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Antagonistic Potential of Iranian Native *Streptomyces* Strains in Biocontrol of *Pythium aphanidermatum*

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Abstract: *Pythium aphanidermatum*, the major phytopathogen induce damping off, root and stem rots, blights of grasses and fruit and in greenhouse cucurbits growing all areas of the world. Soil Actinomycetes including 178 isolates were assayed for assessing antagonistic activity against *P.aphanidermatum*. From tested isolates, 43 isolates were effective but 2 isolates strains 311 and 321 showed high anti-fungal activity revealed by bioassays in agar disk and well diffusion methods. For further biological characterizations, the active strains were grown in submerged cultures to determine growth curve and prepare crude extracts. Preliminary greenhouse studies indicated that amending soil with these strains, 311 and 321; reduce crop losses due to the pathogen. Antifungal activities of both antagonists were of fungicidal type on pathogen with complete inhibitory effects.

Key words: *Pythium aphanidermatum*, antagonist, antifungal, biological control

INTRODUCTION

Pythium diseases of cucurbits are of the important limitations of seedling and quality in growing cucurbits arias in infected fields of the world. Causal agent, *Pythium aphanidermatum*, is an aggressive species causing damping off, root and stem rots and blights of grasses and fruits. *P. aphanidermatum* is a cosmopolitan pathogen with a wide host range. It is of economic concern on most annuals, cucurbits and grasses. *P. aphanidermatum* occurs world wide particularly in warm regions and greenhouses. The fungus prefers temperatures between 27 and 34°C and wet conditions (water potential of 0 to-0.01 bars). It has a very wide host range, including many annuals and bedding plants. It causes economic losses on beets, pepper, chrysanthemum, cucurbits, cotton and grasses. Many of classical management efforts have focused on using chemicals (Agrios, 1998; Loria *et al*., 1997; Parker, 2004). Many non-pathogenic *Streptomyces* demonstrate antagonistic characteristics against the pathogens. These criteria include production of different kinds of secondary metabolites and biologically active substances which some have high commercial values such as enzymes and antibiotics (Lee and Hwang, 2002; Saadoun and Gharaibeh, 2002). The classical control measures have not succeeded in eliminating the disease. With the respect to their role in biological control of soil-borne pathogens, at the present research, 178 isolates of Actinomycetes were isolated from agricultural soils of Kerman and Fars Provinces, Iran and screened against the *P. aphanidermatum* (Fini *et al*., 2003). From tested isolates, 43 showed antagonistic effects against the pathogens. Two *Streptomyces* strains, 311 and 321, had prominent activity against *P. aphanidermatum* the causal agent of Pythium-disease in cucurbits growing areas in Iran which revealed by *In vitro* studies and used to evaluate their potentials in biocontrol of the pathogen as follows.

MATERIALS AND METHODS

Microorganisms and culture media: *Pythium aphanidermatum* the causal agents of Pythium disease in major cucurbit growing areas of Iran were kind gifts from Shiraz Agricultural University of Iran. The Pathogen was grown at 27- 34°C and maintained on Corn Meal Agar (CMA) composed of Corn meal, 17 gr in 1 L of H_2O. All cultures stored at room temperature (24-30 °C) and sub-cultured as needed. Casein Glycerol (or starch) Agar (CGA) was used for screening and isolating of

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Actinomycetes which composed of: glycerol or soluble starch, 10 gr; casein, 0.3 gr; KNO_3, 2 gr; NaCl, 2 gr; K_2HPO_4, 2 gr; $MgSO_4.7H_2O$, 0.05 gr; $CaCO_3$, 0.02 gr; $FeSO_4.7H_2O$, 0.01 gr and Agar, 18 gr in 1 L of distilled H_2O (pH 7.2) (Dhingra and Sinclair, 1995). In submerged cultures, Agar was excluded (CG medium). Actinomycete colonies with different morphologies were selected and transferred to CGA slants for further studies.

