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Biological Control of *Fusarium oxysporum* f. sp. *melonis*, the Causal Agent of Root Rot Disease of Greenhouse Cucurbits in Kerman Province of Iran

[1]Sara Shafii Bafti, [1]Gholam Hosein Shahidi Bonjar, [1]Sonia Aghighi
[1]Sara Biglari, [2]Parvin Rashid Farrokhi and [3]Ataollah Aghelizadeh
[1]Department of Plant Pathology and Biotechnology
College of Agricultural Sciences, Bahonar University of Kerman, Iran
[2]Department of Agronomy and Plant Breeding
College of Agricultural Sciences, Bahonar University of Kerman, Iran
[3]Management and Planning Organization of Kerman Province, Kerman, Iran

Abstract: Antagonistic activity of 178 soil actinomycete isolates was assayed against *Fusarium oxysporum* f. sp. *melonis* Schlecht, Emend (Snyde and Hansen) cause of root rot and fusarium wilt of greenhouse cucurbits in Kerman Province, southeast of Iran. From tested isolates, *Streptomyces olivaceus* (strain 115) showed anti-fusarium activity revealed through screening and bioassays by agar disk and well-diffusion methods. The active strain was grown in submerged cultures for determination of growth curve and preparation of crude extract for further biological characterizations. Antifungal activity was fungistatic type on the pathogen mycelia. It is prominent that amending greenhouse soil mix with the *S. olivaceus* (strain 115) will reduce crop losses by the pathogen.

Key words: *Fusarium oxysporum* f. sp. *melonis*, *Streptomyces olivaceus*, Antifungal, Biological Control, Bioassay

INTRODUCTION

The search for new principles in combating plant pathogens, different from the currently used fungicides, is of worldwide concern. This demand originates from the problems as fungicidal resistance, which results in high application rates of many synthetic fungicides having adverse effects in the environment in consequence [1-3]. Microorganisms play a major source of antibiotic substances [4]. Actinomycetes are one of the most attractive sources of antibiotics and other biologically active substances of high commercial value, such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitors [5-11]. Their ability to parasitize and degrade spores of fungal plant pathogens is well established [12]. They are able to metabolize many different compounds including sugars, alcohols, amino acids and aromatic compounds by producing extracellular hydrolytic enzymes. Their metabolic diversity is due to their extremely large genome which has hundreds of transcription factors that control gene expression, allowing them to respond to specific needs [13]. Streptomyces species are the most widely studied and well known of the actinomycetes. 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. Besides, they have been much studied as potential producers of antibiotics and exert antagonistic activity against wide range of bacteria and fungi [14, 15]. These prokaryotes have been much studied as potential producers of antibiotics worldwide [4, 16]. The results even show that use of streptomycetes enhances growth of the crop plants [17]. With the increased concern about conserving natural resources as air, soil and water, natural or biological control of plant diseases has received increased emphasis. 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 [18]. Phytopathogenic fungi are of major problems in agriculture. *Fusarium oxysporum* is ubiquitous phytopathogen causing root rot, vascular wilt and damping off in many plant species [19]. With extended environmental diversity, however, the actinomycetes microflora of the Iranian soils has not been very well explored with the goal of exploring new means of biocontrol principles. Concerning the merits of their role in biological control of soil-borne fungal-pathogens, at the present research 178 isolates of actinomycetes were isolated from agricultural soils of Kerman province, Iran and screened against *F. oxysporum* f. sp. *melonis*. 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 in biological control of the

plant pathogens. From 178 tested isolates of actinomycetes, one isolate, *Streptomyces olivaceus* (strain 115) showed prominent antifungal activity against *F. oxysporum* f. sp. *melonis*, the causal agent of root rot, vascular wilt and damping off in greenhouse cucurbits of Kerman Province of Iran.

