Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(4): 1257-1261
Received: 04-05-2019
Accepted: 06-06-2019

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Biochemical characterization and antagonistic potential of *Pseudomonas fluorescens* against *Macrophomina phaseolina*

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Abstract
The aim of present investigation was to confirm the biochemical characterization and *in vitro* antagonistic potential of native isolates of *Pseusomonas fluorescens* against *Macrophomina phaseolina*. Among the native isolates tested, isolate Pf2 significantly reduced the mycelial growth, size, number and germination of sclerotia of *M. Phaseolina*. Further, the same isolates tested at different concentrations. Where, isolate Pf2 @ 30% was significantly inhibits the growth (20.45mm) of test pathogen. Also, *P. fluorescens* showed that all the isolates produced similar results with regard to gram staining (negative), motility (possitive), starch hydrolysis (negative), gelatin liquefaction (positive) and fluorescent pigmentation (positive). Among the isolates Pf2 produced more quantity of IAA (3.6 µg/ml), HCN (8.16) and siderophore (0.89).

Keywords: Biochemical characterization, antagonistic potential, *Pseudomonas fluorescens, Macrophomina phaseolina*

Introduction
Sesame is known as the "Queen of oil seeds" because of its nutraceutical qualities. Sesame oil is important in the food and pharmaceutical industry because of its distinct flavor (Elleuch *et al*., 2007) [10]. Sesame seed oil is also a raw material for the production of industrial materials such as paints, varnishes, soaps, perfumes, pharmaceuticals and insecticides (Barut and Cagirgan, 2006) [3]. Sesame seed contain 50-58% of good semi-drying oil, especially with oleic and linoleic triglycerides (Qureshi *et al*., 2003) [20]. It has rich source of vitamins (pantothenic acid and vitamin E) and phosphorus (570mg/100g) (Balasubramaniyan and Palaniappan, 2001) [2]. Among the various diseases in sesame, root rot disease caused by *Macrophomina phaseolina* (Tassi) Goid. is an important pathogen, distributed worldwide and sesame at all stages are susceptible to infection and it attacks crop plants at different stages of plant growth and causes complex disease syndromes like root rot, seedling blight, charcoal rot, ashy stem blight, wilt, collar rot, dry rot, pod rot and seed rot in several crops (Ma *et al.,* 2010) [6]. Biological agent for the control of plant diseases is an alternative method of chemical control (Cook and Baker, 1983) [5]. Since bacterial antagonists have the twin advantage of fester multiplication and higher rhizosphere competence, the potential of bacteria as biocontrol agents against pathogens have been investigated by several workers (Ramamoorthy *et al,* 2002; Zaidi *et al*., 2004; Alice and Sundravadana, 2012; Meena, 2014) [21, 32, 1, 17]. Hence, the present investigation with the aim of isolation, identification and characterization of native Pseudomonas isolates were tested against *M. Phaseolina*.

Materials and methods
Isolation of the pathogen
M. phaseolina was isolated from the diseased roots of sesame plants showing the typical root rot symptoms by tissue segment method (Rangaswami, 1972) [22] on potato dextrose agar (PDA) medium. The axenic cultures of the pathogen were obtained by single hyphal tip method (Rangaswami, 1972) [22] and these were maintained on PDA slants for subsequent experiments.

Isolation of *P. fluorescens*
Four soil samples were collected from rhizosphere of sesame crop of different locations *viz*., Vridachalam, Kothattai, Bhuvanagiri and Palur of Cuddalore district. Further, the soil dilution plate technique was followed for the isolation of *P. fluorescens* from the rhizosphere soil.

From the 10^{-5} soil dilution one ml was transferred into sterile Petri dish under aseptic condition to which 15ml of sterile King's medium was poured, gently rotated for uniform mixing of the soil dilution with the medium and incubated at room temperature (28±2°C) for 48h. Fluorescent colonies were detected by viewing the plates under UV light. These colonies were marked and sub cultured. The pure cultures of these isolates were obtained by single colony method and designated as Pf1, Pf2, Pf3 and Pf4.

Biochemical tests for *P. fluorescens*
All the four native isolates were subjected to biochemical tests for their confirmation according to Bergey's Manual for Determinative Bacteriology (Breed *et al*., 1989) [4].

