Science Letters
2016 | Volume 4 | Issue 1 | Pages 66-70

RESEARCH ARTICLE OPEN ACCESS

Antagonistic Activity of *Pseudomonas fluorescens* against Fungal Plant Pathogen *Aspergillus niger*

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Abstract
Soil-borne plant pathogenic fungi are one of the serious problems in agriculture. *Aspergillus niger* is well-known to cause important plant diseases, resulting in a significant loss of agricultural crops. *Pseudomonas fluorescens* is one of the major bio-control agent found in the rhizospher soil of many crop systems. In the present study, the biocontrol strain *P. fluorescence* Pf4 was isolated from the soil and its antagonistic activity was observed against fungal plant pathogen *A. niger* by using different antifungal assays, i.e., cross streak, pour plate and agar well diffusion assay. *P. fluorescens* showed significant antagonistic potential against *A. niger*, with the formation of 16 mm in diameter zone of inhibition of. In the seed treatment experiment, the chilli seeds treated with *P. fluorescence* Pf4 showed 100% germination index and 50% reduction in seed mortality as compared to treatment with fungal pathogen alone. These results show the potential of *P. fluorescence* Pf4 to be used as a biocontrol agent for the control of diseases caused by *A. niger*. The isolation of new biocontrol strains is necessary to cope with plant diseases.
Keywords Antagonistic effect, biocontrol, fungal plant pathogens, *Pseudomonas fluorescens*.

Received February 10, 2016 **Accepted** March 29, 2016 **Published** April 15, 2016
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To cite this manuscript: Yunus F, Iqbal M, Jabeen K, Kanwal Z, Rashid F. Antagonistic activity of *Pseudomonas fluorescens* against fungal plant pathogen *Aspergillus niger*. Sci Lett 2016; 4(1):66-70.

Introduction

Biological control is an attractive mean for decreasing the threat caused by plant pathogens [1]. The biological control mechanisms include antibiosis, competition, direct parasitism, induction in plant resistance, hypovirulence and predation for plant disease management. The antagonistic activity has been related to the production of secondary metabolites [2, 3]. Eco-friendly biological control was given high importance in the Integrated Disease Management (IDM). Management of soil borne pathogens has turned into one of the significant concerns in agriculture. *Pseudomonas fluorescence* is abundant soil microorganisms and common populations of the rhizosphere. The *P. fluorescence* has ability to diminish the fungal pathogens by bio-control mechanism. It is observed that *P. fluorescence* has ability of producing special antibiotics and other metabolites which not only enhance plant growth, but also protect plants from pathogens, so play an important role in the conservation of soil health and bio-protection of crops from pathogens [4]. The *Pseudomonas* produces several antibiotics with high antimicrobial specificity against several pathogenic microorganisms [5]. Similarly, production of siderophores and hydrolytic enzymes may also be involved in the *P. fluorscence* biological control activity [6,7]. Biological control is a promising approach for managing the plant diseases [8].

There are many pathogens that affect the plant growth, including fungal pathogens. In this study, we selected a pathogenic fungal species, named *Aspergillus niger* that not only affect the plant normal growth but lead to plant death. *A. niger*, a filamentous ascomycete has a capability of fast growth and pH tolerance [9]. This organism is a soil saprobe with an extensive array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocelluloses. Some of them are commonly used in the food industry because of their capability to produce extracellular organic acids [10]. These traits of *A. niger* allow them to cause deterioration of various organic substances, including fruits, vegetables, cereals, beans, nuts, herbs, wood and herbal drugs. The known symptoms are wilting of plants, yellowing of leaves and ultimately plant death. The fungus is notorious to produce pathogenicity like scarce to ample aerial mycelium, and white, pink, salmon, and purple pigmentation on the back side of the colony on culture medium [11]. In the present study, the *P. fluorescence* strains were isolated from different soil samples and identified using biochemical assays. Later, the newly isolated *P. fluorescence* strains were evaluated for their antagonistic potential against *A. niger in vitro*. The most effective *P. fluorescence* strain was further evaluated for its biocontrol potential using seed germination and infection assay.

