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In Vitro Antagonistic Antifungal-Activity of *Streptomyces* Isolate 339 Against *Magnaporthe oryzae*

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ABSTRACT

Biological control offers an environmentally friendly alternative to the use of antimicrobials for controlling plant diseases. A collection of about 100 Actinomycete strains was screened for the ability to produce metabolites that inhibit *Magnaporthe oryzae* growth *in vitro*. The *Streptomyces* isolate 339 showed strong *in vitro* antagonistic activity against *M. oryzae* in Agar disc and Well-diffusion methods by producing extracellular antifungal metabolites. The isolate No. 339 was propagated in submerged cultures and active crude was prepared upon which some biological characterization performed. Antifungal activity of this antagonist was of fungistatic type on the pathogen. The active metabolite (s) is polar, soluble in H_2O and methanol but insoluble in chloroform, dichloromethane or hexane. Thermal inactivation point of active phase of *Streptomyces* isolate 339 was 80°C. Antifungal gene from isolate 339 may be a useful candidate for genetic engineering of agriculturally important crop for increased tolerance against *M. oryzae*.

Keywords: Biological Control, Antifungal, Actinomycete, *Magnaporthe oryzae*, *Streptomyces*

1. INTRODUCTION

In modern agriculture, use of agrochemicals is falling into public disfavor because of food residuals, environmental pollution and detrimental effects on a variety of nontarget organisms. Potential use of microbes based biocontrol-agents as replacement or supplements for agrochemicals has been addressed in many recent reports (Shimizu *et al.*, 2000). 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 (Dhingra and Sinclair, 1995). *Streptomyces* spp. have been shown to have characteristics which make them useful as biocontrol agents against plant pathogens. These characteristics include the production of different kinds of secondary metabolites and biologically active substances of high commercial value such as enzymes and antibiotics and they are of the major contributors to the biological buffering of soils and have roles in decomposition of organic matter conductive to crop production (Champness, 2000; Gottlieb, 1973; Kong *et al.*, 2001; Okami and Hotta, 1988). Rice blast, caused by *Magnaporthe oryzae* (anamorph *Pyricularia oryzae* Cav.), is the most destructive diseases of rice (*Oryza sativa* L) world-wide and can result in significant reductions in yield (Te Beest *et al.*, 2007).

At the present research 100 isolates of Actinomycetes were isolated from agricultural soils of Kerman of Iran and screened against *M. oryzae in vitro*. The objective of the present study was also to isolate *Streptomyces* isolates having antagonistic properties with the aim that they can serve as gene donors in developing resistant transgenic plants and use as soil amendments as biofertilizer or biofungicide in biological control of the tested pathogen. From all tested isolates of Actinomycetes, *Streptomyces* isolate No. 339 showed high *in vitro* anti rice-blast activity.

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2. MATERIALS AND METHODS

2.1. Culture Media and Preparation of Pathogen

Pure culture of *Magnaporthe oryzae* was obtained from Mr. Padasht, Iran Rice Research Institute (IRRI), Rasht. The pathogen was maintained on Potato Dextrose Agar (PDA, Difco-39 g PDA L^{-1} of distilled H_2O, pH 7.2). Casein Glycerol Agar (CGA) was prepared from basic ingredients as described by Kuster and Williams (1964) and used as Actinomycetes culture.

2.2. Soil Sampling and Isolation of Streptomycetes

Soil samples were collected from grassland, orchards and vegetable fields in different localities of Kerman provinces, Iran. Several samples randomly were selected from the 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}-10^{-6} soil dilutions to autoclaved CGA (1, 25 mL^{-1} 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 100 pure *Streptomyces* isolates were collected (Zarandi *et al*., 2009).

2.3. 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 (2003). Antifungal activity around the *Streptomyces* agar disks was evaluated as follows and the ratings used were modified from those of (Lee and Hwang, 2002; 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.

