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Biological Control of *Pythium aphanidermatum*, the Causal Agent of Damping off Disease of Greenhouse Cucurbits in Kerman Province of Iran

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Abstract: Actinomycetes enhance soil fertility and have antagonistic activity against wide range of plant root-pathogens. These micro organisms were isolated from agricultural soils of Kerman and Fars Provinces as pure cultures. *Pythium aphanidermatum*, causes damping off and root and stem rots of cucurbits worldwide. From 178 Actinomycetes isolates, 43 inhibited growth of the pathogen in culture plates and two of the most active isolates exhibited biological control of the pathogen under greenhouse conditions. When plants were grown in sterile soil mix and treated both with Actinomycetes and the pathogen, the number of healthy plants increased dramatically and the symptoms on diseased plants were less severe in comparison with seedlings treated with the pathogen alone. From the collected data it was well conclusive that in greenhouse tests, soil applications of Actinomycetes controlled causal agent of damping off in cucurbit seedlings. Antifungal activity was of fungicidal type on the pathogen mycelia. Regarding biotechnological implications, the results indicate that the active isolates can be investigated for use as biofertilizers, biofungicides and use in future development of recombinant DNA in cucurbits bearing elevated resistance to damping off. Field trials of the active isolates are under investigation.

Key words: *Pythium aphanidermatum, streptomyces*, antifungal, biocontrol, biofertilizer, biofungicide

INTRODUCTION

Streptomyces are one of the most attractive sources of biologically active substances such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitors (Omura, 1986; Shahidi, 2003; Shahdi *et al*., 2004). Soil streptomycetes are of the major contributors to the biological buffering of soils and have roles in decomposition of organic matter conductive to crop production (Gottlieb, 1973; Keiser, *et al.,* 2000). Studies even show that use of streptomycetes enhances growth of the crop plants (Brown, 1974). The search for new principles in biocontrol of plant pathogens different from the classical used fungicides, is of worldwide concern (Cohen and Coffey, 1986; Fruh *et al*, 1996; Knight *et al*., 1997). Biological control of plant diseases is slow, gives few quick profits, but can be long lasting, inexpensive and harmless to life. Biocontrol systems do not eliminate neither pathogen nor disease but bring them into natural balance (Dhingra and Sinvlair, 1995). *Pythium aphanidermatum* is a ubiquitous phytopathogen causing damping off, root and stem rots and blights of grasses and fruit (Parker, 2004; Paulitz and Bakr, 1987b). For evaluation of Actinomycetes microflora of the Iranian soils with the goal of exploring new means for biocontrol of cucurbit diseases, at the present research 178 isolates of Actinomycetes were isolated from agricultural soils of Kerman and Fars provinces of Iran and screened against *P. aphanidermatum* both *in vitro* and greenhouse conditions. The objective of the present study was also to isolate Actinomycete strains having antagonistic properties with the aim that they can serve as gene donors in developing resistant transgenic plants or use as soil amendments as biofertilizer or biofungicide in biological control of the tested pathogen. From all tested isolates of Actinomycetes, 43 isolates showed high *in vitro* antifungal activity and one (strain 311) inhibited the pathogen in artificially infested susceptible seedlings of melon (*Cucumis melo* L.) in greenhouse experiments.

MATERIALS AND METHODS

Culture media and preparation of fungal isolate: Pure culture of *Pythium aphanidermatum* was obtained from Shiraz Agricultural University of Iran. It was maintained on Corn Meal Agar (CMA) and subcultured as needed. Casein Glycerol (or starch) Agar (CGA) prepared from basic ingredients as described by Kuster and Williams, (1964) and used as Actinomycetes culture.

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Soil sampling and isolation of streptomycetes: 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 the 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 m m 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, 25 mL^{-1} 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 on, *Streptomyces* 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 *Streptomyces* isolates were collected.

***In vitro* studies:** To evaluate the antifungal activity of isolated *Streptomyces* against the pathogen, bioassays were performed in agar disk method as used by Shahidi Bonjar (2003). Antifungal activity around the Actinomycetes agar disks was evaluated as follows and the ratings used were modified from those of Lee and Hwang (2002) and El-Tarabily *et al.* (2000: 1) no inhibition = mycelial growth not different from control (-); 2) weak inhibition = partial inhibition of mycelial growth, measured as a diameter of 5-9 mm (+); 3) moderate inhibition = almost complete inhibition of mycelial growth, measured as a diameter of 10-19 mm (++); 4) strong inhibition = complete inhibition, in which most mycelia did not grow, measured as a diameter of >20 mm (+++). Controls included plain agar disks.

Detection of fungicidal and/or fungistatic activity: Small blocks of inhibition zones (1 mm^3) of active isolates against the pathogen were transferred to fresh CMA plates and incubated for 7 days at 25-26°C. During incubation, growth or lack of growth of the pathogen was investigated both visually and microscopically. Rejuvenation of growth would be indicative of fungistatic and lack of growth represents fungicidal properties of the antagonist.

***In vivo* studies:** Seeds of commercial melon, *Cucumis melo* L., grown under greenhouse conditions in plastic pots containing sterilized sand and humus of decayed leaves (4:1, w:w) to produce seedlings. Two seeds were planted 3-4 cm below soil surface per pot. When the seedlings reached 15-20 cm in height, they were separated in four groups with five pots in each and treated in following groups: a) pathogen alone, b) pathogen plus *Streptomyces* strain 311, c) *Streptomyces* strain 311alone and d) control (untreated). For inoculation, sterile pot soil mixed with mycelial mat and/or *Streptomyces* and added on collar and root of each seedling (one Petri dish of the well grown organisms per each pot) and pots filled with sterilized soil mix to the original heights. All pots irrigated regularly. After onset of symptoms, the seedlings were desoiled and examined for lack or development of root rot. Reisolation of the pathogen was aseptically performed from decayed roots on CMA media and the results were recorded. *Streptomyces* strain 311 which showed *in vitro* high antagonistic activity was used in the mentioned sets.

