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First Report of Antifungal Properties of a New Strain of *Streptomyces plicatus* (Strain101) Against Four Iranian Phytopathogenic Isolates of *Verticillium dahliae*, A New Horizon in Biocontrol Agents

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Abstract: This is the first report on antifungal activity of a new strain of *Streptomyces plicatus* (strain101) against four Iranian phytopathogenic isolates of *Verticillium dahliae.* In the recent decades, biological control of plant diseases has received more attention mainly as a response to public concern about the use of hazardous chemicals in the environment. Soil Actinomycetes particularly *Streptomyces* spp. enhance soil fertility and have antagonistic activity against wide range of soil-borne plant pathogens. In search for metabolites of soil Actinomycetes having antifungal activity against four isolates of the cosmopolitan pathogen, *Verticillium dahliae* Klebahn, 110 isolates were screened. Among all, strain101 that was identified as a new strain of *S. plicatus*, showed high level of activity in Agar disk and Well-diffusion methods. *S. plicatus* was propagated in submerged cultures and active crude was prepared upon which some biological characterizations performed. The active metabolite (s) is polar, soluble in H_2O and methanol but insoluble in chloroform, dichloromethane or hexane. Antifungal activity composed of two types, mycelial inhibition, inhibition of microsclerotia and melanin production. Antifungal gene from *S. plicatus* Strain 101 may be a useful candidate for genetic engineering of agriculturally important crop plants for increased tolerance against *V. dahliae*.

Key words: Antifungal, *Streptomyces plicatus*, *Verticillium dahliae*, bioassay

INTRODUCTION

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[1]. *Verticillium dahliae* Kleb. and *V. albo-atrum* Reinke et Berth., stand out in importance both in agriculture and scientific literature. *V. dahliae* is a root pathogen causing vascular infections in more than 300 plant species. Verticillium wilts are overwhelmingly diseases of dicotyledonous plants in temperate regions. Monocotyledonous plants very occasionally have been cited as true hosts for *Verticillium* [2]. Major maladies include vascular wilting (tracheomycosis, hadromycosis) of pistachio, olive, avocado, stone fruits, elm, tomato, eggplant, cotton, alfalfa, mint, strawberry, rose, chrysanthemum, potato early dying and many others[3,4]. Except for soil solarization, there are currently no effective control methods available. The classical control measure of soil fumigation is loosing its popularity because of its adverse effects on the biological buffering of soil [5-7]. Wide host range, long persistence in soil, propagation on rhisosphere of non-host plants and lack of effective control measurements highlights the need for research in evaluation of new methods for control of this pathogen[8-10]. In search for biocontrol agents, several fungi as *Talaromyces flavus, Gliocladium* spp., *Trichoderma* spp. and *Penicillium chrysogenum* and bacteria as *Bacillus subtilis, Pseudomonas fluorescens*, *Stenotrophomonas maltophilia*, Actinomycetes (specially *Streptomyces* spp.) and *Serratia* spp. have shown activity against *V. dahliae*[11-17].

Saprophytic Gram-positive bacteria in the genus *Streptomyces* have been shown to have characteristics which make them useful as biocontrol agents against soil-borne fungal plant pathogens. These characteristics include the production of different kinds of secondary metabolites and biologically active substances of high

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commercial value such as enzymes (which degrade the fungal cell wall directly) and antibiotics [18,19]. Soil Streptomyces 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 [20-23]. Several workers have reported that *In vitro* studies have documented satisfactory results in use of *Streptomyces* against some root pathogens. For example, *Streptomyces* sp. strain 5406 has been used in China for the last 35 years to protect cotton crops against soil-borne pathogens [24]. The results even show that use of *Streptomyces* enhances growth of the crops and vegetables [25, 26]. Some of commercially produced antifungals driven from *Streptomyces* are: Cycloheximide from *S. griseus* used in control of bacterial diseases of lawn and cherry leaf spot; Blastcidin-S from *S. griseochromogenes* and Kasugamycine from *S. kasugaensis* used in control of rice blast pathogen, *Piricularia oryzae* [27], Rhizovit[R] from *S. rimosus* and Mycostop[R] from *S. griseoviridis* that are used in control of wide range of fungi as *Pythium* spp., *Fusarium* spp., *Phomopsis* sp., *Phytophthora* spp. *Rhizoctonia solani, Alternaria brassicola, Botrytis* sp. and *Fusarium* spp. [28, 29].