MATERIALS AND METHODS

Culture Media: Casein glycerol (or starch) agar (CGA) was used for screening and isolating of actinomycetes which composed of: glycerol or soluble starch, 10 g; casein, 0.3 g; KNO_3, 2 g; NaCl, 2 g; K_2HPO_4, 2 g; $MgSO_4.7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4.7H_2O$, 0.01 g and agar, 18 g in 1 L of distilled H_2O (pH 7.2) [18]. In submerged cultures, agar was excluded (CG medium). Actinomycete colonies with different morphologies were selected and transferred to CGA slants for further studies.

Preparation of Fungal Isolate: *Fusarium oxysporum* f. sp. *melonis* Schlecht, Emend (Snyder and Hansen) the causal agent of root rot disease of greenhouse cucurbits was kind gift from Prof. Banihashemi, Mycology Lab., Dept. of Plant Pathology, College of Agriculture, Shiraz University, Shiraz, Iran. The fungus was grown at 25-26°C and maintained on potato dextrose agar (PDA, Difco). All cultures stored at 4°C and sub-cultured as needed.

Isolation of Actinomycetes from Soil: For isolation of actinomycetes, 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 [20]. 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 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, pure actinomycete isolates were collected and maintained refrigerated in stock.

Screening Procedures and *in vitro* Antifungal Bioassays

Agar Disk-Method: 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. Disks were then aseptically transferred to PDA plates having fresh lawn culture of *F. oxysporum* f. sp. *melonis*. Controls included using plain disks from CGA medium. Plates were incubated at 25- 26°C for 4- 6 days and bioactivity was evaluated by measuring the diameter of inhibition zones (DIZ, mm) [18, 21].

Classification of the Active Isolate of Actinomycete: Actinomycete 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) [22]. Identification procedures of the active isolate were done by Saadoun *et al.*, Dept. of biological sciences, University of Science and Technology, Irbid, Jordan [23].

Bioassays: To evaluate the antifungal activity of *S. olivaceus* against the pathogen, bioassays were performed in two ways: agar disk and well methods as used by Aghighi and Shahidi Bonjar and Shahidi Bonjar [7, 8]. Antifungal activity around the *S. olivaceus* agar disk or well was evaluated as follows and the ratings used were modified from those of Lee and Hwang [20] and El-Tarabily *et al.*[24]: (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 or well filled with CG medium.

Monitoring Activity: In submerged cultures, active *S. olivaceus* (strain 115) was grown in CG medium on rotary shakers under 130 rpm at 30°C. To monitor the activity, aseptically small aliquots of culture media were taken every 24 h for 30 days and the activity was evaluated by well diffusion-method [18] against lawn cultures of *F. oxysporum* f. sp. *melonis* and antifungal activity was measured as described. In solid cultures, active *S. olivaceus* (strain 115) was grown in CGA as streaks and to monitor the activity, aseptically 6 mm

agar disks were taken by sterile cork borer every day for 15 days and the activity was evaluated by agar disk-method [21] against lawn cultures of *F. oxysporum* f. sp. *melonis* and antifungal activity was measured as mentioned.

Preparation of Crude Extract: 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 (1 mm^3) of *S. olivaceus* (strain 115) against *F. oxysporum* f. sp. *melonis* was transferred to fresh PDA plates and incubated for 7 days at 26°C. During incubation, growth or lack of growth of the fungus was investigated both visually and microscopically. Rejuvenation of growth was indicative of fungistatic and lack of growth represented fungicidal properties of the antagonist.

Greenhouse Studies: Pathogenisity of *F. oxysporum* f. sp. *melonis* on cucurbit seedlings investigated as follows. Seedlings 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). In two leaves-stage, containers were gently cut to desoil the seedling roots by gentle rinse of soil mix in tap water. Spore suspension of the pathogen was prepared by adding 2-3 ml sterile distilled water to Petri dishes of well grown lawn culture of the pathogen and collecting the liquid in small beakers. Desoiled bared roots were dipped in the spore suspension for 10 min and replanted. Treated pots were irrigated by spore suspension afterwards. Controls included use of tap water instead of spore suspension.

