

Biological Control of *Sclerotinia sclerotiorum*, Causal Agent of Sunflower Head and Stem Rot Disease, by Use of Soil borne Actinomycetes Isolates

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Abstract: Problem statement: High level of biosafety and non adverse effects on the environment of biocontrol strategies of pest management, are priorities of tomorrow's world agriculture. Actinomycetes are active biocontrol agents due to their antagonistic properties against wide range of plant pathogens particularly fungi. Fungal pathogens are liable for a big part of damages in agriculture economy. **Approach:** In the present research antifungal bioactivity of 50 isolates of Actinomycetes collected from soils of Kerman province of Iran was investigated against *Sclerotinia sclerotiorum* (Lib.), the causal agent of stem rot in sunflower, through agar disc method and dual culture bioassays. The *Streptomyces* isolate No. 363 was propagated in submerged cultures and active crude was prepared upon which several biological characterizations performed. Greenhouse studies were achieved to confirm laboratory results. **Results:** Among the tested *Streptomyces* isolates, 10 isolates revealed antagonistic properties in dual culture procedure from which isolate No. 363 showed highest bioactivity. The active metabolite of *Streptomyces* isolate No. 363 was polar and well soluble in H₂O. Using agar-disc method, progressive growth of the pathogen was highly reduced by the antagonist through exhibiting ability to constitute fungus-free zones of inhibitions. The results indicated that isolate No. 363 was a proper candidate for field biocontrol studies. **Conclusion:** Results may open a horizon for production of resistant transgenic plants having antifungal properties originated from biologically active *Streptomyces* spp. recognition and production of effective metabolite(s) of *Streptomyces* spp. which was responsible for antifungal activities will be our commercial goal due to rich reserves of soil borne Actinomycetes in Iran.

Key words: Actinomycetes, biocontrol, *sclerotinia sclerotiorum*, *streptomyces*, sunflower

INTRODUCTION

The population on the earth exceeded over 6.5 billion people in 2008. It will be certainly a great challenge to prepare this volume of population with food. Beside wars, natural disasters and progress of deserts, various pests and plant pathogens harm agricultural crops and thus threaten food resources all over the world. Sunflower (*Helianthus annuus* L.), an

important oilseed plant in food industries to produce vegetable oils, is a good example of plants threatened with pests and pathogens, particularly fungi leading to a significant decrease of crop yield. *S. sclerotiorum* that causes head and stem rot in sunflower, is an aggressive fungal agent which attacks and infects nearly 400 plant species^[16]. Control of this fungal pathogen is of importance due to wide range of its infections in different plants (especially oilseed plants like sunflower

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and canola). But there are few methods which can increase the crop yield and protect food immunity simultaneously. However physical and chemical methods of control, which damage crops with toxins and heat, biological control is a safe way to defy *S. Sclerotiorum* due to antibiosis via production of antibiotics and toxins against pathogen without having any adverse effect on crop. Actinomycetes have been known as efficient biocontrol agents that naturally exist in soil and have the ability to inhibit growth of plant pathogens among which *Streptomyces* spp. have been shown to have characteristics which make them useful as antagonistic agents against soil-borne fungal plant pathogens. These characteristics include the production of different kinds of secondary metabolites and biologically active substances of high commercial value such as enzymes (which degrade the fungal cell wall directly) and antibiotics^[7,9,12]. Soil *Streptomyces* are of the major contributors to the biological buffering of soils and have roles in decomposition of organic matter conducive 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^[4-6]. Biocontrol of soil-born fungal or bacterial pathogens will be of increasing importance for a more sustainable agriculture. However, that the transgenic plants and the produce derived from them gain acceptance in the marketplace remains controversial^[13].

MATERIALS AND METHODS

Culture media: Casein Glycerol (or starch) Agar (CGA) was used for screening and isolating Actinomycetes and composed of: Glycerol or starch 10 g; casein, 0.3 g; KNO₃, 2 g; NaCl, 2g; k₂Hpo₄, 2 g; MgSO₄.7H₂O, 0.05 g; CaCO₃, 0.02 g; FeSO₄.7H₂O, 0.01 g and agar, 18 g in 1 L of distilled H₂O (pH 7.2)^[2]. Actinomycetes colonies with different morphologies were selected and transferred to CGA slants for further studies^[11,12].

