to the test.

Table.1 Characters of pseudomonas isolates

Characters	Pf1	Pf2	Pa3	Pa4	Pp	Pp6
Fluorescent pigment	+ve	+ve	+ve	+ve	+ve	+ve
Growth at 41c	-ve	-ve	-ve	-ve	+ve	+ve
Gelatin hydrolysis	+ve	+ve	+ve	+ve	-ve	-ve
Nacl @ 7% and 14%	+ve	+ve	+ve	+ve	-ve	-ve
Caesin hydrolysis	-ve	-ve	-ve	-ve	-ve	-ve
KOH Test	+ve	+ve	+ve	+ve	+ve	+ve
HCN Production	+ve	+ve	+ve	+ve	+ve	+ve
Starch hydrolysis	-ve	-ve	-ve	-ve	-ve	-ve

Table.2 Cultural and morphological characteristics of *Pseudomonas* spp isolates on kings B medium and NA medium

S. N 0	Isolates	Size	Margin	Colour	Elevation	Surface	Pigmentation	Gram reaction	Shape	Sporulation
1	Pl1	Small	Round	Off white	Convex	Smooth shiny	Light yellow	Negative	Rods	Negative
2	Ps2	Medium	Irregular	Off white	Convex	Smooth shiny	Light yellow	Negative	Rods	Negative
3	Pc3	Medium	Irregular	Yellow	Convex	Smooth shiny	Dark yellow	Negative	Rods	Negative
4	Pt4	Small	Round	Yellow	Convex	Rough	Dark yellow	Negative	Rods	Negative
5	Pm5	Medium	Irregular	Light orange	Convex	Smooth shiny	Dull yellow	Negative	Rods	Negative
6	Pd6	Small	Round	Orange	Convex	Smooth shiny	Dull yellow	Negative	Rods	Negative

Table.3 Biochemical characterization of *Pseudomonas* spp isolates from different rhizospheric crop soils

S.No	Isolate	Catalase	Gelatin Liquefaction	Starch Hydrolysis	KOH test	Casein hydrolysis	Nacl @ 14%	Growth at 41c	HCN production
1	Pl1	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
2	Ps2	+ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve
3	Pc3	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve
4	Pt4	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve
5	Pm5	+ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve
6	Pd6	+ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve

Table.4 Growth promoting characteristics of rhizobacteria

S.No	Test	Pl1	Ps2	Pc3	Pt4	Pm5	Pd6
1	HCN Production	+ve	+ve	+ve	+ve	+ve	+ve
2	Siderophore	+ve	+ve	+ve	+ve	+ve	+ve

Table.5 Effect of isolates of *Pseudomonas* spp on *In vitro* radial growth of *P. aphanidermatum*

S.No	Isolates	Growth after 24hrs		Growth after 48hrs		Growth after 72hrs	
		Radial growth of *Pythium* (mm)	Percent reduction over control(mm)	Radial growth of *Pythium* (mm)	Percent reduction over control(mm)	Radial growth of *Pythium* (mm)	**Percent reduction over control(mm)**
T1	PL1	8.64	43.85	14.32	57.57	26.18	**50.82**
T2	PS2	9.04	41.26	14.09	58.25	27.62	**48.12**
T3	PC3	9.48	38.92	14.91	55.52	27.66	**48.04**
T4	PT4	9.40	38.40	15.01	55.82	28.29	**46.86**
T5	PM5	10.05	34.69	15.13	55.17	28.85	**45.81**
T6	PP6	10.33	32.87	15.29	54.69	29.14	**45.26**
T7	Control	15.39		33.75		53.24	
F(test)		Sig.		Sig.		Sig.	
S.E(m)		0.341		0.27		0.349	
C.d (P=0.05%)		**1.044**		**0.854**		**1.914**	

Table.6 Effect of Pseudomonas isolates on Percent plant height of chilli at different days after transplanting in field conditions

S.No.	Treatments	25DAT (%)	55DAT(%)
T1	**PL1**	**23.02**	**34.46**
T2	**PS2**	**22.78**	**33.64**
T3	**PC3**	**22.38**	**33.26**
T4	**PT4**	**22.76**	**33.84**
T5	**PM5**	**22.40**	**32.92**
T6	**PD6**	**22.24**	**32.68**
T0	**P0**	**17.08**	**30.20**
	F(test)	**18.411**	**3.705**
	S.E(m)	**0.490**	**0.433**
	C.d (P=0.05%)	**1.439**	**1.271**

