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Isolation of *Pseudomonas* Species from Rhizospheric Soil and its Antagonistic Effect on Plant Pathogen

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Abstract: ***The pathogen attack on plants (such as tubers and small plants) has substantially progressed by 60% irrespective of even when the plants have strong immune system for the past five decades. It drastically affects the plant growth, yield and production. Certain pesticides and fertilizers have been tried to control the progressing pathogenic microorganisms. Although these measures have proved worthless due to the resistance shown by those pathogens and resulted in environmental pollution over a period of time. Bio control measures are well appreciated as it is ecofriendly. Antagonist microorganism (bacteria, fungi) will be used to suppress the growth of the invading pathogens. In this study, Pseudomonas species , were screened from rhisozpheric soill from the local region of Srivilliputhur. Eleven isolates from rhizospheric soil were screened and characterized by Gram staining, catalase test, Voges-Proskauer test, and oxidase test. The morphological studies of all the screened Pseudomonas isolates were observed as Gram negative. Further, all the isolates were found to be catalase positive and negative for VP test. The isolates were subjected to antagonistic effect on plant fungal pathogen such as Rhizoctonia solani. Among eleven Pseudomonas isolates four isolates showed antagonistic effect against R.solani. In the present investigation, four isolates showed potent antagonistic effect and could be used as effective bio-control agent against plant fungal pathogen R.solani.***

Keywords: Pseudomonas, Rhizosphere, Serial dilution, Biochemical characterization, Antagonistic activity, Rhizoctonia solani, Dual culture method

I. INTRODUCTION

Losses in crop yields due to disease need to be reduced in order to meet increasing global food demands associated with growth in the human population. There is a well-recognized need to develop new environmentally friendly control strategies to combat bacterial crop disease. Current control measures involving the use of traditional chemicals or antibiotics are losing their efficacy due to the natural development of bacterial resistance to these agents. The overuse of chemical pesticides to cure or prevent plant diseases has caused soil pollution and had harmful effects to the microbiome of soil [1, 2 and 3].

Accordingly, to reduce the use of these chemicals, one possibility is to utilize the activity of microorganisms. It is desirable to replace chemical pesticides with materials that possess the following three criteria such as high specificity against the targeted plant pathogens, easy degradability after effective usage and low cost of mass production

Products produced biologically or the microbial cells themselves are called biological control (bio-control) agents or biological pesticides if they fulfill these criteria. Biological control using introduced microorganisms with the capacity to elicit induced systemic resistance (ISR) against plant diseases. It is ideal if the microorganisms that produce such products stably inhabit the environment as non-dominant species but maintain their effectiveness. Under these circumstances, biocontrol of some soil- borne diseases has been attracting attention recently.As most of the soil-borne plant pathogens are fungi, bio-control by fungi has been attempted intensively. The use of bacteria has also been investigated mainly because genetic and biochemical analyses and the mass production of bacteria or bacterial products are much easier than those of fungi, and thus the advance of bacterial control is expected to have great potential. As bacterial control agents, *Agrobacterium, Pseudomonas, Bacillus, Alcaligenes, Streptomyces,* and others have been reported. Different mechanisms are involved in the actions of these microorganisms against plant pathogens, such as parasitism, cross protection, antibiosis, and competition[4, 5 and 6].

Pseudomonas strains are one of the most active and dominant bacteria in the rhizosphere and have been intensively investigated as bio-control agents. Among them, *P. fluorescens*, *P. putida* and *P. cepacia* were the predominant focus of research for practical applications. The strains of Pseudomonas produce several kinds of antibiotics, such as pyrrolnitrin, pyoluteorin and phenezine-1-carboxylate, all of which are closely related to the suppression of plant diseases. *Pseudomonas fluorescens* encompasses a group of common, non-pathogenic saprophytes that colonize soil, water and plant surface environments Pseudomonas fluorescens has simple nutritional requirements and grows well in mineral salts media supplemented with any of a large number of carbon sources. Because they are well adapted in soil, *P. fluorescens* strains are being investigated extensively for use in applications that require the release and survival of bacteria in the soil.