Some workers have reported biological control of *Verticillium* by *Streptomyces* spp. Chi and Hanson [30] reported *In vitro* antifungal activity of *S. rimosus* against *V. dahliae* and *V. albo-atrum*. There are also reports on control of root fungal-pathogens by use of seed and seedling treatments with spore suspensions of some *Streptomyces* isolates [31].

With extended environmental diversity, however, the *Streptomyces* microflora of the Iranian soils has not been very well explored with the goal of exploring new means of biocontrols. With the respect to their role in biological control of soil-borne fungal-pathogens, at the present research 110 isolates of Actinomycetes were isolated from agricultural soils of Kerman province, Iran and screened against four isolates of *V. dahliae* through which strain No. 101 was the most active demonstrated by *In vitro* studies. Preliminary characterization and biological properties of this strain is being reported here.

MATERIALS AND METHODS

Culture media: A synthetic medium, Casein glycerol (or starch) agar (CGA) was used for screening and isolating of 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)[1]. In submerged cultures, Agar was excluded (CG medium). Actinomycetes colonies with different morphologies were selected and transferred to CGA slants for further studies[32,33]. All *V. dahliae* isolates were grown at 26°C and maintained on potato dextrose agar (PDA) (Difco).

Preparation of *V. dahliae* isolates: Four registered phytopathogenic isolates of *V. dahliae* were used. One pistachio isolate was obtained from the Laboratory of Plant Pathology of Agricultural Research Center, Ministry of Agriculture, Kerman, Iran. It had been isolated from roots of diseased pistachio trees in Bahramjerd region of Kerman, having widespread infection by this pathogen. Three others, cotton (Shiraz, Iran), potato (Kerman, Iran) and olive (Gorgan, Iran) isolates were obtained from Prof. Banihashemi, Mycology Laboratory of the Department of Plant Pathology, College of Agriculture, Shiraz University, Shiraz, Iran.

Soil sampling and 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[18]. 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, Actinomycetes colonies were isolated on CGA, incubated at 28°C for one week and stored refrigerated as pure cultures before use. For screening studies 110 pure Actinomycetes isolates were collected.

Screening procedures and *in vitro* antifungal bioassays agar disk-method: Each Actinomycetes 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 Actinomycetes colony mass was prepared by using sterile cork borers. Disks were then

aseptically transferred to PDA plates having fresh lawn cultures of *V. dahliae* isolates. Controls included using plain disks from CGA medium. Plates were incubated at 27°C for 4-6 days and bioactivity was evaluated by measuring the diameter of inhibition zones (DIZ, mm) [1, 34].

Dual culture bioassay: Fungal mycelial-disks (diameter of 6mm) prepared from growing margin of cultures of test *V. dahliae* isolates and placed in the center of PDA plate and at 30 mm distance from it, the *Streptomyces* disks (prepared as mentioned) were placed. Plates incubated at 27°C for 2-3 weeks [1]. Antifungal activity was indicative as mycelial growth of *V. dahliae* isolates was prohibited in the direction of active *Streptomyces* isolate. The level of inhibition at dual cultures was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist colony (γ) from the fungal growth radius ($\gamma°$) of a control culture to give $\Delta\gamma = \gamma° - \gamma$. The ratings used were modified from those of Lee *et al.* [18] and El-Tarabily *et al.*[35], where $\Delta\gamma$: 5 – 9 mm, + (weak inhibition); $\Delta\gamma$: 10 - 19 mm, ++ (moderate inhibition) ; and $\Delta\gamma > 20$ mm, +++ (strong inhibition). Controls included *V. dahliae* mycelial plugs in center of non- *Streptomyces* inoculated PDA plates.