RESULTS

Screening and Bioassays: In screening for actinomycetes having antagonistic activity against *F. oxysporum* f. sp. *melonis,* 178 isolates of soil actinomycetes from Kerman Province, southeast of Iran, were screened from which one isolate showed strong activity against the tested pathogen. Bioassay results are indicated in Fig. 1 and 2.

Identification of Active Streptomycetes: Taxonomic criteria and identity of the active streptomyces was kind work by Saadoun *et al.*, Dept. of biological sciences, University of Science and Technology, Irbid, Jordan. The isolate was determined as *Streptomyces olivaceus* (strain 115) as a new record from Iran having prominent activity against *F. oxysporum* f. sp. *melonis,* the causal agent of root rot disease of greenhouse cucurbits.

Monitoring Activity: The results of activity versus time in submerged and solid media cultures are indicated in Fig. 3. Since the activity reaches its maximum after 10-15 days in rotary submerged cultures, this period was used to harvest cultures for preparation of crude extract for use in future investigations.

Fungicidal and/or Fungistatic Activity: Transfer of blocks of inhibition zones of *S. olivaceus* (strain 115)

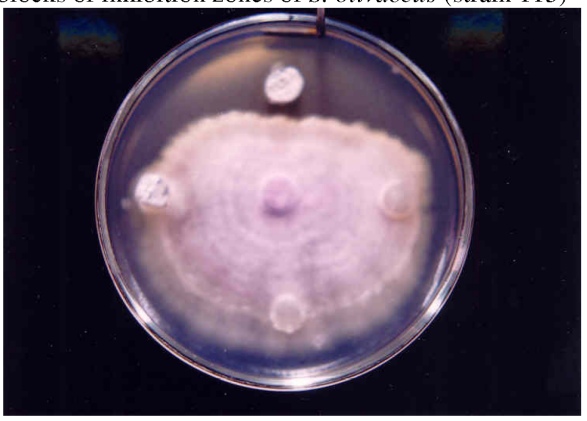

Fig. 1: Test Result of Screening Using Agar Disk-Method. Center, Agar Plug of *Fusarium oxysporum* f. sp. *melonis* with Radial Growth; Top, Agar Plug of *Streptomyces olivaceus* (Strain 115) Showing Inhibitory Effect upon the Mycelia Mat of *F. oxysporum* f. sp. *melonis;* Right, Plain Agar Plug as Control; Bottom and Left are two Inactive Streptomyces Isolates Showing no Inhibitory Effect Against the Pathogen

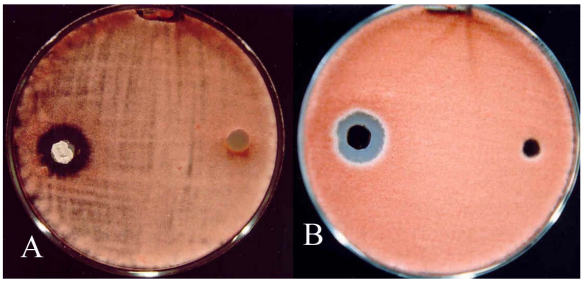

Fig. 2: Bioassay Results of Antifungal Activity of *Streptomyces olivaceus* (strain 115) Against *Fusarium oxysporum* f. sp. *melonis* Performed in Agar Disk Method (A) and Well Method (B). Left A, Agar Disk of *S. olivaceus* (Strain 115) Indicating Complete Inhibition; Right A, Plain Agar Disk as Control; Left B, Well Containing 13th Day Aqueous Submerged-Culture of *S. olivaceus* (Strain 115) Indicating Complete Inhibition of the Pathogen and Right B, Blank Well as Control. In Both Methods, Antifungal Activity was of Fungistatic Type

against *F. oxysporum* f. sp. *melonis* to fresh PDA plates revealed afterward growth of the pathogen which was indicative of fungistatic activity of the active strain.