Materials and methods

Collection of soil samples

Samples were collected from the rhizospheric soil of Jinnah garden and field areas of the University of the Punjab, Lahore. Thirty soil samples were collected from the roots and nodules of plants in sterilized zip-lock polythene bags. These samples were labeled and immediately stored at 4°C in the refrigerator for further processing.

Isolation of *Pseudomonas fluorescens*

One gram soil sample was added in 10 ml of sterilized nutrient broth and after gently mixing kept it for 30 minutes at room temperature. Ten-fold serial dilution was prepared from supernatant after sample processing from 10^{-1} to 10^{-6} and spread 100 µl from each dilution on petri plates in triplicate containing King's B medium (Sigma) which is selective medium for *P. fluorescens* [12, 13]. The plates were then incubated at 30°C for 48 hours. After incubation, all the isolates were checked for fluorescence under UV light and representative types of colonies were further selected on the basis of its morphology and microscopy on King's B agar medium. The purified isolates were preserved in slants for further study.

Biochemical tests

For biochemical identification of *P. fluorescens*, certain biochemical tests were performed according to Bergey's Manual for determinative Bacteriology [14].

Catalase test

The catalase test was performed for the presence of catalase enzyme which is produced by *P. fluorescens* as described by Olutiola et al. [15]. Isolates containing the catalase enzyme, produced oxygen gas bubbles when exposed to 3% hydrogen peroxide, showed a positive reaction for *P. fluorescens*.

Oxidase test

This test depends on the presence of certain oxidases in bacteria that will catalyze the transport electrons between electron donors in bacteria. This test is used for the identification of *Pseudomonas*, which produce oxidase enzyme. Oxidase test was performed as described by Olutiola et al. [15]. The positive reaction for *Pseudomonas* showed the smear turned purple within 10-30 seconds.

Starch hydrolysis

For starch hydrolysis test, the filter paper was dipped in the culture suspension and placed it in petri dishes containing starch agar medium and then incubated for two days at 30°C. Then it was flooded with iodine solution (1%). A colorless halo around the growth and blue color of the rest of the plates showed utilization of starch by the microorganism [16].

Gelatin liquefaction

For gelatin liquefaction, the filter paper was dipped in the culture suspension and placed it in petri dishes containing gelatin nutrient agar medium. Then kept petri dishes at 30ºC for two days and flooded with $HgCl_2$ solution (12.5%). The development of yellow halo around growth indicated utilization of gelatin [16].

Cultivation of *Aspergillus niger*

Pure cultures of *A. niger* (Accession No. 764) was procured from first fungal culture bank, University of the Punjab, Lahore. The culture was subcultured on potato dextrose agar (PDA), which is known as the best medium for fungal culture growth and incubated at 25±2°C for 3 to 4 days.

Antifungal assays

Cross streak assay

For cross streak assay, a heavy inoculum from fresh grown cultures of *P. fluorescens* strains were inoculated on PDA plate, then cross-streaked with culture of *A. niger* and kept at 28ºC for 3 days. Antagonistic activity of *P. fluorescens* was observed by inhibition of the growth of *A. niger*.

Pour plate assay

For pour plate assay, freshly grown cultures of *P. fluorescens* strains and pathogen *A. niger* were added simultaneously into 5ml of molten agar, mixed by shaking the tubes and then poured on PDA plates. Antagonistic activity of *P. fluorescens* was observed by inhibition of the growth of *A. niger* culture.