Well diffusion method: For evaluation of antifungal activity of aqueous samples, by use of sterile cork borer wells (6 x 4 mm, 2 cm apart) were punctured in fresh lawn cultures or at 30 mm distance from plugs of *V. dahliae* isolates. Respective concentrations in dimethyl sulfoxide: methanol (1/1: v/v) solvent (DM solvent) were then administered to fullness in each well. Plates were incubated at 26°C for 4- 6 days for lawn cultures and 14 days for dual culture disk-plugs. Bioactivity was determined by measuring inhibitory zones (mm). Each experiment was repeated three times and the mean of inhibitory zones recorded. Controls included use of blank wells and use of DM solvent without test compounds [1].

Submerged cultures and preparation of crude extract: Strain101, the most active among other isolated *Streptomyces* strains, was grown in submerged cultures of CG medium on rotary shakers under 130 rpm at 30°C. To monitor the activity versus post seeding time, aseptically small aliquots of culture media were taken every 24 hr for 20 days and the activity was evaluated by well diffusion-method[1,34]. To prepare crude extract, after 9-10 days of post seeding which the activity reached its 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, pulverized and kept refrigerated before use.

Classification of *Streptomyces* strain 101: Streptomyces colonies were characterized morphologically and physiologically following the direction mentioned in the methods manual of international cooperative project for description and deposition of cultures of *Streptomyces* (ISP) [36].

Morphological characterization: Streptomyces colonies on glycerol-nitrate-casein agar were transferred onto oatmeal agar and streaked across the plate and incubated in the dark at 27°C for 21 days.

Color determination: This made for: a) Mass color or mature, sporulating aerial surface growth, b) The color of substrate mycelium as viewed from the reverse side and c) Diffusible soluble pigments other than melanin. Observation was made after 21 days and was limited to mature cultures with heavy spore mass surface using code for determining the color of aerial mycelium of Streptomycetes composed by Prauser [37] for color tabs of Baumann Farbtonkarte Atlas I.

Determination of morphological characteristic of the sporebearing hyphae: The spore-bearing hyphae characteristics were determined by direct microscopic examination of the culture surface (21 days old) on opened dishes of the crosshatched cultures using 100 x magnification. The species involved in the genus Streptomyces divide into sections: Rectus (R) or straight, flexible (F) or flexeous, Retinaculum-Apertum (RA) and spiral (S).

Spore morphology surface: The spore surface of the isolate was examined under scanning electron microscope at a magnification of 8000 to 100000 x. Preparation for the scanning electron microscope consisted of scratching the culture of 14-21 day old growing on oatmeal agar by a sterile needle and depositing the specimen onto specimen aluminum stubs which held by a piece of double stick scotch tape. The stubs were placed in a sputter coater (Polaron Equipment Ltd. E 5000) for 2-3 min. (approximately 150°A of gold deposited). The gold sputterer was set at 1-4 kv, 20 mA and 0.1 torr. After coating, the specimens were viewed with a Lutz 100 A scanning electron microscope with an accelerating voltage of 20 kv. Secondary electron images were recorded with black and white film.

Melanin production: Peptone iron agar was used for the detection of deep brown to black diffusible pigment (+). Absence of the color was recorded as negative(-).

Carbon utilization: The following sugars were tested, L-arabinose, D-xylose, meso- inositol, D-mannitol, D-fructose, rhamnose, raffinose and sucrose. Preparation was done as described in the ISP [36].

Characterization of *Streptomyces* Strain 101 to species level was based on morphological, cultural and physiological characteristics following the directions given for the International *Streptomyces* project (ISP) [36]. General morphology was determined on oatmeal agar plates, incubated in the dark at 27°C for 21 days, by direct light microscopy examination of the surface of crosshatched cultures. Colors were determined according to the scale adopted by Prauser [37] and melanin reactions were detected by growing the isolate on at least one of the ISP media (Nos. 6 and 7) [36]. Strain 101 was identified as a new strain of *Streptomyces plicatus*.