Isolation of actinomycetes from soil: For isolation of Actinomycetes, soil samples were collected from grasslands and vegetable fields in different localities of Kerman province (Iran). Several samples were randomly 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^[9]. Soil samples were taken from a depth of 8-12 cm below the soil surface. 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 were mixed with sterile distilled water (100 mL). Mixtures were shaken vigorously for 15 min and then allowed to settle for 15 min. 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 10⁻³-10⁻⁶ soil dilution to autoclaved CGA (1 mL in 25 mL CGA) at 50°C before pouring the plates and solidifications. Three replicates were considered for each dilution. Plates were incubated at 28°C for up to 7 days^[12]. From eighth day on, 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 in stock.

Preparation of fungal isolate: Pure culture of *S. sclerotiorum*, the causal agent of stem and head rot disease in sunflower was obtained from Dr. Nikkhab, Mycology Lab, Dep. of Plant Pathology, college of Agriculture, Tehran University, Iran. The fungus was grown at 25°C and maintained on potato dextrose agar (PDA, Difco, 39 g PDA L⁻¹ of distilled H₂O, pH = 7.2). All cultures stored at 4°C and sub-cultured as needed.

Screening procedure and *in vitro* antifungal bioassays: To evaluate the antifungal activity of isolated *Streptomyces* against the pathogen, bioassays were performed in agar disk method as used by Shahidi Bonjar and Aghigh^[1,14]. Antifungal activity around the *Streptomyces* agar disks was evaluated as follows and the ratings used were modified from those of Lee and Hwang^[9] and El-Tarabily *et al.*^[3].

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 Actinomycetes colony mass was prepared by using sterile cork borers. Disks were then aseptically transferred to PDA plates having fresh lawn culture of *S. sclerotiorum*. Controls included using plain disks from CGA medium. Plates were incubated at 28°C for 6 days and bioactivity was evaluated by measuring the Diameter of Inhibition Zones, (DIZ mm)^[2].

Dual culture bioassay: Fungal mycelial-disks (diameter of 6 mm) prepared from growing margin of cultures of test *S. sclerotiorum* isolates and placed in the center of PDA plates and at 30 mm distance from it, the *Streptomyces* disks (prepared as mentioned) were

placed. Plates incubated at 28°C for 8 days^[2]. Antifungal activity was indicative as mycelial growth of *S. sclerotiorum* isolates was prohibited in the direction of active *Streptomyces* isolate. Antifungal activity around the Actinomycetes agar disk was evaluated as follows and the ratings used were modified from those of Lee and Hwang and El-Tarabily *et al.*^[1] no inhibition = mycelia growth not different from control (-); (2) weak inhibition = partial inhibition of mycelia growth, measured as a diameter of 5-9 mm (+); (3) moderate inhibition = Almost complete inhibition of mycelia 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^[3,9].

Submerged cultures and preparation of crude extract: *Streptomyces* isolate 363, the most active among other Actinomycetes isolates, was grown in submerged cultures of CG medium on rotary shakers under 130 rpm at 28°C. To monitor the activity versus post seeding time, aseptically small aliquots of culture media were taken every 24 h for 30 days and the activity was evaluated by well diffusion-method^[2]. To prepare crude extract, after 7 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.

Ascertainment of curve of bioactivity in submerged culture: From day 3rd to 20th, aseptically small aliquot samples were taken consecutively from the active isolate which was seeded in submerged CG medium that had been placed on rotary shaker at 130 rpm at 29 C. All samples kept frozen before bioassays. To monitor the bioactivity, all samples were evaluated in well diffusion method against the pathogen in two replications as described previously.

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, 0.312, 0.156, 0.078 and 0.039 mg ml⁻¹ of the crude extract were prepared in Dimethyl sulfoxide: Methanol (1:1 v/v) (DM solvent) and assayed by well-diffusion method as described by Shahidi Bonjar^[15]. The MIC was defined as the lowest concentration that is able to inhibit any visible fungal growth.

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 H₂O, methanol and chloroform were added to 10 mg pulverized-crude samples separately and vortexed for 20 min. Each sample was then centrifuged at 3000 rpm for 15 min using a bench low speed centrifuge. Supernatants and pellets were separated, dried under reduced air at 50°C and bioassayed at concentration of 10 mg ml⁻¹ by Agar diffusion-method^[2].

Determination of shelf life or stability of active crude: To measure the stability of the active crude in soluble state, 5 mg mL⁻¹ samples were prepared in distilled water and placed in small vials. These samples were kept at room temperature and tested using Agar diffusion-method for anti *S. sclerotiorum* activity at 14 days intervals as long as the activity persisted.

