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Laboratory Preparation of a New Antifungal Agent from *Streptomyces olivaceus* in Control of *Fusarium oxysporum* f.sp. *melonis* of Cucurbits in Greenhouse

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Abstract: In greenhouse cucurbits of Kerman Province, *Fusarium oxysporum* f.sp. *melonis* Schlecht, Emend (Snyder and Hansen) causes root rot and fusarium wilt. To investigate for new biofungicides, antagonistic activity of soil Actinomycetes isolates were assayed against the pathogen from which *Streptomyces olivaceus* strain 115 showed anti-fusarium activity both *in vitro* and *in vivo* experiments. The active strain was grown in aqueous media on rotary shakers to monitor activity versus time and prepare active dry crude for further biological and physical studies. Antifungal activity was of fungistatic type on the pathogen mycelia. From the results of our studies it is clear that usage of *S. olivaceus* strain 115 as a biofungistatic natural product applied as an amendment in greenhouse soil mix will lead to inhibition or reduction of the pathogen effects.

Key words: *Streptomyces olivaceus*, antifungal, biological control, *Fusarium oxysporum* f.sp. *melonis*

INTRODUCTION

It is well established that Actinomycetes are one of the most attractive sources of biologically active substances such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitors[1-3]. *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[4,5]. The results even show that use of Streptomycetes enhances growth of the crop plants[6]. The search for new principles in combating plant pathogens, different from the currently used fungicides, is of worldwide concern[7-9]. 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[10]. *Fusarium oxysporum* f.sp. *melonis* is ubiquitous phytopathogen causing root rot, vascular wilt and damping off in many plant species[11]. For evaluation of Actinomycetes microflora of the Iranian soils with the goal of exploring new means of biocontrol principles, 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 or biofungicide in biological control of the plant pathogens. From all tested isolates of Actinomycetes, one isolate, *Streptomyces olivaceus* strain 115, showed high 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 and preparation of fungal isolates: *F. oxysporum* f. sp. *melonis* was kind gift from Prof. Banihashemi, Mycology Laboratory, Department of Plant Pathology, College of Agriculture, Shiraz University, Shiraz, Iran. It was maintained on potato dextrose agar (PDA) (Difco), 39 g PDA L^{-1} of distilled H_2O (pH 7.2). Casein glycerol (or starch) agar (CGA) prepared from basic ingredients as described by Kuster and Williams[12] and used as Actinomycetes culture.

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Screening procedures and antifungal bioassays: To evaluate theantifungal activity of *S. olivaceus* against the pathogen, bioassays were performed in two ways: Agar disk and well methods as used by Shahidi Bonjar[2]. 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[13] and El-Tarabily *et al.*[14]: (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: *S. olivaceus* strain 115 was grown in CG medium in rotary cultures with 130 rpm at 30°C throughout the experiment. 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 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 against lawn cultures of *F. oxysporum* f.sp. *melonis* and antifungal activity was measured as mentioned.

Preparation of crude extract: In rotary cultures, when the activity reached maximum, the cultures were harvested. Spores and mycelia were excluded by filtration through two layers of cheese cloth and 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* against *F. oxysporum* f.sp. *melonis* was transferred to fresh PDA plates and incubated for 7 days at 25-26°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.

Monitoring activity: Activity reached maximum after 10-15 days in rotary cultures. This interval was used to harvest cultures to prepare crude extract for use in future investigations. Activity versus post seeding time in rotary cultures is presented in Fig. 1.

Fungicidal and/or fungistatic activity: Transfer of blocks of inhibition zones of *S. olivaceus* against *F. oxysporum* f.sp. *melonis* to fresh PDA plates revealed afterward growth of the pathogen which was indicative of fungistatic activity of *S. olivaceus*.

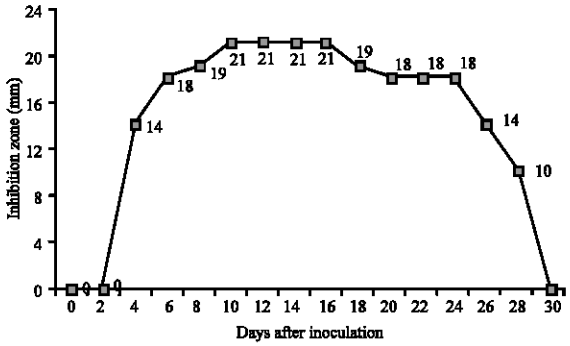

Fig. 1: Bioactivity of *Streptomyces olivaceus* strain 115 in rotary cultures indicative of production time versus inhibition zones against *Fusarium oxysporum* f.sp. *melonis* measured *in vitro* by well diffusion method

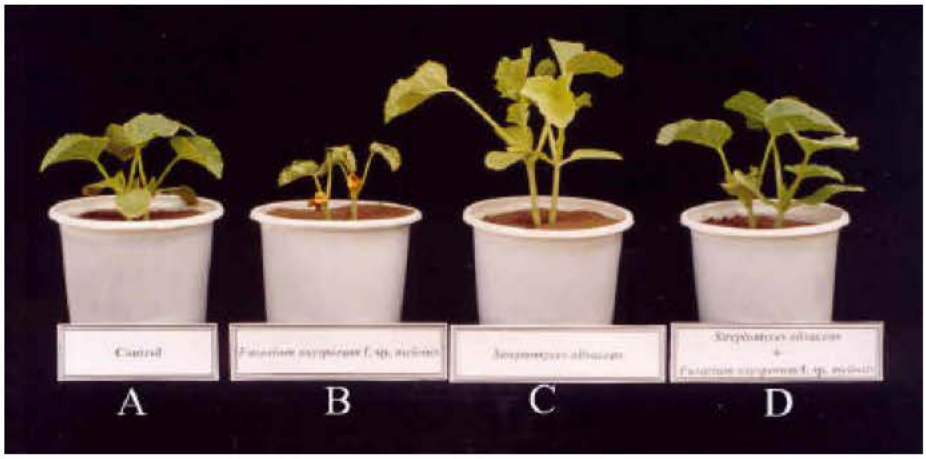

Fig. 2: Antifungal activity of *Streptomyces olivaceus* strain 115 in control of *Fusarium oxysporum* f.sp. *melonis* of cucurbits under greenhouse conditions. Left to right: A) control pot, received no treatment; B) pot received root treatment with *F. oxysporum* f.sp. *melonis*; C) pot received only *S. olivaceus* strain 115 and D) pot received both *F. oxysporum* f.sp. *melonis* and *S. olivaceus* strain 115

Greenhouse studies: Pathogenisity of *F. oxysporum* f.sp. *melonis* on vascular wilting of melon *Cucumis melo* L. revealed symptoms in the 7th day after inoculation showing wilt and damping off. Controls remained healthy. Fig. 2 represents antifungal activity of *Streptomyces olivaceus* strain 115 in controlling *Fusarium oxysporum* f.sp. *melonis* of cucurbits under greenhouse conditions in different treatments.

DISCUSSION

As a worldwide perspective, an environmentally safe measure in control of plant diseases in the field or greenhouses is use of no synthetic fungicide, instead, use of natural biofungicides if possible. One approach is 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 Actinomycetes in restricted soils 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[2]. 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[1-5]. 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[1-6]. We believe that the results of these findings can form the avenue for production of resistant transgenic-plants with recombinant DNAs having antifungal genes cloned from biologically active *S. olivaceus* strain 115.

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