Determination of minimum inhibitory concentrations (MIC): To measure the MIC values, two-fold serial dilutions of 20, 10, 5, 2.5, 1.25, 0.625 and 0.312 mg ml^{-1} of the crude extract were prepared in DM solvent and assayed by well diffusion-method as described by Shahidi Bonjar [38]. The MIC was defined as the lowest concentration able to inhibit any visible fungal growth. All data represent average of three replicated experiments.

Solubility studies of active crude in organic solvents: To evaluate the relative polarity of the active principle (s) present in the crude, 2 ml of each of H_2O, methanol, DMSO: Methanol (1:1, v/v), chloroform, dichloromethane and hexane were added to 20 mg pulverized-crude samples separately and vortexed for 20 min. Each sample was then centrifuged at 3000 rpm for 15 min. Supernatants and pellets were separated, dried under reduced air at 50°C and assayed at concentration of 10 mg ml^{-1} by Agar diffusion-method [1].

Determination of shelf life or stability of active crude: To measure the stability of the active crude in both soluble and dry states, 5 mg ml^{-1} of each sample was prepared in DM solvent and 5 mg dry samples placed in small vials. These samples were kept at room temperature and tested using Agar diffusion-method for anti *Verticillium* activity at 14 days intervals as long as the activity persisted.

Effect of culture filtrate on spore germination of *V. dahliae* isolates: To evaluate of the ability of culture filtrate of strain101 to inhibit conidial germination of *V. dahliae* isolates, to each well of depression slides, 0.2 ml of the culture filtrate of strain101 was placed and dried at room temperature. Spore suspension (5×10^{-3} ml) from each of *V. dahliae* isolates prepared in distilled water and added over each of the dried culture filtrates separately. Slides incubated in a humid chamber at 26°C for 24 h. Controls included CG medium plus spore suspension. Spore germination of *V. dahliae* isolates and germ tube characteristics were monitored and compared to the control with a light microscope [1, 39].

RESULTS

Screening and bioassays: In screening for metabolites of soil Actinomycetes having antifungal activity against four isolates of the cosmopolitan pathogen, *Verticillium dahliae* Klebahn, 110 isolates were screened from which strain101 showed high level of activity as indicated in Fig. 1 and 2.

Determination of MIC: In well diffusion-method, MIC of the crude was determined as 2.5 mg ml^{-1} against all of the four *V. dahliae* isolates.

Solubility active crude in organic solvents: Solubility results are indicated in Table 1. As the results show, apparently the active principle (s) has a polar nature since activity is recoverable only in H_2O, methanol supernatants and pellets of chloroform, dichloromethane and hexane treatments

Shelf life or stability of active crude: Stability of the active crude in DM solvent and dry form at room temperature (12- 30°C) was 2 and 6 months respectively, assayed by using Agar diffusion-method against four *Verticillium dahliae* isolates.

Antifungal activity of submerged cultures: Activity versus post seeding time in submerged media cultures is indicated in Fig. 3. Since the activity reaches its maximum after 9- 10 of post seeding, this time was used to harvest cultures for preparation of crude extract.

Table 1: Bioassay results of solubility tests of the antifungal principle (s) of *Streptomyces plicatus* Strain 101 against *Verticillium dahliae* in fractions of different solvents indicated by well diffusion-method at 10 mg ml^{-1} of dry crude

Solvent	Fraction	Yield (%)	Activity
H_2O	S*	90	+
	P*	10	-
Methanol	S	15	+
	P	85	-
DMSO: Methanol	S	95	+
(1:1, v/v)	P	5	-
Chloroform	S	0.5	-
	P	99.5	+
Dichloromethane	S	2	-
	P	98	+
Hexane	S	1	-
	P	99	+

S*: supernatant, P*: pellet.