Gram staining
A loopful bacterial culture was transferred on a clean slide and a smear was made which was air dried and heat fixed. The smear was flooded for one min. with ammonium oxalate crystal violet. Excess strain was poured off and the slide was washed in a gentle stream of water. Lugol's iodine solution was applied and allowed to remain for one min decolorized with 95 per cent ethyl alcohol. The smear was washed in gentle stream and counter stained with safranin for 30 seconds. The gram negative cells appeared red in colour and gram positive cells appeared violet in color (Cyrabree and Hindshill, 1975) [6].

Motility
Semisolid agar medium was prepared and the bacterial isolates were incubated at 30 ˚C for 72 h. and observed for motility. The distance of growth from the point of stab showed motility.

Starch hydrolysis
Filter papers were dipped in a dry old culture suspension and were placed on Petri dishes containing starch agar medium and were incubated for two days. The plates were than flooded with one per cent iodine solution. A colourless halo around the growth and blue colour in the rest of the plates showed utilization of starch by the microorganism (Stolpe and Godkeri, 1981) [27].

Gelatin liquefaction
Filter paper discs were dipped in a day old culture suspension and were placed on Petri dishes containing gelatin nutrient agar medium. The Petri dishes were incubated at 30°C for two days and then flooded with 12.5 percent $HgCl_2$ solution. The development of yellow halo around the growth utilization of gelatin (Stolpe and Godkeri, 1981) [27].

Fluorescent pigment
The test tube containing sterilized King's B medium were inoculated with the isolates of *Pseudomonas* spp. Incubated for five days and observed. Yellowish green fluorescent pigment observed under UV light (366 nm) indicate positive results.

Estimation of IAA
Indole acetic acid (IAA) in the methanol fraction was determined by employing Salper reagent (Gordon and Paleg, 1957) [11]. To 1.5ml distilled water in a test tube 0.5ml of methanol residue was mixed, four ml fresh salper reagent was rapidly added, kept in complete darkness for one hour and read in spectrophotometer at 535 nm. From a standard curve prepared with known concentration of IAA, the quality of IAA in the filtrate was calculated (1 division = 0.307 µg of IAA).

Extraction of Siderophore from the medium
The spent culture fluid was separated from the cells by centrifugation at 7000 rpm for 15 min. The supernantant was concentrated to one fifth of the original volume by the flash evaporation at 45 ˚C. Catechol type phenolates were extracted with ethyl acetate from the culture supernantant twice with an equal volume solvent at pH 2.0.The ethyl acetate layer was removed and evaporated to dryness and the residues were dissolved in a minimum quantity of distilled water, while hydroxamate types were measured from the untreated culture supernatant.

HCN production
Production of HCN was determined by (Wei *et al*., 1996) [31]. Bacteria were grown on TSA supplemented with 4.4 g/l of glycine, white filter paper strips soaked in picric acid solution (2.5 g of picric acid, 12.5 g of Na2CO3 and 1 lit. of water) were placed in the lid of each Petri dish. Petri dishes were sealed with parafilm and incubated for two to three days at (28±2 ˚C). After incubation HCN production was indicated by the presence of a coloured zone around the bacteria.

Dual culture technique
The antagonist activity of all the four native isolates of *P. fluorescens* against *M. phaseolina* was tested by dual culture technique (Dennis and Webster, 1971) [8]. At one end of the sterile plate dish containing 15ml sterilized and solidified PDA medium a 9mm culture disc of the pathogen *M. phaseolina* was placed and incubated under aspetic conditions. Similarly at the opposite end approximately 75 mm *P. fluorescens* one cm long streak was gently made onto the medium using two days old culture. A control was maintained by inoculating *M. phaseolina* alone at one end of the Petri dish. The plates were incubated at room temperature (28±2 ˚C) for three days. The radial growth (in mm) of the pathogen and the antagonist and the extent of the inhibition zones (in mm) developed between the two colonies were measured. The effective antagonists were identified based on the inhibition of the growth of the pathogen. The radial mycelia growth of the pathogen and percent reduction over control was calculated by using the formula (Vincent, 1927) [29].

Percent inhibition (I) = C-T/C × 100

Where,
C- Mycelial growth of pathogen in control
T- Mycelial growth of pathogen in dual plate.

Poisoned food technique
The culture discs (four discs of 9mm size) obtained at the point where the pathogen and antagonist interacted from the above experiment were used for the estimation of the sclerotial number and size. The culture discs cut from the plates inoculated with *M. phaseolina* alone served as control. For assessing the effect of antagonists on the sclerotial germination, twenty five uniform sized sclerotia from dual culture plates were picked and placed on a cavity slide containing sterile water. The observations on the germination and the number of germ tubes produced were recorded 24h (Dhingra and Sinclai, 1978) [9].