2.4. Monitoring Activity in Shaked Culture

Streptomyces isolate 339 was grown in submerged culture of CG medium on rotary shakers under 130 rpm at 29°C. To monitor the activity, small aliquots of culture media were taken every 24 h for 18 days and the activity was evaluated by well diffusion-method (Dhingra and Sinclair, 1995; Acar and Goldstein, 2005).

2.5. Classification of the Active Antagonists

From ten active *Streptomyces* isolates one showed high antagonistic activity and their colonies were characterized morphologically and physiologically to the genus level (Shirling and Gottlieb, 1966; Saadoun and Gharaibeh, 2002).

2.6. Determination of Minimum Inhibitory Concentrations (MIC)

To measure the MIC values, two-fold serial dilutions of 50, 25, 12.5, 6.25, 3.125, 1.562 and 0.781 mg ml^{-1} of the crude extract were prepared in DM solvent and assayed by well diffusion-method as described by Shahidi Bonjar (2004a). The MIC was defined as the lowest concentration able to inhibit any visible fungal growth. All data represent average of three replicated experiments.

2.7. 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 and chloroform were added to 50 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, at 50°C and assayed at concentration of 50 mg mL^{-1} by Agar diffusion-method (Shahidi Bonjar, 2004b).

2.8. Determination of Shelf Life or Stability of Active Crude

To measure the stability of the active crude in soluble state, 50 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 *M. oryzae* activity at 14 days intervals as long as the activity persisted.

2.9. Determination of Thermal Inactivation Point (TIP)

Small aliquots (50 mg mL^{-1}) of soluble crude were exposed to each of 30, 40, 50, 60, 70, 80 and 90°C for 10 min and cooled on ice afterwards to monitor the effect of temperature on bioactivity. Bioactivity of treated samples was evaluated using well diffusion method. Control included incubation of an untreated sample at 29°C (Nawani and Kapadnis, 2004).

2.10. Detection of Fungicidal and/or Fungistatic Activity

Small blocks of inhibition zones (1 mm^3) of *Streptomyces* isolate 339 against *M. oryzae* was transferred to fresh PDA plates and incubated for 7 days at 26-28°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.

3. RESULTS AND DISCUSSION

3.1. Antifungal Bioassays

From tested Actinomycete isolates, ten isolates were active in dual culture methods from which two *Streptomyces* isolates of 339 and 328 showed the highest activity. **Figure 1** shows bioassay results of antifungal inhibitory effects of these two isolates against *M. oryzae* measured in agar disk-method.

3.2. Monitoring Activity and Growth Curve

Activity reached maximum after 11 days in rotary cultures. In shaked cultures, this interval was used to harvest cultures to prepare crude extract for use in further investigations. Activity versus post seeding time in rotary cultures is presented in **Fig. 2**. Colony morphology of *Streptomyces* isolate 339 in submerged culture of CG medium is indicated in **Fig. 3**.

Fig 1. Antifungal inhibitory effects of two *Streptomyces* isolates on *Magnaporthe oryzae*. Top: *Streptomyces* isolate 339 and Bottom: *Streptomyces* isolate 328. Center disk is *Magnaporthe oryzae* agar disk which its growth towards the antagonists is inhibited clearly

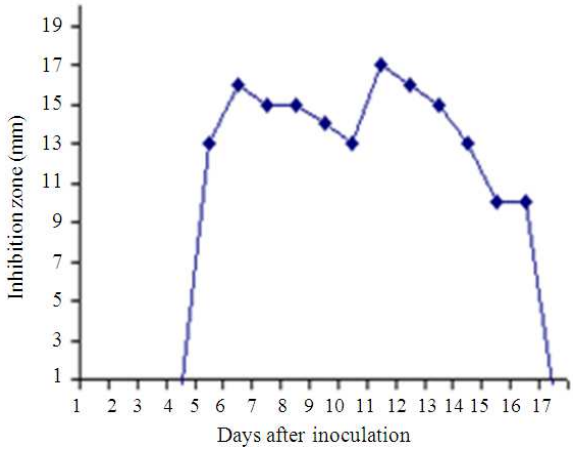

Fig. 2. *In vitro* bioassay results of *Streptomyces* isolate 339 against *Magnaporthe oryzae* in rotary cultures indicative of production time versus inhibition zones

3.3. Taxonomy of *Streptomyces* Isolate 339

Figure 4 shows scanning electron micrograph of spore chains of *Streptomyces* isolate 339.

