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Contribution of phosphate solubilizing activity to plants by *Pseudomonas* sp. having antifungal activity

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Abstract

The application of chemical fungicides has caused health hazards in animals and humans due to their residual toxicity. The objective of this research work is to find antagonistic bacteria having plant growth promoting activity for the control of some plant pathogenic fungi. Isolation of antagonistic bacteria from rhizosphere of rice, colonial and microscopic morphology and biochemical characterization were done. For antagonistic activity, dual culture and well diffusion method were employed. P-solubilizing activity was detected by plate screening and Vogel method. Isolated bacteria were assumed as Pseudomonas sp. according to its characteristic. This bacteria exhibited antagonistic activity against eight plant pathogenic fungi. The highest activity gave against fungi infected on green gram (26 mm) by dual culture method and against Fusarium sp. in giving 21 mm of inhibition zone by well diffusion method. 264.20 ppm and 235.77 ppm of soluble phosphate was detected by this isolate. Isolated bacteria exhibited antagonistic activity against eight plant pathogenic fungi by dual culture and well diffusion methods. Its activity was higher on PDA media than on nutrient media. In addition, isolated bacteria also possess P-solubilizing activity.

Keywords: Antagonistic activity, *Pseudomonas* sp., Fungi, Dual culture method, Well-diffusion method, P-solubilizing.

Introduction

Background: Soil borne pathogens are complex not only in their behavioral pattern but also in their biochemical constituents. Hence, it is not very easy to control these pathogens. These pathogens are *Fusarium sp., Rhizotonia sp., Pythium sp., Alternaria solani, Curvularialunata, Bipolaris sp., Helminthosporium sp*., and *Penicillium digitatium* etc. The pathogen, *Penicillium digitatium,* destroyed crops (citrus fruit) up to 50% of the total production[1]. To increase crop yields, it is necessary to apply agrochemicals, which has several negative side effects[2]. At present, with an effective management of plant diseases and microbial contamination in several agricultural commodities is generally achieved by the use of synthetic fungicides.

The application of these chemical fungicides has caused health hazards in animals and humans due to their residual toxicity. Long term uses of these chemical fungicides has resulted in the accumulation of toxic compounds potentially in soil and has harmful effect to humans and environment also in the buildup of resistance of pathogens[3]. In addition, repeated use of certain systemic fungicides has led to the appearance of fungicides-resistance population such as *Penicillium digitatum* infected in citrus fruit[4,5]. In order to tackle these national and global problems, alternative of chemical control are investigated by the use of antagonistic microbes[6]. The use of plant growth-promoting bacteria (PGPB) with antifungal properties is an attractive alternative to the use of such xenobiotic compounds[7].

Biocontrol, or the use of microorganisms, or their secretion to prevent plant diseases, is eco-friendly, normally safe, may provide long term protection to the crops[8]. Plant-beneficial bacteria isolated from soil, rhizosphere or from within the plant are increasingly being used to improve plant productivity. Plant growth and yield can also be improved by organisms which control soil-borne plant pathogens. Disease control can occur as a result of pathogen inhibition or through the initiation of plant defense responds. It is important to consider the role of the whole community of soil and plant-associated micro-biota both in disease control and plant growth stimulation[9,10]. Biological control by using antagonistic bacteria instead of chemical pesticides to suppress crop disease, offer a powerful contribution to environmental conservation. In recent year, efforts to control plant diseases with antagonistic bacterial agents have been made successfully and some commercial strains of bacteria have been marketed as biocontrol agent for fungal diseases of crops[2]. Srivasta and Shalini[11] studied antifungal activity of *P. fluorescens* by suppressing upon *Alternaria solani, Curvularia lunata, Fusarium* spp*., Bipolaris* spp. and *Helminthosporium* spp.

The mechanisms resulting in biocontrol are competition for substrates[12], ability to colonize the niche favored by the pathogens, antagonism by antibiotics, antibiosis[13] and action of cell wall degrading enzymes[14]. Another potential mode of action may lie with the production of antifungal metabolites[15]. Species belonging to *Pseudomonas* and *Bacillus* are frequently used as biocontrol agents, since they excrete hydrolytic enzymes able to

degrade cell walls[16], iron-chelating siderophores, and several cyclic lipodepsipeptides (LDP)[17], and due to their nutrition or site competition.