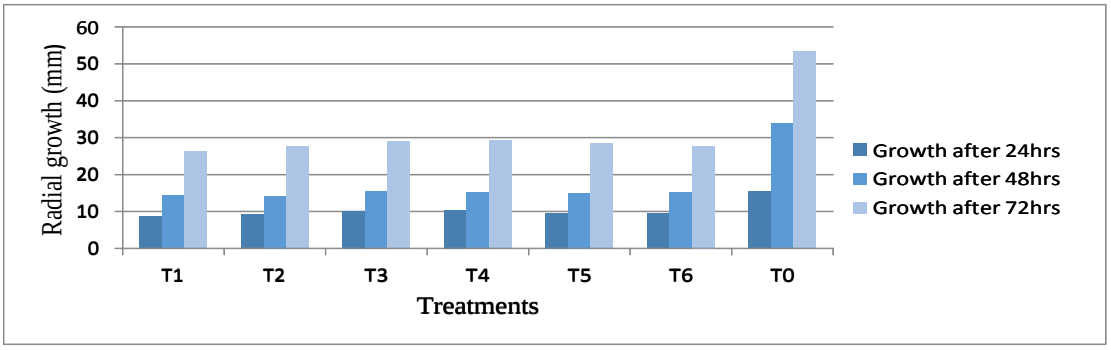

Fig 3.1 Effect of isolates of *Pseudomonas* spp on *In vitro radial* growth of *Pythium (mm)*.

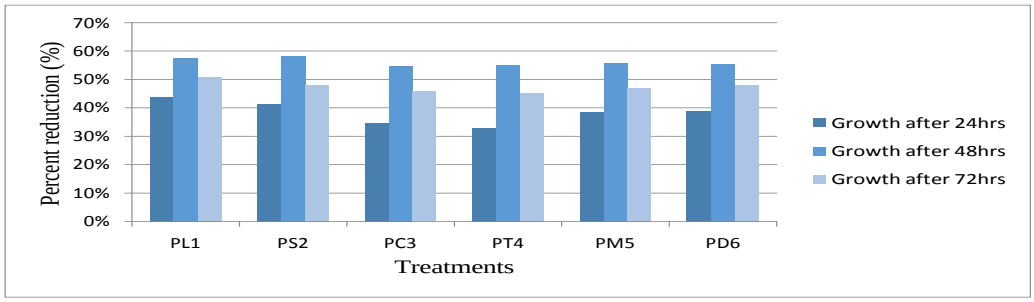

Fig 3.2 Effect of isolates of *Pseudomonas* spp on *invitro* percent reduction over control.

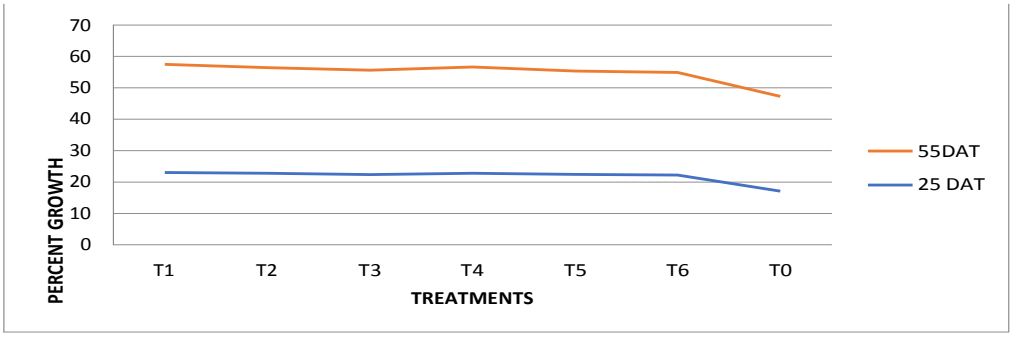

Fig 3.3 Effect of *Pseudomonas* isolates on increasing plant height of chilli over control at different days after transplanting in field conditions.