RESULTS

Preparation and screening of streptomycetes: In screening for *Streptomyces* having antagonistic activity against *P. aphanidermatum* the causal agent of damping off and root and stem rot of cucurbits, 178 isolates of soil *Streptomyces* from Kerman and Fars Provinces were screened from which over forty isolates showed strong activity against the tested pathogen. Figure 1 shows the screening results of some active isolates.

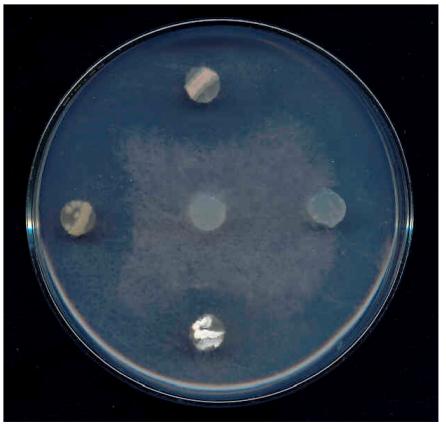

Fig. 1: *In vitro* Agar disk bioassay of three *Streptomyces* isolates (top, left and bottom plugs) against *Pythium aphanidermatum* (center plug) indicating antifungal inhibition. Right plug is control (plain agar plug)

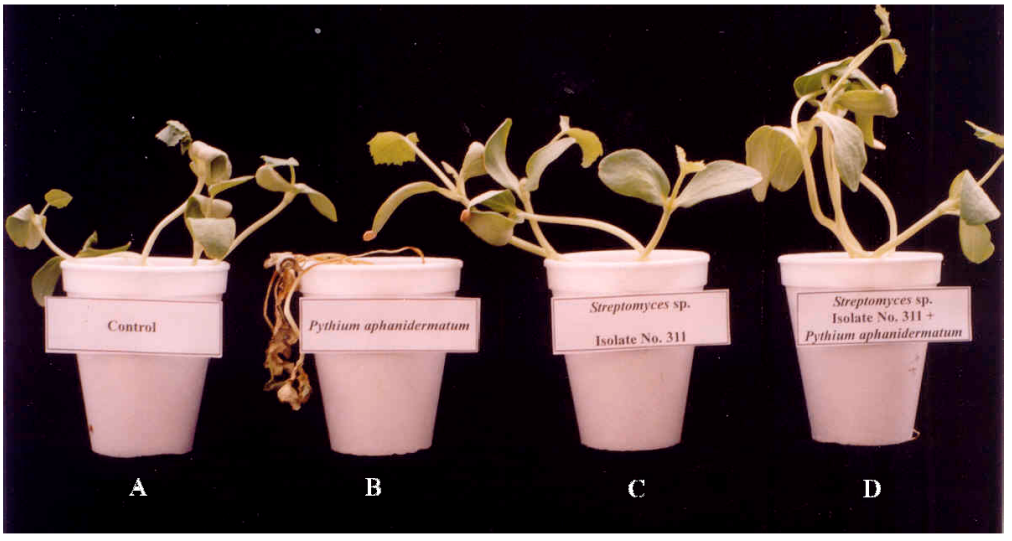

Fig. 2: *In vivo* greenhouse results in melon (*Cucumis melo* L.) seedlings indicative damping off (B) in pots inoculated with the pathogen alone and (C) pots inoculated with the *Streptomyces* sp. isolate 311alone, (D) pots inoculated with both pathogen and the antagonist (*Streptomyces* sp. isolate 311) showing prominent suppressive effect of the antagonist upon the pathogen and (A) contains untreated control seedlings

Fungicidal and/or fungistatic activity: Transfer of blocks of inhibition zones to fresh CMA plates revealed no afterward growth of the pathogen which was indicative of fungicidal activity of both tested *Streptomyces* isolates.

***In vivo* greenhouse studies:** The results of biological control of *Streptomyces* isolate 311 against *P. aphanidermatum* the causal agent of damping off and root and stem rot in cucurbits seedlings are indicated in Fig. 2 which is indicative of clear suppression of the pathogen in pots which received the antagonist.

DISCUSSION

Using natural biofungicides is an approach to environmentally safe method in management of plant diseases in the field or greenhouse. In infested soils with *P. aphanidermatum*, it is an ideal goal that amending the soil with selected natural antagonists and attaining a sustainable long-lasting biocontrol. 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. This is one of the main aims of pest management in sustainable agriculture. In this study, we attempted to isolate and perform a preliminary screening of Streptomycetes from restricted soils of Kerman and Fars Provinces and perform greenhouse studies to validate this prospective goal. The results may be considered for further studies of *Streptomyces* microflora in native Iranian soils with the goal to find new agents in biocontrol of soil born diseases of plants. The genes encoding many antifungal characteristics are currently being used by agribusiness to create genetically modified plants that have increased fungal resistance in the field. Nearly all private investments in biological control today are for transformation of plants to express genes from microorganisms. In these examples, the plant rather than the microorganism becomes the biological control agent. We believe that the results of these findings can form the avenue for production of resistant transgenic-plants with recombinant DNA having antifungal genes cloned from biologically active *Streptomyces* isolates which would lead to environmentally safer measures in plant-pest management.

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