Bacillus strains produce important antibiotics (e.g.iturin, surfactin, fengycin) that are useful for plant disease control, enzymes that degrade fungal structural polymers (e.g. chitinase, beta-1,3 glucanse), and antifungal volatiles[15].

Pseudomonas sp. also excrete a great variety of antibiotics such as 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin, pyrrolnitrin and hydrogen cyanide[18]. Some *Pseudomonas sp.* have been recognized as antagonists of plant fungal pathogens and antibiotic producers. This is probably due to the abundance of this diverge group of bacteria and their obvious importance in the soil. In some situations, volatile organic compounds (VOCs) secreted by some antagonistic bacteria have been associated with increased plant growth and the induction of plant systemic resistance mechanisms[7].

Aim of the research: The aim of this research work is to obtain effective plant growth promoting rhizobacteria (PGPR) for antagonistic activity against some pathogenic fungi in my country.

Materials and methods

Isolation of bacteria from collected soil samples: Soil samples were collected from different places of rhizosphere of rice. One gram of soil was mixed in the test tube containing 10 ml autoclaved normal saline in a test tube. The suspension was shaken and stands for 1 hour. After 1hour standing, a loopful of the supernatant was spread on King's B medium and the plates were incubated at 30ºC until colony development was observed.

Study on phenotypic characteristics: The phenotypic characteristics of isolated strain were studied and characterized such as colonial and microscopic morphology, some biochemical characteristics and antibiotic sensitivity patterns.

Screening on antifungal activity of isolated bacteria: Antifungal activity was screened by dual culture method in which one loopful of pathogenic fungi were inoculated in Potato Dextrose Agar (PDA) broth medium and incubated in water batch shaker at 27ºC for two days. After two days incubation, the growth of fungi in broth medium was visually checked and the PDA medium containing the spores of test fungi was spread on PDA and nutrient agar medium. Then, test bacterial culture from King's B was cultured on the same media and incubated at 30ºC for three days. After three days incubation, the growth inhibition of pathogenic fungi by isolated bacteria was recorded.

Influence of different broth media on the antifungal activity of A-19 isolate: A-19 strain was inoculated in four different broth media such as nutrient broth (NB), peptone broth (PB), King's B broth (KB), and Pikovskaia's broth media in water batch shaker at 30ºC.

One loopful of test fungi on PDA media was spread over the agar surface of PDA medium. Then, 7 mm diameters of four wells were made with the help of a sterilized core borer. Bacterial supernatant from four different broth media (50µl), was prepared by centrifuging the bacterial broth at 6000 rpm for 30 minutes and poured into the wells. The plates were incubated at 30ºC for two weeks. After three days incubation, the hyphal growth inhibition was initially recorded until two weeks.

Study on antifungal activity of A-19 isolate on PDA medium by agar well diffusion method: A-19 strain was inoculated in 10 ml King's B broth and incubated at 30°C for two days. And, pathogenic fungi was also inoculated in PDA broth medium and incubated in water batch shaker at 27°C for two days.

After incubation, the spores of fungus was swabbed on PDA media and made a well (7 mm diameter) on the surface of the media. Then, bacterial supernatant (50µl), was prepared by centrifuging at 6000 rpm for 30 minutes, poured into the well and the plates were incubated at 30° C for three days. After incubation, the hyphal growth inhibition zone diameter of the strain against pathogenic fungi was recorded.

Study on P-solubilizing activity: Plate screening method: Selected bacterial strain was tested on both solid and liquid media for their phosphate solubilizing activity. Cultures were spotted separately on Pikovskaia's media (PVK) and National Botanical Research Institute Phosphate's medium (NBRIP) media supplemented with Bromothymol Blue (BTB). Tricalcium phosphate was used as phosphate source. After 5 days of incubation at 30˚C, the halo zone around the colony was used to assess the potential of PSB.

Quantitative determination of P solubilizing activities of bacterial isolates: Bacterial isolates were further evaluated for their P solubilizing ability in PVK liquid medium. Phosphate solubilization in liquid media was quantified in a flask (100ml) and incubated at 30˚C and 150rpm on a rotary shaker. Uninoculated medium served as the control. P solubilizing activities of A-19 strain was measured by spectrophotometric method. Sodium molybdate solution and hydrazine sulphate solution were used to form blue color complex and measured at 830nm by Vogal method[19].