Soil culture procedure and isolation of streptomycete antagonists: For isolation of Actinomycetes, soil samples were collected from grasslands, orchards and vegetable fields in different localities of Kerman and Fars provinces, Iran. Several samples randomly were selected from mentioned localities using an open-end soil borer (20 cm in depth, 2.5 cm in diameter) as described by Lee and Hwang (2002). Soil samples were taken from a depth of 10-20 cm below the soil surface. The soil of the top region (10 cm from the surface) was excluded. Samples were air-dried at room temperature for 7-10 days and then passed through a 0.8 mm mesh sieve and were preserved in polyethylene bags at room temperature before use. Samples (10 g) of air-dried soil were mixed with sterile distilled water (100 mL). The mixtures were shaken vigorously for 1 h and then allowed to settle for 1 h. Portions (1 mL) of soil suspensions (diluted 10^{-1}) were transferred to 9 mL of sterile distilled water and subsequently diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Inocula consisted of adding aliquots of 10^{-3} to 10^{-6} soil dilutions to autoclaved CGA (1 in 25 mL CGA) at 50°C before pouring the plates and solidification. Three replicates were considered for each dilution. Plates were incubated at 30°C for up to 20 days. From day 7 after, Actinomycete colonies were isolated on CGA, incubated at 28°C for one week and stored refrigerated as pure cultures before use. For screening studies, 178 pure Actinomycete isolates were collected and maintained refrigerated in stock.

***In vitro* bioassay for anti-fungal activity:** From the refrigerated stocks, each Actinomycete isolate was smeared on CGA medium as a single streak and after incubation at 28°C for 4- 6 days, from well-grown streaks 6 mm agar disks of Actinomycete colony mass was prepared by using sterile cork borers with 6 mm in diameter. Disks were then aseptically transferred to CMA plates having fresh disk culture of *P. aphanidermatum*. Controls included using plain disks from CGA medium. Plates were incubated at 29-31°C for 4-6 days and bioactivity was evaluated by measuring the Diameter of Inhibition Zones (DIZ, mm) (Dhingra and Sinclair, 1995; Acar and Goldstein, 1996).

Classification of the active antagonists: From 43 active Actinomycete isolates two showed high antagonistic activity and their colonies were characterized morphologically and physiologically to the genus level following the direction mentioned in the methods manual of international cooperative project for description and deposition of cultures of *Streptomyces* (ISP) (Shirling and Gottlieb, 1996).

Bioassays: To evaluate the antifungal activity of *P. aphanidermatum* against the pathogen, bioassays were performed in two ways: agar disk and well methods as used by Shahidi Bonjar (2003) and Aghighi *et al.* (2004). Antifungal activity around both strains, 311 and 321; agar disks or wells, was close to the ratings applied by Lee and Hwang (2002) and El-Tarabily *et al.* (2000, 2006).

Monitoring activity: The antagonist strains, 311 and 321; were grown in CG medium on rotary shakers under 130 rpm at 30°C in submerged cultures. To monitor the activity, aseptically small aliquots of culture media were taken every 24 h for four months and the activity was evaluated by well diffusion-method (Dhingra and Sinclair, 1995) against disk culture of *P. aphanidermatum* and antifungal activity was measured as described. In solid cultures, active strains of 311 and 321 were grown in CGA medium as streaks and to monitor the activity, aseptically 6 mm agar disks were taken by sterile cork borer every day for 25 days and the activity was evaluated by agar disk-method (Acar and Goldstein, 1996) against disk culture of the pathogen and antifungal activity was measured as mentioned.

Preparation of crude extracts: In submerged cultures, when the activity reached maximum, the cultures were harvested; spores and mycelia were excluded by filtration through two layers of cheese cloth. The clarified sap was then dried to dark crude under reduced air at 50°C and kept refrigerated for further studies.

Detection of fungicidal and/or fungistatic activity: Small blocks of inhibition zones ($1mm^3$) of both strains on *P. aphanidermatum* were transferred to fresh CMA plates and incubated for 7 days at 30°C. During incubation, growth or lack of growth of the pathogen was investigated visually and microscopically.

RESULTS

Screening and bioassays: In screening for Actinomycetes being antagonists of *P. aphanidermatum*, 178 isolates of soil Actinomycetes of Kerman and Fars Provinces were

screened from which 43 isolates showed antifungal activity against the tested pathogen. Bioassay results are indicated in Table 1.