Table 2: Morphologicaland physiological characterization of Streptomyces plicatus strain 101

Morphological criteria									Physiological criteria							
Spore chain	Sporulation of aerial mycelia	Aerial mass color	Fragmentation of substrate mycelia	Spore surface	Selerotia formation	Melanoid pigment	Reverse side pigment	Soluble pigment	Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Surcose	Raffinose
S	LC	Gy	0	S m	0	0	0	0	-	+	+	+	+	+	-	-

S: Spiral LC: Long chain Gy: Grey 0: Negative Sm: Smooth -: No utilization +: Utilization

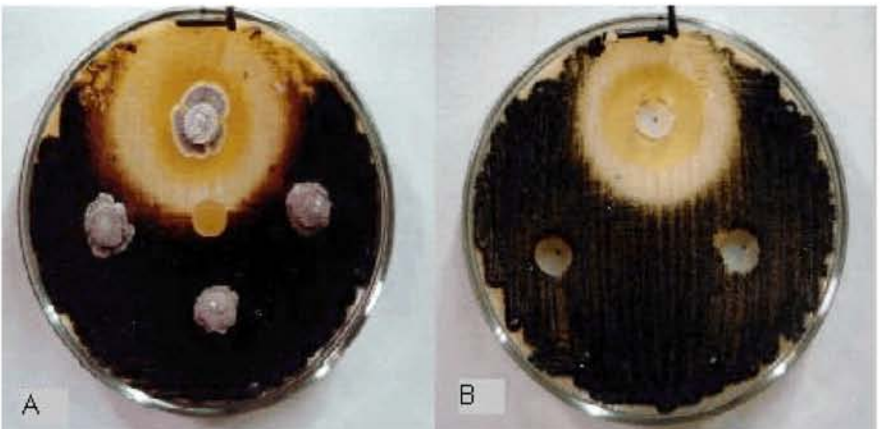

Fig. 1: Antagonistic activity of *Streptomyces plicatus* Strain 101 against pistachio root-isolate of *Verticillium dahliae* investigated by two methods. A) Agar disk-method, clockwise from top: Strain101, next three disks are from inactive isolates and middle represents blank agar disk (controls). B) Well diffusion-method, clockwise from top: Crude extract of *S. plicatus* Strain 101 at 5 mg ml $^{-1}$, DM solvent and blank (controls).

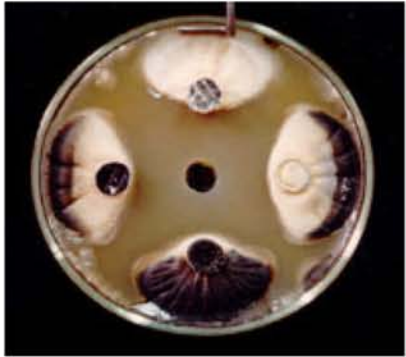

Fig. 2: Antifungal activity of culture filtrate of *Streptomyces plicatus* Strain 101 against four isolates of *Verticillium dahliae*, clockwise from top: pistachio, olive, potato and cotton isolates tested by well-diffusion method at 10mg ml^{-}.

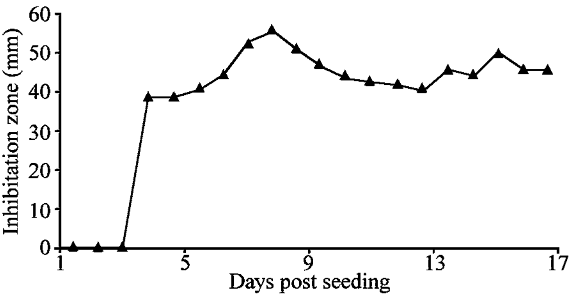

Fig. 3: Activity versus post seeding time in submerged media cultures of *Streptomyces plicatus* strain 101 against pistachio root-isolate of *Verticillium dahliae* monitored by well diffusion-method

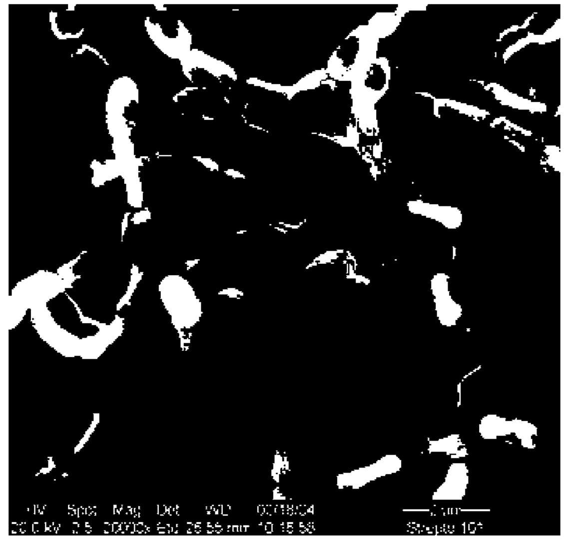

Fig. 4: Scanning electron micrograph of spore chains of *Streptomyces plicatus* Strain 101

Spore germination: Culture filtrate of strain101 inhibited spore germination of all four *V. dahliae* isolates whereas controls showed no inhibition.