The intensity of the blue color is proportional to the amount of phosphate initially corporate in the heteropoly acid. Total P accumulation in cultures of bacterial isolate grown in insoluble mineral phosphate ($Ca_3(PO_4)_2$) was determined[19].

Results and discussion

Isolation of bacteria from collected soil samples: Twenty-four bacterial strains were isolated from different soil sources on King's B medium at 30ºC for 24 hrs incubation and their antifungal activity were screened on PDA medium and nutrient medium at 30˚C for three days.

Study on phenotypic characteristics of A-19 isolate: Some phenotypic characteristics of selected strain were studied. According to colonial and microscopic morphology, and biochemical characteristics, A-19 isolate may be *Pseudomonas* species. Its some phenotypic characteristics were showed in Table-1 and Figure-1.

Influence of different broth media on the antifungal activity of A-19: Among four different broth media, the antifungal activity of A-19 strain gave the best result with King's B media. The antifungal activity of the strain with four different broth media was shown in Table-2.

Table-1: Some phenotypic characteristics of A-19 isolate.

Colonial morphology		Some biochemical characteristics		Antibiotic sensitivity	
King's B	0.5-1 mm in diameter, non-mucoid, pale yellow color	Motility	+	Ampicillin	Sensitive
		Catalase	+		
		Citrate utilization	+	Penicillin G	Resistant
Nutrient	0.5-1mm in diameter, non-mucoid, pale yellow color	Nitrate reduction	+		
		Starch hydrolysis	+	Kanamycin	Sensitive
Pikovaskaia's	1-1.5 mm in diameter, non-mucoid, yellow color	Gelatin agar	+		
		Indole test	-	Chloramphe-nicol	Sensitive
		Urease	+		
NBRIP	1-1.5 mm in diameter, non-mucoid, creamy white color	Methyl red	+	Tetracycline	Sensitive
		Voges-proskauer	-		

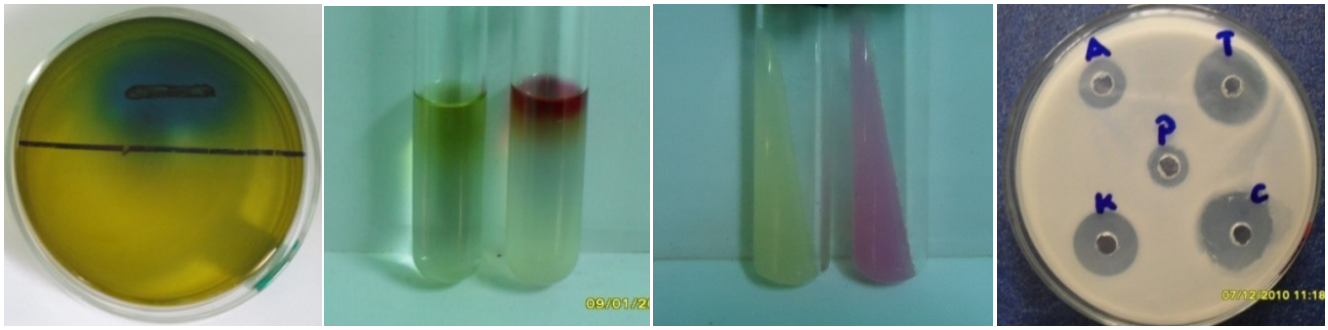

Figure-1: Some phonotypic characteristics such as citrate utilization, methyl red, urease and antibiotic sensitivity tests.

Table-2: Influence of different broth media on antifungal activity of A-19 isolate.

Test Media	Inhibition zone diameter (mm)		
	Against *Fusarium oxysporum*	against *Rhizotonia solani*	against *Pythium* sp.
King's B	21	17	11
Peptone	13	14	11
Nutrient	15	14	ND
Pikovaskia's	ND	ND	ND

ND=Not Detected.