All the native isolates further tested at different concentration (10, 20 and 30 %) against test pathogen were inoculated into Erlenmeyer flasks containing 50ml of sterilized King's B broth and kept on a rotary shaker at 100 rpm for 48h. Then the cultures were filtered through bacteriological filter under vacuum and the filtrates thus obtained were used for the studies.

The culture filtrate of the antagonist were separately incorporated into sterilized PDA media at 10, 20 and 30 per cent by adding the calculated quantity of the culture filtrates to the medium by means of a sterile pipette. The PDA medium without the culture filtrate served as control. The amended media were transferred to sterile Petri dishes separately @15ml and allowed to solidify. Each plate was inoculated at the centre with seven days old (9mm) PDA culture disc of *M. phaseolina*. Three replications were maintained for each treatment. The diameter of the mycelia growth (mm) of test pathogen was measured when the mycelia growth fully covered the control plates.

Result

Biochemical tests for *P. fluorescens*

The results of the gram reaction and biochemical tests performed for the identification of the effective native isolates of *P. fluorescens* showed that all the isolates produced similar results with regard to gram staining (negative), motility (positive), starch hydrolysis (negative), gelatin liquefaction (positive) and fluorescent pigmentation (positive). All the isolates showed positive results in IAA production. Among the isolates Pf_2 produced more quantity (3.6) of IAA followed by Pf_1, Pf_3 and Pf_4 (3.2, 2.7 and 2.1 respectively) in the decreasing order of merit. All the isolates recorded positive results with regard to hydrogen cyanide production. The isolate Pf_2 recorded maximum production of siderophore (Table 1). The identified isolates were designated as Pf_1, Pf_2, Pf_3 and Pf_4.

Effect of *P. fluorescens* isolates against *M. phaseolina* (Dual culture)

The result presented in table 2 revealed varying degree of antagonism by the isolates of *P. fluorescens* against *M. phaseolina.* Among the isolates, Pf_2 produced significantly the maximum inhibition zone (9.16 mm)and minimum mycelia growth (23.70 mm) accounting for 73.66 percent reduction on the mycelia growth of *M. phaseolina* over control. This was followed by isolate Pf_1 which recorded an inhibition zone of 8.14 mm accounting for 70.55 per cent reduction on the mycelial growth over control. The isolate Pf_3 was the least effective as it produced only the minimum inhibition zone of 5.25 mm and 64.44 per cent reduction on the mycelial growth of *M. phaseolina.*

The isolate Pf_2 was found to be more inhibitory to the size, number and germination of sclerotia of *M. phaseolina,* which recorded the minimum size (68.10 μm) number (68.32) and germination (40.49 %) of sclerotia and accounted for 39.19, 57.56 and 56.46 per cent reduction over control respectively. This was followed by the isolate Pf_1 recorded minimum size, number and germination of sclerotia (73.51, 72.23 and 46.35) respectively and the least inhibitory effect (38.71% reduction) was recorded with the isolate Pf_3 (Table 3).

The mycelial growth of *M. phaseolina* was found reduced with an increase in the concentration of culture filtrates of all the isolates of the antagonists tested (Table 4) and the reduction was significantly the maximum in the case of *Pseudomonas* isolate Pf_2 with 58.45, 36.22 and 20.45 mm at 10, 20 and 30 per cent concentration of the culture filtrate (Pf_2) respectively as against the maximum growth of 90 mm in the control. This was followed by Pf_1, Pf_4 and Pf_3 in the decreasing order of merit. The least reduction of the growth was recorded by the culture filtrate of isolate Pf_3 (71.06, 41.87 and 27.67 mm at 10, 20 and 30 % conc., respectively).

