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Biological Control of *Phytophthora drechsleri* Tucker, the Causal Agent of Pistachio Gummosis, under Greenhouse Conditions by Use of Actinomycetes

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Abstract: Actinomycetes enhance soil fertility and have antagonistic activity against wide range of plant root-pathogens. These microorganisms were isolated from agricultural soils of Kerman as pure cultures. *Phytophthora drechsleri* Tucker, causes gummosis and root rot of pistachio trees worldwide. From 130 Actinomycetes isolates, 12 inhibited growth of the pathogen of pistachio gummosis in culture plates and four 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 root rot of pistachio seedlings. Antifungal activity was of fungicidal type on the pathogen mycelia. From the stand point of biotechnological goals, the results indicate that the active isolates can be investigated for use as biofertilizers, biofungicides and use in future development of recombinant DNA in pistachio trees bearing elevated resistance to gummosis. Field trials of the active isolates are under investigation.

Key words: *Phytophthora drechsleri,* biofungicide, *Streptomyces*, antifungal, biological control, biofertilizer

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 Bonjar, 2003; Shahidi Bonjar *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). The results even show that use of streptomycetes enhances growth of the crop plants (Brown, 1974). The search for new principles in combating plant pathogens, different from the currently 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 Sinclair, 1995). *Phytophthora drechsleri* Tucker is ubiquitous phytopathogen causing gummosis, root rot and damping off in pistachio orchards and many other plant species (Lamour *et al*., 2003; Saberi-Riseh *et al*., 2004). For evaluation of Actinomycetes microflora of the Iranian soils with the goal of exploring new means for biocontrol of pistachio gummosis, at the present research 130 isolates of Actinomycetes were isolated from agricultural soils of Kerman province, Iran and screened against *Phytophthora drechsleri* both *in vitro* and greenhouse conditions. The objective of the present study was also to isolate Actinomycetes 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 pathogens. From all tested

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isolates of Actinomycetes, 12 isolates showed high *in vitro* antifungal activity and four inhibited the pathogen in artificially infested susceptible seedlings of pistachio in greenhouse experiments.

MATERIALS AND METHODS

Culture media and preparation of fungal isolate: Pure culture of *Phytophthora drechsleri* Tucker which had been isolated from pistachio trees showing gummosis in Rafsenjan region was obtained from Agricultural Research Center, Ministry of Jahad Keshavarzi, Kerman. It was maintained on cornmeal 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.

Soil sampling and isolation of streptomycetes: Soil samples were collected from grasslands, orchards and vegetable fields in different localities of Kerman province, 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 mL/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 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 130 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 was indicative of fungistatic and lack of growth represented fungicidal properties of the antagonist.

***In vivo* studies:** Seeds of commercial pistachio *Pistachia vera* var. *Fandoghi* grown under greenhouse conditions in plastic pots containing sterilized sand and humus of decayed leaves (4:1 w/w) to produce seedlings. Seeds were treated with fungicide (Carboxin Tiram, 2/1000: w/w) prior to planting. Two seeds were planted 3-4 cm below soil surface/pot. When the seedlings reached 15-20 cm in height, they were separated in four groups with five pots in each and inoculated in following groups: a) pathogen alone, b) pathogen plus *Streptomyces*, c) *Streptomyces* alone and d) control (untreated). For inoculation, 3-5 cm of top soil was removed, by a sterile razor blade 3-4 cuts (1 mm in depth) was made on collar and root of each seedling, crushed mycelial math (one Petri dish of the well grown organisms per each pot) of the pathogen and/or *Streptomyces* were applied on the cuts 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. Isolates of 30, 43, 44 and 95 of *Streptomyces* which showed high *in vitro* antagonistic activity, were used in each of the mentioned set of experiments.

RESULTS

Preparation and screening of streptomycetes: In screening for *Streptomyces* having antagonistic activity against *P. drechsleri* the causal agent of gummosis and root rot of pistachio trees, 130 isolates of soil *Streptomyces* from Kerman Province were screened from which over forty isolates showed strong activity against

Table 1: The screening results of active isolates of pure soil cultures of Actinomycetes against *P. drechsleri*, the causal agent of root rot gummosis of pistachio, assessed by *in vitro* dual culture bioassays

Actinomycete isolate No.	Inhibition zone (mm)	Actinomycete isolate No.	Inhibition zone (mm)	Actinomycete isolate No.	Inhibition zone (mm)
L	23	78	17	57	14
89	20	43	17	27	13
Z	20	28	17	102	13
31	20	32	16	100	13
44	19	90	16	91	12
103	19	26	16	36	12
U	19	12	16	80	12
55	19	56	15	69	12
77	19	48	15	62	11
82	19	58	15	N	10
103	18	54	15	58	10
30	18	35	15	01	9
95	18	M	15	87	8
R	18	G	14	70	7
24	18	96	14	88	6
81	18	101	14		
93	17	76	14		

Fig. 1: A) *In vitro* Agar disk bioassay of three *Streptomyces* isolates (peripheral plugs) against (*Phytophthora drechsleri*) (center plug) indicating antifungal inhibition and *in vivo* greenhouse results in pistachio seedlings indicative of root rot (B) in pots inoculated with the pathogen alone and (C) pots inoculated with both pathogen and the antagonist (*Streptomyces* sp. isolate 30) showing prominent suppressive effect of the antagonist upon the pathogen, however, other three isolates produced similar results

the tested pathogen. Table 1 shows the screening results of the active isolates. Fig. 1-A shows the result of *in vitro* bioassay in Agar disk method.

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 tested *Streptomyces* isolates.

***In vivo* greenhouse studies:** The results of biological control of *Streptomyces* isolate 30 against *P. drechsleri* the causal agent of gummosis and root rot in pistachio seedlings are indicated in Fig. 1-B and C. As the data shows, there is clear suppression of the pathogen in pots received the antagonists. However, the other three isolates (*Streptomyces* isolate 43, 44 and 95) produced similar suppressive effect upon the pathogen.

DISCUSSION

An approach to environmentally safe method in control of plant diseases in the field or greenhouse is use of no synthetic fungicide, instead, using natural biofungicides. It is possible to amend the soil mix with selected natural 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 perform a preliminary screening of Streptomycetes from restricted soils of Kerman Province. 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 (Shahidi Bonjar, 2003). 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 (Mathre *et al*., 1999). 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.

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