Inhibition of mycelial growth, microsclerotia formation and melanin production: As revealed by microscopic examinations, antagonistic activity of *S. plicatus* isolate No. 101 against isolates of *Verticillium dahliae* consists of two inhibitory effects. Clear zones adjacent to the wells which represent complete mycelial growth (fungicidal activity) and in their periphery, there is microsclerotia inhibition zone (Fig. 1) which indicates inhibition of melanin formation in the pathogen.

Taxonomy of *Streptomyces* Strain 101: Strain 101 was identified as *Streptomyces plicatus* which is a new record from Iran.

Table 2 shows the complete identification of this isolate based on morphological and biochemical characterization. Scanning electron micrograph of spore chains of strain 101 is indicated in Fig. 4.

DISCUSSION

Although the nature and number of active antifungal principles involved in the present study are not clear, but the prominent anti *Verticillium* activity of *S. plicatus* strain No. 101 highlights it as a candidate for further investigation in biological control of this pathogen.

Control of *Verticillium* wilt has largely been based on soil fumigation with broad-spectrum biocides, most of which will soon be banned from the market because of their adverse effects on the environment. With this worldwide perspective, to help the soil ecosystem environmentally safe and non chemical-measures are to be developed for combating plant diseases. In ideal biological control measures, proper micro organisms are those having well adaptation in soil and rizosphere exerting effective antagonistic activity against soil pathogens persistently. In this regard soil-driven *Streptomyces* do not have adverse effect or alter the biological buffering of soils as chemical measures do. They should receive higher attention in research for biological controls worldwide.

Phytopathogenic fungi are of major problems in agriculture. Genetic engineering provides an opportunity to protect plants from fungal diseases and to reduce the use of synthetic fungicides. The genes for antifungal metabolites can be engineered into plants to increase the resistance of crop plants to fungal attack, decreasing the use of environmentally unfriendly fungicides. The major factor limiting the application of this technology is the identification and isolation of useful genes that code for antifungal metabolites. Plants themselves are a potential source of new antifungal activities. For example, two antifungal proteins have been isolated from radish. The genes for these proteins were cloned and constitutively expressed in transgenic plants, which showed enhanced resistance to pathogenic fungi [40, 41]. The main objective in our study was to identify biologically active *Streptomyces* isolates against the cosmopolitan plant pathogen, *Verticillium dahliae*. Preliminary data in this paper describes the presence of potential antifungal metabolite (s) in *Streptomyces plicatus* (strain101) against four Iranian phytopathogenic isolates of *V. dahliae.* This strain prohibits mycelial growth and formation of melanin in the tested pathogen. Lack of melanin renders the pathogen more susceptible to antagonists and hence favors its biocontrol in soil. Having special environmental

characteristics and being rich in Actinomycete population, the microbiology of the Iranian soils has to be further explored for new active isolates of *Streptomyces*.

Results of these findings may form the avenue for production of r esistant transgenic-plants with recombinant DNA having antifungal genes cloned from biologically active *Streptomyces* spp. Expression of cloned genes in transgenic plants has provided evidence in plant defense [19]. The genes encoding many antifungal proteins are currently being used by agribusiness to create genetically modified plants that have increased fungal resistance in field [42]. Expression of cloned chitinase genes in transgenic plants has provided evidence of their role in plant defense [43]. Thus it may be assumed that the antifungal-metabolite gene from *Streptomyces plicatus* Strain 101 may be a useful candidate for genetic engineering of agriculturally important crop plants for increased tolerance against *V. dahliae*.

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