Screening on antifungal activity of A-19 isolate by dual culture method and well-diffusion method: Antifungal activity of isolated strains was screened on PDA and nutrient medium. Among all isolates, one strain (A-19) gave higher antagonistic activity against eight plant pathogenic fungi than other strains. Inhibition zone diameters of A-19 strain were shown in Table-3 and Figure-2. Its activity was slightly higher on PDA media than on nutrient media by observing inhibition zone diameter.

Table-3: Inhibition zone diameters of A-19 against eight plant pathogenic fungi on potato dextrose agar (PDA) and nutrient media.

Test pathogenic fungi	Inhibition zone diameter (mm) on PDA	Inhibition zone diameter (mm) on nutrient medium
Fusarium oxysporum	20	8
Rhizotonia solani	14	13
Pythium sp.	10	5
Fungi infected on onion	20	13
Fungi infected on green gram	26	25
Fungi infected on chili	21	19
Fungi infected on cotton	24	10
Fungi infected on tomato	25	14

After selecting the best media for antifungal activity, A-19 isolate was studied its antifungal activity by agar well-diffusion method. The widest inhibition zone diameter of A-19 was observed against *Fusarium oxysporum.* Inhibition zone diameter of A-19 by agar well-diffusion method was shown in Table-4 and Figure-3.

Visual observation and inhibition zone diameter from both methods showed that isolated *Pseudomonas* sp. belonging to antagonistic activity.

Table-4: Antifungal activity of A-19 against eight plant pathogenic fungi by agar well-diffusion method after three days incubation.

Test Pathogenic Fungi	Inhibition zone diameters (mm)
Fusarium oxysporum	21
Rhizotonia solani	13
Pythium sp.	13
Fungi infected on onion	18
Fungi infected on green gram	16
Fungi infected on chili	13
Fungi infected on cotton	13
Fungi infected on tomato	16

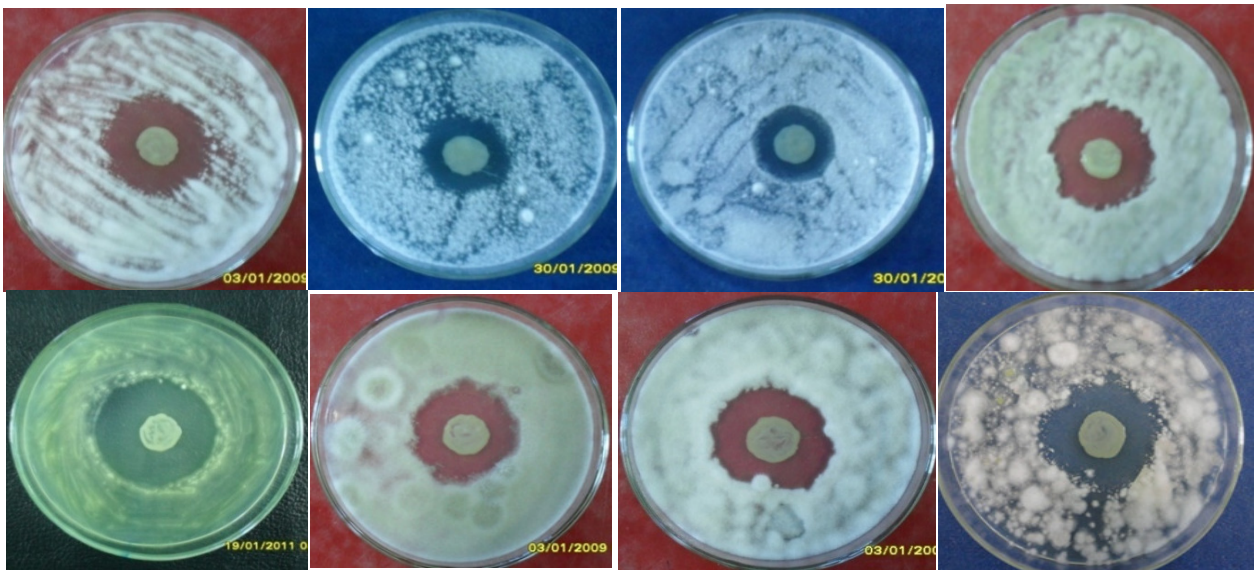

Figure-2: Antifungal activity of A-19 on PDA media against eight plant pathogenic fungi by dual culture method after three days incubation, (a) *Fusarium oxysporum* (b) *Rhizoctonia solani* (c) *Pythium* sp. (d,e,f,g and h) fungi infected from leaves of onion ,green gram, chilli, cotton and tomato respectively.