Identification of active *streptomyces* species: The isolates were determined as strain 311 and strain 321 according to the methods described in manual of international cooperative project for description and deposition of cultures of *Streptomyces* (ISP) (Shirling and Gottlieb, 1996).

Table 1: Screening results of active Actinomycetes isolated from soils of Kerman and Fars Provinces with anti-fungicidal effects on *Pythium aphanidermatum*. Scaling rates of inhibition intensities are as: no inhibition or pathogen growth not different from control (-); weak inhibition or partial inhibition of 5-10 mm (+); moderate inhibition of 10-30 mm (+ +) and strong inhibition of >30 mm (+ + +). Control includes plain agar disk in each bioassay

SInhibition Intensity	Actinomycete isolates No.	Inhibition Intensity	Actinomycete isolates No.	Inhibition Intensity	Actinomycete isolates No.
++	287	++	149	++	34
++	301	++	150	+	57
+	303	++	151	+	118
+	304	++	157	++	119
++	305	++	166	++	122
++	306	+	212	+	124
++	309	+	215	++	126
+++	311	++	216	++	127
+	312	++	222	++	128
+++	321	++	223	++	129
+	322	++	227	+	133
++	323	+	229	++	135
+	324	+	236	+	136
–	Control	++	238	++	139
		++	247	+	140

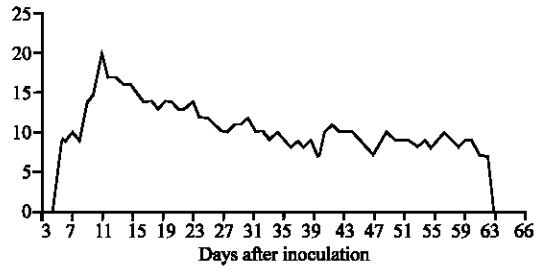

Fig. 1: Activity versus time in submerged cultures of strain 311 against *P. aphanidermatum*

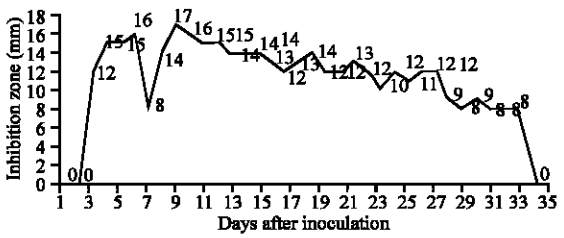

Fig. 2: Activity versus time in submerged cultures of strain 321 against *P. aphanidermatum*

Monitoring activity: Activity versus time in submerged and solid media cultures of strains 311 and 321 against *P. aphanidermatum* is indicated in Fig. 1 and 2, respectively. The results were recorded as follows. In rotary submerged cultures, activity of both strains of 311 and 321 reached maximum at 11th day post seeding against *P.aphanidermatum*. The submerged cultures at 11th day were harvested for preparation of crude extract for use for future investigations.

Detection of fungicidal and/or fungistatic activity: After transfer of small blocks from inhibition zones to fresh medium, lack of growth of pathogen represented fungicidal properties of the antagonists.

DISCUSSION

P. aphanidermatum is the principal casual agent of Pythium-diseases. The pathogen infects seeds, juvenile tissue, lower stems, fruit rot and roots. The symptoms and extent of damage caused depend on the region infected. Application of antagonist Streptomyces isolates to infected fields can effectively control reduction of crop values and could be significant in terms of safety and economy. The results may be considered for further studies of Actinomycete microflora in native Iranian soils with the goal to find new agents in biocontrol of this disease (Shahidi, 2003). An ideal and environmentally safe measure in control of this pathogen in Iran is to amend the soil mix with selected antagonists. However, this requires investigation of conditions which favor the survival of the antagonists, because soil is very complex substrate in which numerous factors influence the number of microorganisms as well as the qualitative composition of its microflora. In this study, we attempted to isolate and study a preliminary screening of Actinomycetes in restricted area of Kerman and Fars Provinces, Iran and believe that the results of these findings can form the avenue for production of resistant transgenic-cucurbit plants with recombinant DNA having anti-fungal genes cloned from biologically active strain 311 and strain 321.

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