Agar well diffusion assay

In agar well diffusion assay, 100 µl of freshly grown cultures of *P. fluorescens* strains were spread on PDA plates. A 5mm plug disc culture was taken by cork borer from 7 days old *A. niger* culture and placed in the center of each petri dish to observe the antifungal activity of *P. fluorescens*. The percentage

inhibition of mycelial growth in all of the above methods was calculated by as per formula: Growth inhibition (%) = Growth of pathogen in control – growth of pathogen in the presence of *P. fluorescens* / growth of pathogen in control

Seed germination and infection assay

Chilli seeds were used to study the antagonistic activity of *P. fluorescens* against *A. niger*. In this method, chilli seeds were surface-sterilized with 1% sodium hypochlorite and 4% clorox, rinsed with sterile water and then treated with *P. fluorescens* Pf4 and test fungus. The four treatments were as follows: T1 = control (no treatment); T2 = seeds treated with pathogen *A. niger*; T3 = seeds treated with pathogen *A. niger* and *P. fluorescens* Pf4; T4 = seeds treated with *P. fluorescens* Pf4. The plants were cultivated in sterilized vermiculite and sand (1:1 wt/wt) at 30°C with a 16-h light/8-h dark photoperiod in a green house and irrigated regularly with sterile half strength Hoagland solution. Seed germination, growth index as root dry weight and shot dry weight and infection levels were determined after 8 days.

Statistical analysis

The data were subjected to statistical analysis by employing SPSS ver. 19.0 statistical software (SPSS, Chicago, IL).

Results and discussion

The control of plant pathogens using biocontrol microbial strains is a cheap and environmentally friendly method for the management of crop diseases. In the present study, we used fungal plant pathogen, *A. niger* which is one of the most common species of the genus *Aspergillus*. As a strong pathogen, *A. niger* can cause the decomposing of several fruits and vegetables and causes a disease called black mold on certain fruits and vegetables such as grapes, onions and peanuts [17-19].

In the present study, seventeen isolates of *P. fluorescens* were isolated from thirty rhizosphere soil samples on King's B selective medium. Biochemical confirmatory tests such as catalase, oxidase, starch hydrolysis and gelatin liquefaction were performed for the identification of *P. fluorescens* strains. All seventeen isolates were identified as *P. fluorescens*; However, 16S rRNA identification required for confirmation. Seventeen isolates were subjected for their antagonistic potential through different antifungal assays whereas, seed treatment were also used to check the antagonistic nature of isolates against *A. niger*. The inhibitory effects were tested by three different antifungal assays; cross streak, pour plate and agar well diffusion assay. The zone of inhibition diameter was measured in mm in relation to growth of pathogen in control treatment and results were recorded. In the cross streak assay, the results showed significant antagonistic potential of *P. fluorescens* against *A. niger* with the reduction of 65.1%-81.3% in the fungal colony diameter (Table 1). The highest percentage of fungal growth inhibition was obtained by isolate Pf15. In pour plate assay, the results showed low antagonistic potential of *P. fluorescens* against *A. niger* than that of cross streak assay with the reduction of 63.9%–70.9% in

Table 1 The growth inhibition of *Aspergillus niger* by different isolates of *Pseudomonas fluorescens* using differnet antifungal assays.

Isolates of *Pseudomonas fluorescens*	Cross streak assay		Pour plate assay		Agar well diffusion assay	
	Mycelial growth (mm)	Growth inhibition (%)	Mycelial growth (mm)	Growth inhibition (%)	Mycelial growth (mm)	Growth inhibition (%)
Pf1	24	72.0	31	63.9	29	66.2
Pf2	27	68.6	28	67.4	26	69.7
Pf3	28	67.4	28	67.4	27	68.6
Pf4	18	79.0	20	76.7	14	83.7
Pf5	29	66.2	30	65.1	28	67.4
Pf6	30	65.1	24	72.0	34	60.4
Pf7	26	69.7	27	68.6	25	70.9
Pf8	23	73.2	21	75.5	18	79.0
Pf9	20	76.7	27	68.6	22	74.4
Pf10	24	72.0	29	66.2	29	66.2
Pf11	28	67.4	31	63.9	27	68.6
Pf12	29	66.2	26	69.7	21	75.5
Pf13	22	74.4	22	74.4	24	72.0
Pf14	23	73.2	19	77.9	29	66.2
Pf15	16	81.3	21	75.5	24	72.0
Pf16	21	75.5	21	75.5	18	79.0
Pf17	26	69.7	24	72.0	22	74.4
Control	86	-	86	-	86	-