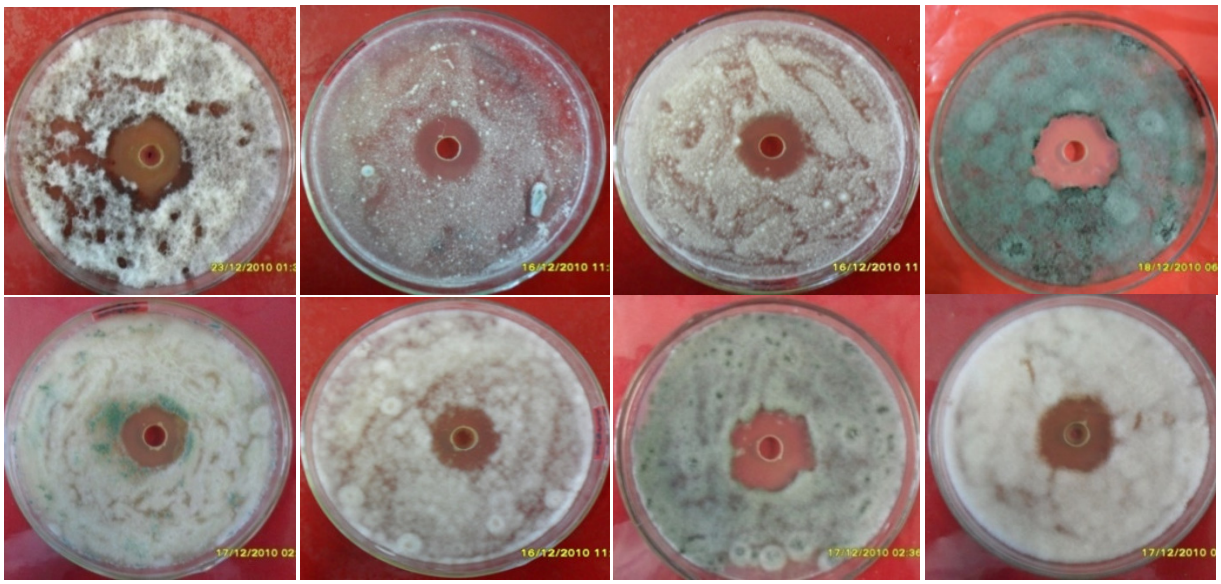

Figure-3: Antifungal activity of A-19 against eight plant pathogenic fungi on PDA media by well-diffusion method after three days incubation, (a) *Fusarium oxysporum* (b) *Rhizoctonia solani* (c) *Pythium* sp. (d,e,f,g and h) fungi infected from leaves of onion, green gram, chilli, cotton and tomato respectively.

Study on P-solubilizing activity of A-19 isolate: P-solubilizing activity of A-19 had been screened according to two methods.

Plate screening method: When phosphate solubilizing activity of A-19 was studied on PVK and NBRIP media by plate screening method, its P-solubilizing activity was found in terms of index of clear zone and halo zone formation around its colonies. It was showed in Table-5 and Figure-4.

Table-5: P-Solubilizing activity of A-19 by plate screening method.

Media	P-solubility Index	
	Without BTB (clear zone)	With BTB (halo zone)
PVK	1.3520	0.8
NBRIP	1.3076	0.0625

Quantative Determination by UV-Vis spectrophotometric method: P-solubilizing activity of A-19 was also measured by spectrophotometric method at 830 nm. Solubilized P-amount of selected strain was shown in Table-6. P-solubilizing activity was slightly higher in PVK broth than in NBRIP broth.