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Chief among these are biocontrol of pathogens in agriculture and bioremediation of various organic compounds. Certain members of the *P. fluorescens* have been shown to be potential agents for the bio control which suppress plant diseases by protecting the seeds and roots from fungal infection. They are known to enhance plant growth promotion and reduce severity of many fungal diseases. [7, 8 9 and 10]The secondary metabolites from the *Pseudomonas sp.* are reported that they are potential antagonist against various fungal diseases. The root knot diseases caused by the fungal organisms are inhibited by the *Pseudomonas sp.* in rhizosphere of the soil. They are producing various types of phenolic compounds to make inhibitory action against the fungal diseases. These compounds are said to be anti-fungal compounds. Examples of anti-fungal compounds produced by the *Pseudomonas sp.* are pyrrolnitrin, 2,4-diacetyl phloroglucinol, and phenazine. Hence, the *in-vivo* test of *Pseudomonas* against any disease causing fungal would done in laboratory will find a way to the effective extraction method of these antifungal compounds. The current study was aimed at isolation of *Pseudomonas* species from rhizosphere of the soil, characterization of *Pseudomonas* species by various biochemical tests and In-vitro evaluation of antagonistic activity against plant pathogen [11 to 18].

II. MATERIALS AND METHODS

A. Isolation of Pseudomonas Species from Soil

Serial Dilution was performed with the 1gm of soil collected sample which was taken from the rhizosphere. The Soil was diluted at the rate of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} in separate test tubes. The test tubes containing the dilution rate of 10^{-5}, 10^{-6} and 10^{-7} was taken for spread plate culture. The LB agar is used as a medium for spread plate culture. The Modified King's B Media was used to isolate the *pseudomonas sp.* from the spread plate. The Modified King's B Media have the composition of 2g/100ml of peptone, 0.15g/100ml of dihydrogen potassium phosphate, 0.15g/100ml sodium dihydrogen phosphate, 0.15g/100ml of magnesium sulphate and 2g/100ml of agar-agar.

B. Biochemical Characterization

1) *Gram Staining:* The King's B Broth was prepared and the twelve colonies was inoculated in twelve different test tubes. Smear was applied on the slides by using inoculation loop from the overnight culture of Kings B Broth. Gram Staining was performed by using Gram Staining Kit. Results were observed under Light Microscope (100X).
2) *Catalase Test:* Few drops from the broth containing overnight culture were dropped on the slides. Two Drops of Hydrogen Peroxide is poured on the slide. The breakdown of hydrogen peroxide H_2O_2 into oxygen and it leads to the production of oxygen bubbles.
3) *Methyl Red Test:* The MR-VP broth was prepared and pure culture of bacteria was streaked. After incubation of 24 hours, few drops of methyl red were added. The sudden change of deep red color will be a positive.
4) *Voges Proskauer Test:* The MR-VP broth was prepared and pure culture of bacteria was streaked. After incubation of 24 hours, few drops of Baritt's reagent was added. The Change in red color of the medium within 15 minutes is said to be positive results in Voges Proskauer test.
5) *Indole Test:* The test tubes containing 9ml of distilled water is mixed with 1ml of overnight broth of bacterial culture. Then it was added with 0.5ml of Kovac's Reagent. Formation of a red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent leads to the bacteria is an indole positive and no color change even after the addition of appropriate reagent is said to be indole negative bacteria.
6) *Oxidase Test:* The Overnight Broth of bacterial culture was dropped on the oxidase discs. The change in deep blue color within 10 to 15 secs was noticed for confirming the presence of oxidase producing bacteria.

C. Antagonistic activity against fungi - Dual culture method

The Potato Dextrose Agar (PDA) medium was prepared. The Overnight Culture of *Pseudomonas* species from 3,5,8 and 11 (colonies) was selected and inoculated in one side of the four plates by simple streaking. The other side of the four plates was inoculated with the fungi *Rhizoctonia solani*

III. RESULT AND DISCUSSION

Among twelve isolates, the isolates 1,3,4,5,6,7,9,11 observed to be rod shaped and the isolates 2,8,10,12 was observed to be round shaped. Due to the thin layer of peptidoglycan of the cell results in pink color. From the results, it was confirmed that the bacteria were gram negative. Interestingly all the isolates were found to be catalase positive because all the cultures produced bubbles. It is due to the production of catalase enzyme in which they react with hydrogen peroxide to evolve oxygen bubbles. It confirms that the bacteria is an catalase positive bacteria.