Plate.1 Isolate plates of *Pseudomonas* spp

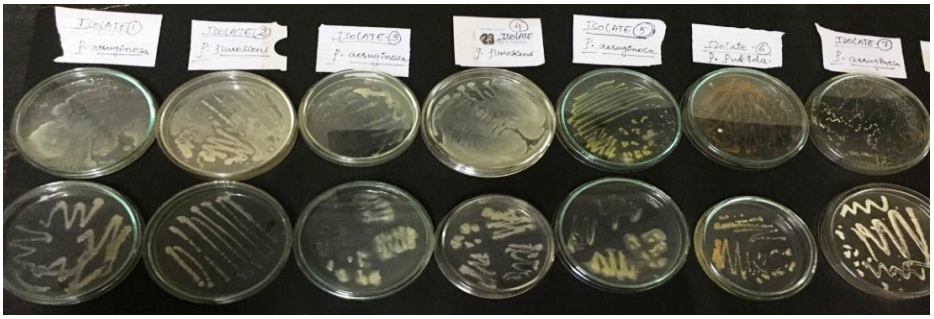

Plate.2 Microscopic view of the culture sample on glass slide

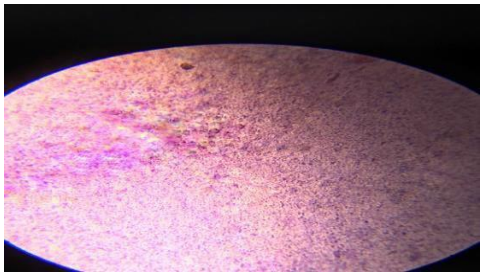

Plate.3 Bubbles observed on the glass slide for Catalase test

Plate.4 Observation of mucoid thread for KOH Solubility test

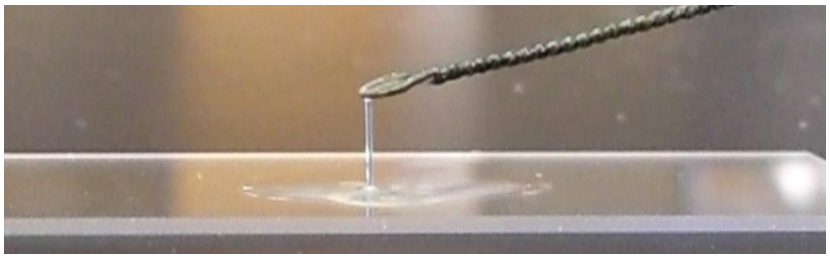

Plate.5 Test tubes of the test gelatine hydrolysis

Plate.6 Test plates for HCN production

Plate.7 *Pseudomonas* spp isolates

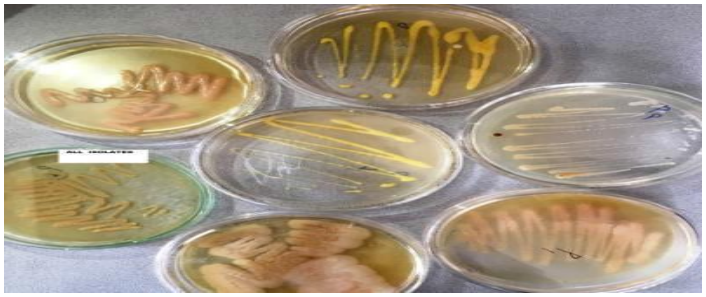

Plate.8 *Pythium aphanidermatum* pure culture plates

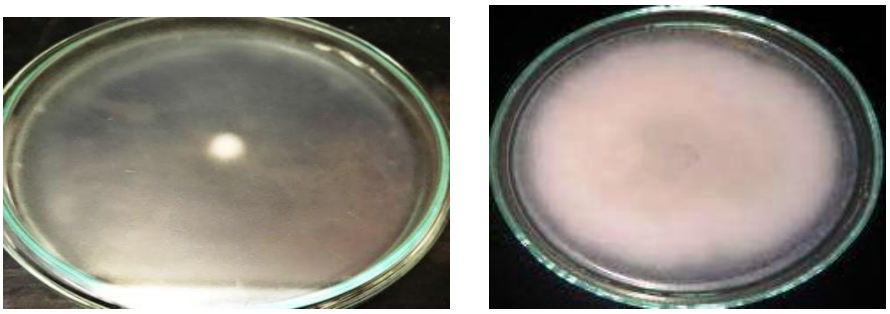

Plate.9 Field layout at one week of transplantation and after 55 days of transplanting

Antagonistic activity of *Pseudomonas* spp against *Pythium* sp by dual culture inhibition assays

The method used to evaluate the antagonistic potential of the six *Pseudomonas* spp. which able to inhibit fungal growth on agar plates. Observations are taken on the basis on Days after incubation (DAI) were at 24hrs, 48 hrs, 72 hrs of the bacteria over growth of *Pythium aphanidermatum.* The data indicated that all the isolates are effective on the mycelial growth of the *Pythium* except in the control.

From this present investigation it is concluded that all *Pseudomonas* isolates performed better but the treatment with PL1 was superiorly significant among all the treatments respect to the fungal antibiosis and on the growth parameters of chilli. Moreover all the isolates have shown the growth promoting characteristics on the chilli seedlings in the field trials with vigorous growth and height of the plants with height of

35.6cm after 55 days of transplanting Thus *Pseudomonas* spp *play* an important role in controlling soil borne fungal pathogen which can be used as bio control agent rather than a chemical fungicide which are environmentally hazardous.

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