Discussion

The fungistatic activity of the fluorescent *Pseudomonas* based on the inhibition of mycelial growth of the pathogen was well established by earlier workers (Vivekanandhan *et al*., 2004; Surjit Sen *et al*., 2006) [30, 28]. *P. fluorescens* strain Pf_1 effectively inhibited the mycelial growth of *M. phaseolina,* causing dry root rot in blackgram and sesame (Jayashree *et al.,* 2000) [13]. *Pseudomonas* isolate GRC2 showed necrotropic antibiosis against *M.phaseolina* (Gupta *et al.,* 2001) [12]. The efficacy of *P. fluorescens* against *S. rolfsii* (Pande and Chaube, 2004) [18] and *F. oxysporum* f.sp. *pisi* (Shalini Verma and Dohroo, 2005) [25] have also been reported under *in vitro* conditions (dual culture). Several workers have reported about the inhibitory effect of *P. fluorescens* on the number, size and sclerotial germination of *M. phaseolina* (Samiyappan, 1988; Krishnaveni, 1991; Laha *et al.,* 1996) [23, 14, 15]. *P. fluorescens* formed inhibition zones and teduced sclerotial formation, sclerotial size, germination and germ tube formation by *M. phaseolina* (Selvarajan and Jeyarajan, 1996) [24]. *P. fluorescens* strain Pf1 effectively inhibited the mycelial growth and sclerotial production of *M. phaseolina* causing root rot in groundnut (Shanmugam *et al.,* 2003) [26]. Das *et al.,* (2008) [7] have found that the cell free culture filtrates of fluorescent *Pseudomonas* strains at 20 per cent conc. significantly reduced the formation and germination of microsclerotia of *M. phaseolina* as observed in the present study. The results of the present investigations were confirmed by the above reports.

Table 1: Biochemical test for *P. fluorescens* native isolates

S. No.	Parameter	Isolates of *P. Fluorescens*			
		Pf_1	Pf_2	Pf_3	Pf_4
1.	Gram staining	Negative	Negative	Negative	Negative
2.	Motility	Positive	Positive	Positive	Positive
3.	Starch hydrolysis	Negative	Negative	Negative	Negative
4.	Gelatin liquefaction	Positive	Positive	Positive	Positive
5.	Fluorescent pigment	Positive	Positive	Positive	Positive
6.	Estimation of IAA	3.2^b	3.6^a	2.7^c	2.1^d
7.	Siderophore production (hydroxamate)	0.84^b	0.89^a	0.83^b	0.76^c
8.	Hydrogen cyanide production	8.05^b	8.16^a	7.82^c	7.39^d

Values not sharing a common superscript differ significantly at $P<0.05$ (DMRT)

Table 2: Efficacy of *P. fluorescens* isolates against *M. phaseolina* (Dual culture)

Isolates	Growth of *M. Phaseolina*		
	Linear growth (mm)	Percent inhibition	Inhibition zone (mm)
Pf_1	26.55^{b}	70.55	8.14^{b}
Pf_2	23.70^{a}	73.66	9.16^{a}
Pf_3	32.47^{d}	64.44	5.25^{d}
Pf_4	29.45^{c}	67.24	6.83^{c}
Control	90.00^{e}	-	-

Values not sharing a common superscript differ significantly at *P*<0.05 (DMRT)

Table 3: Effect of *P. fluorescens* isolates on number, size and sclerotial germination of *M. phaseolina*

Isolates	Number of sclerotia	Percent reduction	Sclerotial size (µ)	Percent reduction	Sclerotial germination (%)	Percent inhibition	No. of germtubes per sclerotium	Percent reduction
Pf_1	73.51^{b}	54.34	72.23^{b}	35.50	46.35^{b}	50.16	6.73^{b}	58.58
Pf_2	68.32^{a}	57.56	68.10^{a}	39.19	40.49^{a}	56.46	5.51^{a}	66.09
Pf_3	98.67^{d}	38.71	85.76^{d}	23.43	59.73^{d}	35.77	9.47^{d}	41.72
Pf_4	85.74^{c}	46.74	80.49^{c}	28.13	53.16^{c}	42.83	8.14^{c}	49.90
Control	161.00^{e}	-	112.00^{e}	-	93.00^{e}	-	16.25^{e}	-

Values not sharing a common superscript differ significantly at *P*<0.05 (DMRT)

Table 4: Effect of culture filtrate of *P. fluorescens* on the growth of *M. phaseolina*

Isolates	Growth of *M. phaseolina* (mm)			Mean
	Concentration of culture filtrate (%)			
	10	20	30	
Pf_1	62.67^{b}	37.73^{b}	21.51^{b}	40.63
Pf_2	58.45^{a}	36.33^{a}	20.45^{a}	38.41
Pf_3	71.06^{d}	41.87^{d}	27.67^{d}	46.86
Pf_4	67.24^{c}	39.93^{c}	23.64^{c}	43.60
Control	90.00^{e}	90.00^{e}	90.00^{e}	90.00

Values not sharing a common superscript differ significantly at *P*<0.05 (DMRT)

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