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The results showed that, there was no color change in the medium and the reagents was settled on the top because there was no mixed acid fermentation occurs there. Those observation proved the negative result in Methyl Red Test.

Further , there was no color change in the medium and the reagents was settled on the top. It is due to there is acetyl methyl carbinol produced which is the one of the products of fermentation of glucose, in presence of α-Naphthol, the acetyl methyl carbinol is converted into diacetyl which results in formation of red color. Those observation proved the negative result in Voges Proskauer Test.

From the obtained results, it was observed that theisolates except 2 and 3 was found to be indole negative bacteria. It was due to the bacteria lacks the ability to degrade the tryptophan to from indole.

The isolates 3,5,8,11 shows sudden change in deep blue color within 10 seconds whereas other culture changed their color within 30 secs. It proofs that culture 3,5,8,11 are oxidase positive bacteria. It is because of in a positive reaction the enzyme cytochrome oxidase combines with N,N-dimethyl-p-phenylenediamine oxalate and a-naphthol to form the dye, indophenol blue.

The *Pseudomonas* isolate 3, 5, 8 and 11 showed the zone of inhibition, and it was proved that *Pseudomonas i*solate 8 showd intense antagonistic activity compared with remaining cultures. The zone of inhibition is may be due to the metabolite 2,4-diacetyl phloroglucinol because the *Pseudomonas fluroscens* resulted in antifungal activity against several fungi with this molecule. It is also reported that the several antibiotic compounds from different strains of *Pseudomonas fluorescens* like pyrollnitrin and phenazine-1-carboxylic acid which supresses the fungal growth. Those metabolite compounds from the *Pseudomonas* are suspectible for the Zone of inhibition. For inhibtion of the growth of *Rhizoctonia solani*, it was reported that pyrollnitrin and phenazine are the potential compounds, the secondary metabolites produced by *Pseudomonas sp.* in the presence of glucose in growth media [6, 7, 8, 14 and 18]

Table No 1: Biochemical Characterization of soil isolates

Biochemical Characteristics	Results (for 12 isolates)
Gram Staining	Negative
Oxidase Test	Positive (isolates 3,5,8,11) Negative (isolates 1,2,4,6,7,9,10,12)
Catalase Test	Positive
Methyl Red Test	Negative
Voges Proskauer Test	Negative
Indole Test	Positive (2,3) Negative (1,4,6,7,8,9,10,11,12)

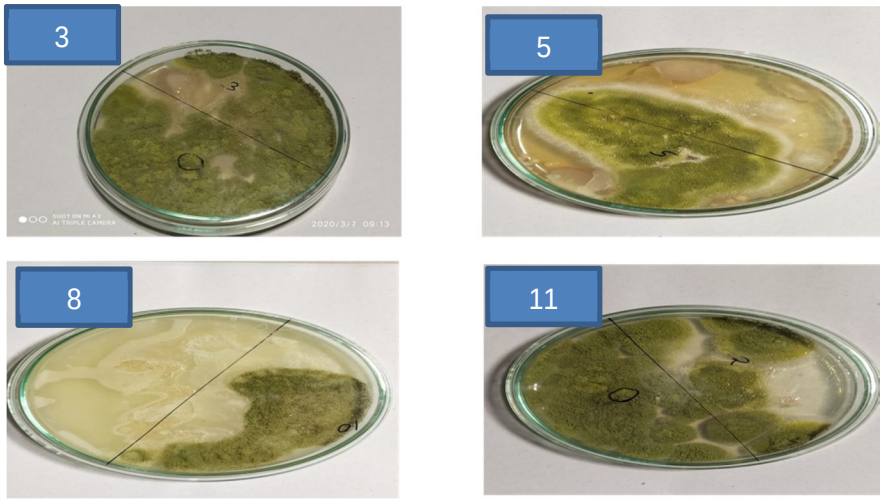


Figure 1. Antagonistic activity of *Pseudomonas* isolates against fungi (isolaes 3, 5 8 and 11)