Greenhouse Studies: Pathogenisity of *F. oxysporum* f. sp. *melonis* on vascular wilt of melon, *Cucumis melo* L., revealed symptoms in the 7 th day after inoculation showing wilt and damping off. Controls remained healthy. The result is shown in Fig. 4.

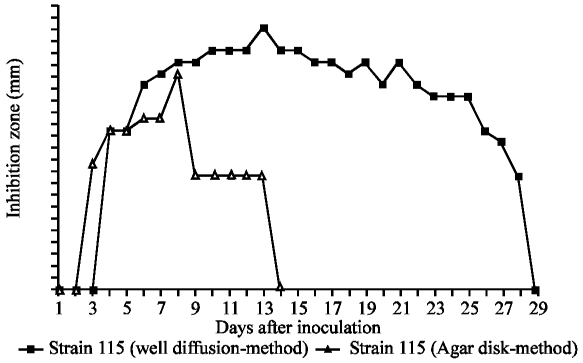

Fig. 3: Monitoring the Antifungal Activity of *Streptomyces olivaceus* (strain 115) Against *Fusarium oxysporum* f. sp. *melonis* in Submerged and Solid Media Cultures Indicated as Inhibition Zone (mm) Versus Days After Inoculation. Optimum Production Time of Active Principle in Submerged Culture was 10 - 15 Days of Post Seeding and 8th Day in Solid Medium.

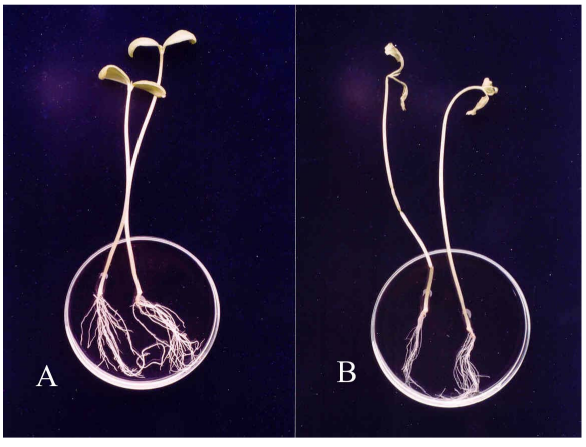

Fig. 4: Pathogenisity of *Fusarium oxysporum* f. sp. *melonis* on Seedlings of Commercial Melon, *Cucumis melo* L., Under Greenhouse Conditions. Left (A) Show Controls and Right (B) are Treated Seedlings Showing Symptoms as Root Rot, Collar Necrosis, Wilt, Damping off and Death

DISCUSSION

Strains of *Streptomyces* by virtue of their wide distribution in soil and antibiotic production, may participate actively in establishing the microbial equilibrium of cultivated soils and may be a factor in affecting the incidence of certain soil-borne plant pathogens [25]. An environmentally safe measure in control of important diseases as fusarium wilts in greenhouses 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 actinomycete in restricted area of Kerman Province, southeast of Iran. 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 soil born diseases of plants [8]. 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. Whether these transgenic plants and the crops derived from them gain acceptance in the marketplace remains to be seen [26]. If the definition of biological control includes the plant induced or genetically modified to defend itself, then biological control has been the most significant approach to plant health management during the 20th century and promises though modern biotechnology to be even more significant in the 21st century. Indeed, the promise of the new tools of biotechnology for crop-based agriculture has arrived, all based on the use of transgenes from microorganisms. Furthermore, the many facets of genomics research, applied to plants and microorganisms, can be expected to reveal entirely new biological approaches to plant health management-approaches likely to make the current use of transgenes for resistance seem crude by comparison [27]. Nearly all private investments in biological control today, at least in the United States, are for transformation of plants to express genes from microorganisms. In these examples, the plant rather than the microorganism becomes the biological control agent [28]. 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 *S. olivaceus* (strain 115).

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