Table-6: P-solubilizing activity of A-19 by spectrophotometric method at 830 nm

Media	Solubilized P-amount (ppm)
PVK	264.20
NBRIP	235.77

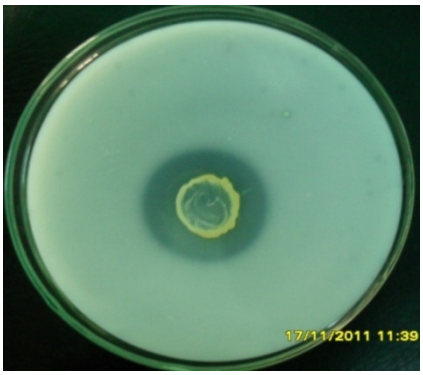

Figure-4: P-solubilizing activity of A-19 on NBRIP medium by clear zone formation after three days incubation.

Discussion: *Pseudomonas* are gram-negative, strictly aerobic, polarly flagellated rods. They colonizes aggressively the rhizosphere of various crop plants, and have a broad spectrum anatagonistic activity for many plant pathogens by antibiosis (the production of inhibitory compounds)[20,21], siderophores production (iron-sequestering compounds) and nutrition or site competition[22]. Although *Pseudomonas fluorescens* have been found associated with empyema, urinary tract infections and septicemia, but it is rarely pathogenic for humans. Some *Pseudomonas* have been known as biocontrol agents against plant fungal pathogens and antibiotic producers[23]. This is probably due to that these groups of bacteria are diverse and their importance in soils is obvious.

In the present study, isolated strains was studied its morphological, some biochemical characteristics and antibiotic sensitivity. According to some phenotypic characteristics, this isolate was assumed as *Pseudomonas* sp. Isolated *Pseudomonas*

sp. exhibited different levels of antifungal activity against eight plant pathogenic fungi. Two different media were used, it exhibited the activity on both media. But on PDA media, the observed data showed that the inhibition activity of isolates were higher.

In this study, inhibition zone diameter was almost the same from dual culture method. The present data was given after inoculating isolated bacteria in King's broth for two days. Antagonistic bacteria control the growth of bacteria by antibiosis, sidephore production and others etc. Together with these substances, their activity can be different at different time. So it is also needed to find the best time for antagonistic activity of bacteria.

Bacteria such as *Bacillus* and *Pseudomonas* have a broad-spectrum antagonistic activity and were widely reported, isolated from the rhizosphere, against wide range of fungal pathogens[24]. There are many reports for the use of *Pseudomonas* spp. as antagonistic bacteria. Vanitha et al.[25] studied that six isolates of *Pseudomonas fluorescens* were screened against *Alternaria chlamydospora* Mouchacca causing leaf blight diseases in *Solanum nigrum* L. It was found that *P. fluorscences* showed 100% inhibition of mycelia growth and culture filtrate of bacteria significantly reduced the mycelial growth of *A.chlamydospora*. Adhikari et al.[26] also found tha two isolates of *P. fluorescens* PF-8 and PF-7 effectively inhibited the mycelial growth of *Rhizoctonia solani* in dual culture method. Jonathan and Stefan[27] found that several strains *P. fluorescens* known to produce antifungal metabolites were able to induce substantial laccase production by *Rhizoctonia solani*. *P. aeruginosa* has been reported as strong antagonist of *Fusarium oxysporum*[28] and *Sclerotinia sclerotiorum*[29].

In the biological control of plant pathogens with antagonistic bacteria, bacteria such as *Pseudomonas* spp. also having plant growth promoting activity have been paid much attention due to their dual activity[30]. *Pseudomonas* sp. used in this study possesses phosphate solubilizing activity according to the observation by qualitative and quantative determination. Therefore, this strain can also be used as plant growth promoting bacteria besides antagonistic bacteria.

Conclusion

Among all isolated bacterial isolates, only one isolate showed strong inhibition against *Fusarium oxysporum*., *Rhizotonia solania* and *Phythium* sp.. Some phenotypical characteristics such as colonial and microscopic morphology, some biochemical characteristics and antibiotic sensitivity tests were said that selected isolate was assumed as *Pseudomonas* sp. Its antagonistic activity was higher when inoculated in King's B broth than those in other culture broth. The antagonistic activity of isolated strain was extended in studying against isolated plant pathogenic fungi that were not still identified. A-19 isolate gave strong inhibition against eight plant pathogenic fungi from dual culture and well diffusion methods. In addition, A-19 isolate also possess phosphate solubilizing activity. So A-19 isolate was suitable strain in applying field because this isolate also possessed plant growth promoting activity.

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