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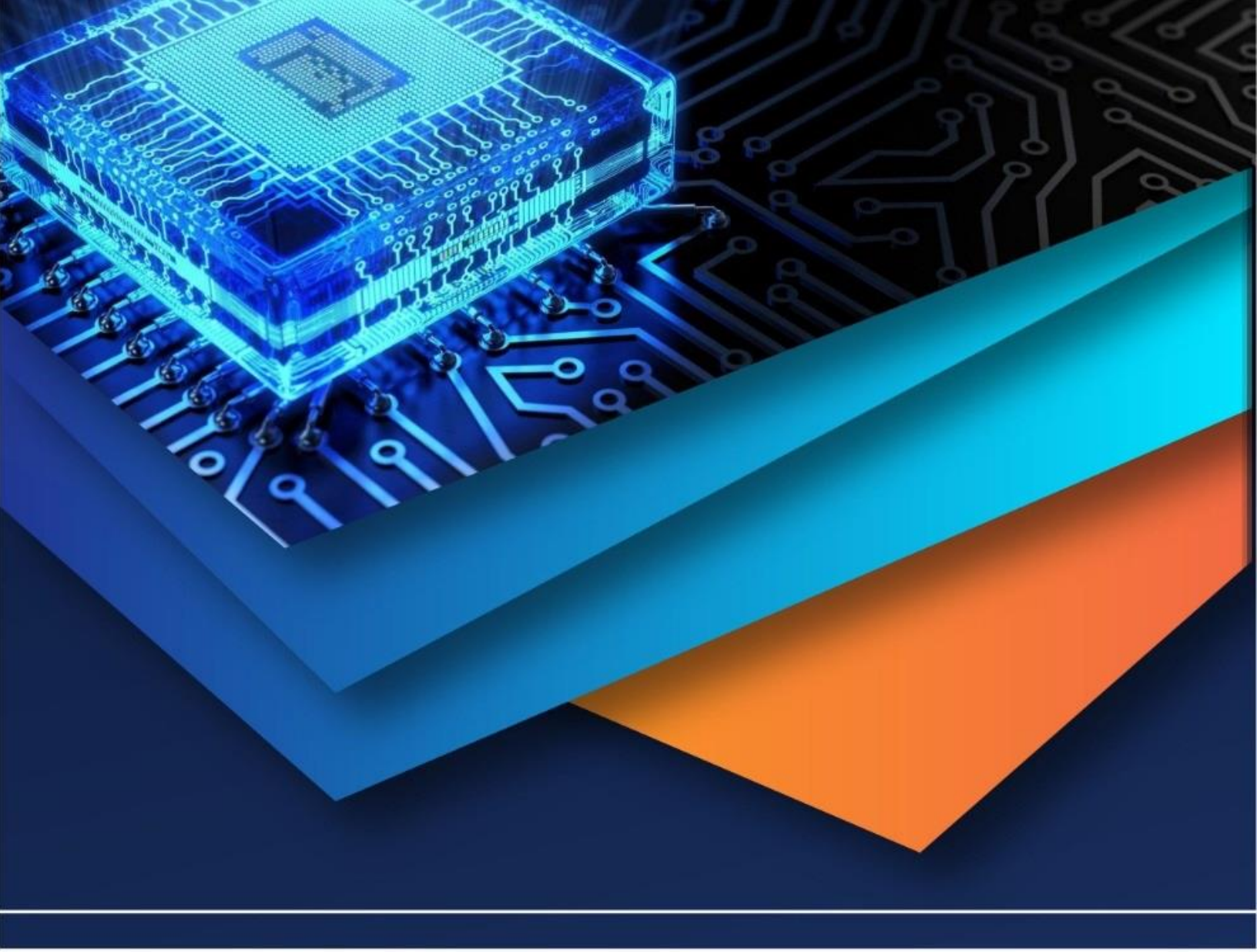
IV . CONCLUSION

The efficacy of bacterial antagonism in controlling diseases was often better than with chemical fertilizers and pesticides. The rhizosphere of the soil is enriched with useful microorganism, while using biocontrol agents as bacterial antagonist it will improve the quality of soil and it is ecofriendly. The protection of agricultural and horticultural crops against pathogens have to be controlled by biocontrol agents is an alternative way to protect, in which they are environment friendly.

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