3.4. Determination of MIC

In well diffusion-method, MIC of the crude was determined as 50 mg mL^{-1} against *M. oryzae*.

Fig. 3. Colony morphology of *Streptomyces* isolate 339 in submerged culture of CG medium

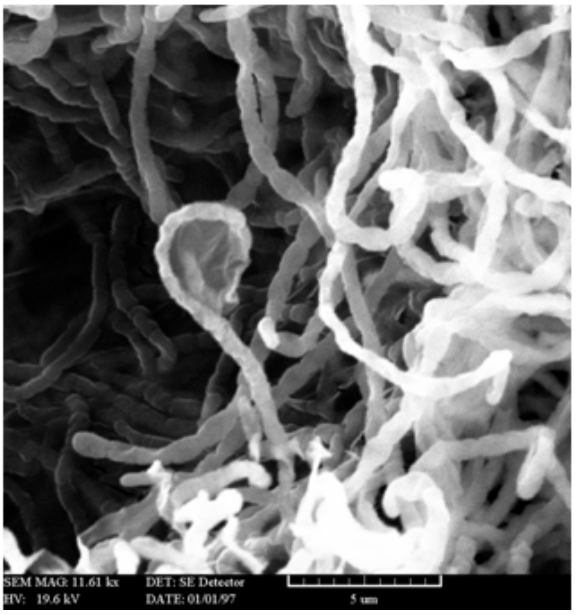

Fig. 4. Scanning electron micrograph of spore chains of *Streptomyces* isolate 339

3.5. Solubility Active Crude in Organic Solvents

The results show, apparently the active metabolite (s) is polar, soluble in H_2O and methanol but insoluble in chloroform, dichloromethane or hexane.

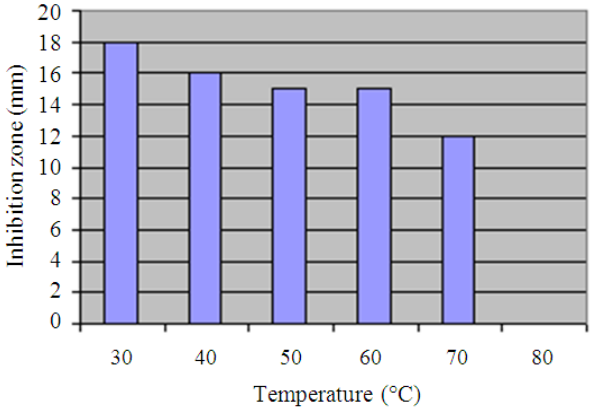

Fig. 5. Temperature effect on antifungal bioactivity of crude preparation of *Streptomyces* isolate 339 at 50 mg mL^{-1} against *Magnaporthe oryzae*

3.6. Shelf Life or Stability of Active Crude

Stability of the active crude in distilled water at room temperature (12-30°C) was about 30 days, assayed by using Agar diffusion-method against *M. oryzae*.

3.7. Determination of TIP

Bioactivity of active isolate diminished to zero at 80°C (**Fig. 5**).

3.8. Fungicidal and/or Fungistatic Activity

Transfer of blocks from inhibition zones to fresh PDA plates revealed afterward growth of the pathogen which was indicative of fungistatic activity of *Streptomyces* isolate 339.

4. CONCLUSION

Actinomycetes produce more than half of the world's antimicrobials and are consequently becoming valuable tools in the field of biological control. Antifungal activity of the isolate found in this study highlights its importance as candidate for further investigation in biological control of tested pathogen.

M. oryzae is an important problem in world 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 chemicals. The major factor limiting the application of this technology is the identification and isolation of useful genes that code for

antifungal metabolites. We are foreseeing that *Streptomyces* isolate 339 receives research attention as a proper candidate for genetic engineering of rice plants for increased tolerance against *M. oryzae*.

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