Determination of Thermal Inactivation Point (TIP): Small aliquots (5 mg mL⁻¹) of soluble crude were exposed to each of 30, 40, 50, 60, 70, 80, 90 and 95°C for 10 min and cooled on ice afterwards to monitor effect of temperature on bioactivity. Bioactivity of treated samples was evaluated using well diffusion method. Control included incubation of an untreated sample at 28°C^[10].

In vivo greenhouse studies: Greenhouse tests were accomplished in a conventional greenhouse using 7-10 day old sunflower seedlings with approximately 10 cm uniform stem sizes grown in sterilized soil mix in Styrofoam containers. Tests performed on a complete randomized plan with the repetition of ten pots for each treatment. Four treatments included as follows: (A) *S. sclerotiorum*, (B) *Streptomyces* isolate No. 363, (C) *S. sclerotiorum* plus *Streptomyces* isolate No. 363 and (D) Control. To prepare inocula, *Streptomyces* isolate No. 363 was cultured on CGA and *S. sclerotiorum* was grown on PDA media. All seedling stems were wounded as surface slashes using a sterile razor blade. Wounds in control received sterile wet cotton pads and covered with Parafilm[®] to avoid dryness. Similarly other treatments received a 2 cm² of the media mat of the well grown pathogen and/or the antagonist and covered similarly. All treated plants covered with transparent plastic bags for 48 h to prevent accidental dryness and provide high relative humidity for onset of pathogen/antagonist activities. Symptoms recorded 10 days after inoculation at 25°C. To compare the relative growth of the plants in the four treatments, seedlings were carefully desoiled through detailed rinsing in tap water, stems and roots cut apart and dried at 60°C for 48 h and weighed with accuracy. The experiment was repeated twice and means were recorded.

Scanning electron microscope studies: Ornamentation of spore chains and mycelial morphology of the *Streptomyces* isolate 363 were examined with use of a scanning electron microscope model CAMSCAN-MV2300. Preparation for the scanning electron microscope consisted of using the culture of 21-28 day old growing on CGA and depositing the specimen onto specimen aluminum stubs which held by a piece of double stick scotch tape. The stubs were coated in a sputter coater for 2 min. Afterwards the specimens were viewed and digital electron micrographs were prepared at magnification of 6000-20000 X with an accelerating voltage of 20 kv accordingly.

RESULTS

Screening and bioassays: In screening for Actinomycetes with antifungal activity, 50 isolates were screened from which 10 isolates showed activities against *S. sclerotiorum* (Fig. 1 and 2). Based on the screening results, in comparison to others, *Streptomyces* isolate No. 363 had maximum inhibition zone against *S. sclerotiorum* and selected for further evaluations.

Monitoring activity in shaken culture: The results of activity versus time in submerged culture are indicated in Fig. 3. In rotary submerged culture, activity of *Streptomyces* isolate No. 363 against *S. sclerotiorum* reached the maximum at 7th post inoculation day. Activity versus post seeding time in submerged media cultures is shown in Fig. 3. Since the activity reaches its maximum after 7th day of post seeding, this time was used to harvest cultures for preparation of crude extract for future investigations.



Fig. 1: Bioassay results of *Streptomyces* isolates against *Sclerotinia sclerotiorum*. Clockwise from top: Isolate 139, isolate 347, 246 and blank agar disk (control). Center disk is *S. sclerotiorum* agar inoculum disk. Clear inhibition zones around the *Streptomyces* agar disks are representatives of lack of growth and bioactivity against the pathogen

Determination of MIC: In well diffusion-method, MIC of the crude of *Streptomyces* isolate No. 363 against *S. sclerotiorum* was determined as 1.25 mg ml⁻¹.

Solubility of active crude in organic solvents: Solubility results are shown in Table 1. As the results show, the active principle (s) has a polar nature and well soluble in distilled water.

Shelf life or stability of active crude: Stability of the active crude in DM solvent was 75 days, assayed by using Agar diffusion-method against *S. sclerotiorum*.

Table 1: Bioassay results of solubility tests of the antifungal principle (s) of *Streptomyces* isolate No. 363 against *Sclerotinia sclerotiorum* in fractions of different solvents evaluated by well diffusion-method at 10 mg ml⁻¹ of dry crude

Solvent	Fraction	Activity
Chloroform	S*	-
	P*	+
Methanol	S	+
	P	+
H ₂ O	S	+
	P	+

*S: Supernatant, *P: Pellet, +: Soluble, -: Insoluble



Fig. 2: Bioassay result in Agar Disk-Method of *Streptomyces* isolate No. 363 against *Sclerotinia sclerotiorum*. Center disk is *S. sclerotiorum* agar inoculum disk