the fungal colony diameter, whereas the maximum zone formation for cross streak assay was calculated as for Pf14 isolate. In agar well diffusion method, the results showed less antagonistic potential of *P. fluorescens* against *A. niger* than that of cross streak assay but slightly higher than pour plate assay with the reduction of 60.4%–83.7% in the fungal colony diameter. The isolate Pf4 showed maximum antifungal activity by the agar diffusion assay. Considering all three antifungal assays, the isolate Pf4 showed maximum average antifungal activity (79.8%) compared to other isolates of *P. fluorescens* so it was selected for further seed germination and infection assay. Many *P. fluorescens* strains have been reported for their antifungal potential for example *P. fluorescens* UM270 showed antifungal and plant growth promoting activity [20]. Jayaswal et al. [21] isolated *P. fluorescens* strains effective against many phytopathogenic fungi. The antifungal activity of *P. fluorescens* strains is mainly attributed to the production of secondary metabolites like 2, 4-diacetylphloroglucinol as has been reported by Defago [22].

The seed germination and infection assay results showed that pathogen alone (T2) decreased the final plant stand by 51%, while decreased the root and shoot dry weights by 31% and 36% over the control. On the other hand, the coinoculation of *P. fluorescens* Pf4 with pathogen (T3) improved the plant stand by 40% and increased the shoot and root dry weight of plants over the controls by 16% and 4.3%, respectively (Table 2).

Table 2 Antagonistic activity of *P. fluorescence* Pf4 against *Aspergillus niger* as determined by seed treatment method.

Treatments	Final plant stand	Root dry weight (mg)	Shoot dry weight (mg)
T1	85±2	124±17	208±11
T2	42±2	85±15	134± 15
T3	70±3	144.6±10	217.5 ±18
T4	80±3	197±11	239.5 ±13

T1 = control (no treatment); T2 = seeds treated with pathogen *A. niger*; T3 = seeds treated with pathogen *A. niger* and *P. fluorescens* Pf4; T4 = seeds treated with *P. fluorescens* Pf4.

The inoculation of seeds with *P. fluorescens* Pf4 alone (T4) improved the root and shoot dry weights by 59% and 15%, respectively, over control treatment. The seed germination assay results showed that the *A. niger* (T3) caused a 12.5% reduction in the germination of chilli seeds while all seed were germinated in other treatments. The infection percentage was 50% in T3 treatment, while it was reduced to 12.5% in the presence of *P. fluorescens* Pf4. In other two treatments, no infection was observed.

These results showed the excellent potential of *P. fluorescens* Pf4 to not only improve the growth of plants, but also control the infection of *A. niger*. The *P. fluorescens* strain Pf4 should be evaluated further for its biocontrol mechnims of action and should be tested under field conditions before the commertial application to explore its true potential.

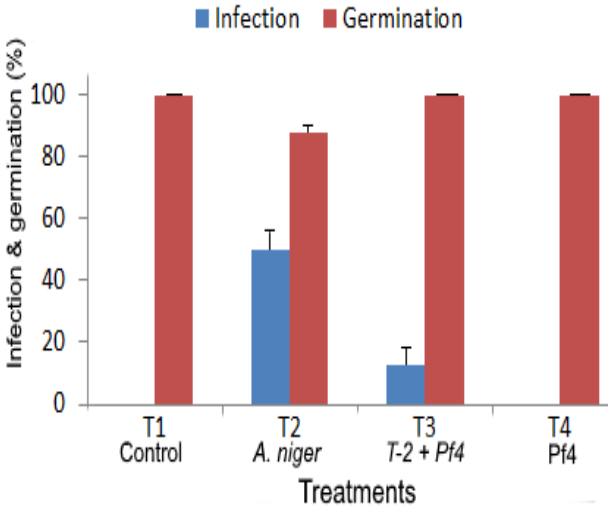

Fig. 1 Effect of *P. fluorescens* Pf4 on chilli seed germination index and infection of *Aspergillus niger*.

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