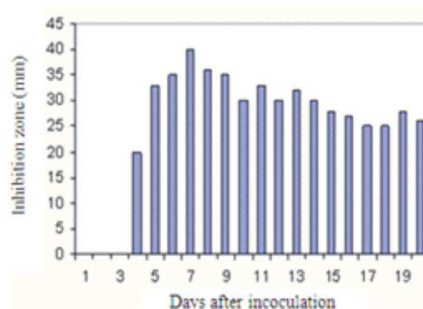


Fig. 3: *In vitro* bioassay results of *Streptomyces* isolate 363 against *Sclerotinia sclerotiorum* in rotary cultures indicative of production time versus inhibition zones



Fig. 4: *In vivo* greenhouse results in sunflower seedlings (P): In plants inoculated with the pathogen alone and (A+P): Plants inoculated with both pathogen and the antagonist *Streptomyces* isolate 363, (A): Plants inoculated with *Streptomyces* isolate 363 alone and (C): Untreated control plants



Fig. 5: Digital scanning electron micrograph of spore chains of *Streptomyces* isolate 363

Determination of TIP: Bioactivity of active isolate diminished to zero at 90°C.

***In vivo* greenhouse studies:** Symptoms as stem rot and wilt appeared 4-6 days after inoculation in seedlings inoculated with *S. sclerotiorum*, while other treatments did not develop signs of the disease. The results of this experiment which indicates promising biological control of *Streptomyces* isolate No. 363 against *S. sclerotiorum* the causal agent of head and stem rot in sunflower in greenhouse are shown in Fig. 4.

Scanning electron microscope studies: Scanning electron micrograph of spore chains of *Streptomyces* isolate 363 is shown in Fig. 5.

DISCUSSION

Overdose usage of chemicals as pesticides and fertilizers is common in farming in most parts of the world which threatens food safety of individuals. Some pesticides can be hardly cleaned from nature and

have a potential capability to have adverse effect or destroy useful microorganisms which have positive effects in fertility of soil and growth of plants. To lower or avoid side effects, biological control is an alternative and proper choice in pest management. In ideal biological control measures, proper microorganisms are those having well adaptation in soil and rizosphere exerting effective antagonistic activity against soil pathogens persistently. In the *in vitro* study to fight sunflower stem rot, a natural agent (Actinomycete) was used that had effective antagonistic characteristics against *S. sclerotiorum*. In green house study, similar to *in vitro* tests, *Streptomyces* isolate 363 suppressed fungal diseases in inoculated plant without any contamination in the soil and plant. It also increased growth of plants. A commercial product containing *Streptomyces* isolate 363 or its effective metabolite is suggested to avoid head and stem rot in sunflower. Identification the effective metabolite (s) and search genes responsible for its function can be the topic for future and perfecting researches which also consider Actinomycetes (like *Streptomyces* isolate 363) as both biological control agents and biological fertilizers. Several workers have reported that *In vitro* studies have documented satisfactory results in use of *Streptomyces* against some pathogens^[8]. Some other workers have reported biological control of *S. sclerotiorum* by *Streptomyces* spp. Tahtamouni reported *In vitro* antifungal activity of some of *Streptomyces* isolates against *S. sclerotiorum* which confirmed earlier findings by El-Tarabily *et al.*^[3] who had reported such activity against *S. minor in vitro*, as well as those of Gupta *et al.* against several phytopathogenic fungi and Saadoun *et al.*^[11,12] against several food-associated fungi and molds. Findings confirm importance of the *Streptomyces* isolates as biocontrol agents and also emphasize the importance of indigenous *Streptomyces* spp. as biocontrol agents against wide range of fungal phytopathogens^[1,17] with the future perspective of replacement with chemical control measures. 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^[1]. Performing genetic modifications of plants to resist phytopathogens is of high importance that related specialists do pay attention to. Translation of genes from an antagonist of the pathogen to plant, without making new damages or problems for plant and environment, may be a choice by which we can control diseases of plants from the

early stages of growing to the end of plant life. After recognition of biocontrol agents, projects should be designed to find their effective principle(s) and gene(s) encoding related traits and at the end, translate gene(s) to the target plant. Thus it may be assumed that the antifungal-metabolite gene from *Streptomyces* isolate 363 may be a useful candidate for genetic engineering of agriculturally important crop plants such as sunflower for increased tolerance against *S. sclerotiorum*.

CONCLUSION

Results may open a perspective for production of resistant transgenic plants having antifungal properties originated from biologically active *Streptomyces* spp.

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