

Biological Control of Rice Blast (*Magnaporthe oryzae*) by use of *Streptomyces sindeneusis* isolate 263 in Greenhouse

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Abstract: Soil Actinomycetes particularly *Streptomyces* spp. have antagonistic activity against wide range of plant pathogens. In the recent decades they have attracted high interests as biocontrol agents. In search for finding such principles, *in vitro* suppression of *Magnaporthe oryzae* the causal agent of rice blast disease was studied by use of *Streptomyces sindeneusis* isolate 263 in greenhouse. Spray of rice seedling-leaves with of mixed spore suspension of the pathogen and *S. sindeneusis* isolate 263 resulted in strong inhibition of the pathogen and suppression of leaf symptoms. Propagation of the antagonist crude sap was performed in aqueous cultures and bioactivity was monitored in shaken cultures. Ongoing goals of this research include isolation, characterization and identification of the active metabolites and future goals include identification of active genes for use in development of recombinant DNAs in transgenic rice varieties bearing elevated resistance to infections by *M. oryzae*.

Key words: *Magnaporthe oryzae*, *Pyricularia oryzae*, *Streptomyces*, Actinomycetes, Biocontrol

INTRODUCTION

Streptomyces are one of the most attractive sources of biologically active substances such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitors^[1-3]. 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^[4,5]. Studies even show that use of Streptomyces enhances growth of the crop plants^[6]. The search for new principles in biocontrol of plant pathogens different from the classical used fungicides, is of world wide concern^[7-9]. Biological control of plant diseases is slow, gives few quick profits, but can be long lasting, inexpensive and harmless to life. Rice blast, caused by *Magnaporthe oryzae* (anamorph *Pyricularia oryzae* Cav.), is considered the most important disease of rice because of its world-wide distribution and the resulting severe yield loss^[10]. 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, 10 *Streptomyces* isolates showed high *in vitro* anti rice-blast activity.

MATERIALS AND METHODS

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 H₂O, pH 7.2). Casein glycerol agar (CGA) was prepared from basic

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ingredients as described by Kuster and Williams^[11] and used as Actinomycetes culture.

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^[12]. 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.

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^[2]. Antifungal activity around the *Streptomyces* agar disks was evaluated as follows and the ratings used were modified from those of Lee and Hwang^[12] and El-Tarabily *et al.*^[13]: (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.

Monitoring activity in shaken culture: *Streptomyces* isolate 263 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 108 days and the activity was evaluated by well diffusion-method^[14,15].

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 following the direction mentioned in the methods manual of international cooperative project for description and deposition of cultures of *Streptomyces* (ISP)^[16]. Identification procedures of the active isolates were done by Saadoun *et al.*, Dept. of biological sciences, University of Science and Technology, Irbid, Jordan as described by Saadoun and Gharaibeh^[17].

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^[18]. 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 H₂O, methanol and chloroform 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 using a bench low speed centrifuge. Supernatants and pellets were separated, at 50°C and assayed at concentration of 20 mg mL⁻¹ by Agar diffusion-method^[19].

Effect of chloroform on active principle (s): To confirm the result of chloroform sensitivity, 15-20 mL of chloroform was added to 300 mg pulverized-crude and were maintained on rotary shakers under 130 rpm at 29°C for 24 h. Then vortexed and divided to three small tubes, supernatants and pellets were separated and dried as above. Equal volumes of chloroform were added to each tube, vortexed and centrifuged and dried under reduced air, the quantitative experiment was conducted as above two times. Then tubes were placed in a desiccator for 24 h. All samples were tested by well diffusion method.

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 *M. oryzae* activity at 14 days intervals as long as the activity persisted.

Determination of thermal inactivation point (TIP): Small aliquots (10 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. For temperatures over than 90°C heat oil is used. Bioactivity of treated samples was evaluated using well diffusion method. Control included incubation of an untreated sample at 29°C^[20].

Detection of fungicidal and/or fungistatic activity: Small blocks of inhibition zones (1 mm³) of *S. sindeneusis* 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.

Greenhouse studies: Seeds of commercial rice *Oryza sativa* L. (cv. Kazemi) grown under greenhouse conditions in plastic pots containing sterilized sand and humus of decayed leaves (3:1 w/w). Seeds were planted 3-4 cm below soil surface per pot. In three leaves-stage, they were separated in four groups with ten pots in each and treated in following groups: (a) pathogen alone, (b) *S. sindeneusis* isolate 263, (c) pathogen plus *S. sindeneusis* isolate 263 and (d) control (untreated). 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. For inoculation, a conidial suspension of *M. oryzae* (4×10⁵ conidia mL⁻¹) with Triton X-100 (250 ppm) was sprayed on the treated rice plants. At the same time selected mixtures of culture filtrates from *S. sindeneusis* isolate 263 plus pathogen and isolate 263 alone were sprayed on rice plants separately in b and c treatments. Controls included use of tap water instead of spore suspension. Percentage of diseased leaf area of fifty plants per treatment was determined according to the method developed by International Rice Research Institute (IRRI). The rice seedlings were desoiled, washed and incubated for 48 h at 60°C to measure the dry weights. The experiment was repeated twice and means recorded.

RESULTS

Antifungal bioassays: From tested Actinomycete isolates, ten isolates were active in dual culture methods. Figure 1 shows two antagonists and the suppressive reaction of *M. oryzae*. Figure 2 shows bioassay results of *S. sindeneusis* isolate 263 against *M. oryzae* measured in agar disk-method. In both methods, *S. sindeneusis* isolate 263 show antifungal inhibitory effects on the pathogen.



Fig. 1: Suppressive reaction of two *Streptomyces* isolates on *Magnaporthe oryzae*. Left: *Streptomyces* isolate 339 and right: *Streptomyces* isolate 328. Center disk is *Magnaporthe oryzae* agar disk which its growth towards the antagonists is inhibited clearly



Fig. 2: Bioassay results of *Streptomyces* isolates against *Magnaporthe oryzae*. Clockwise from top: *Streptomyces sindeneusis* isolate 263, blank agar disk (control), the *Streptomyces* isolate 202. Center disk is *Magnaporthe oryzae* agar disk

Table 1: Morphological and physiological characterization of *Streptomyces sindeneusis* isolate 263

Arial mycelium color	Melanoid pigments	Reverse color	Soluble pigments	Spore chain	Spore surface	L- Arabinose	Xylose D-	Inistol I-	Mannitol D-	Fructose D-	Rhamnose	Sucrose	Raffinose
Yellow/White	0	0	0	F	Sm	+	+	-	+	+	-	-	-

F: Flexuous; Sm: Smooth; 1: Positive; 0: Negative; -: No utilization; +: Utilization

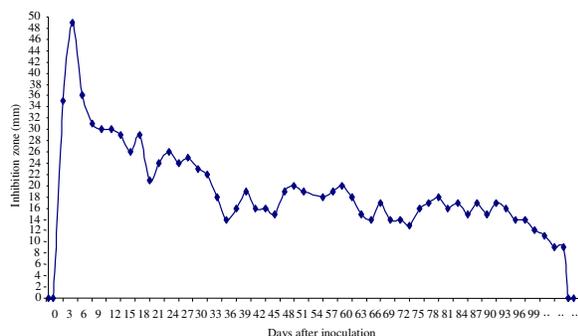


Fig. 3: *In vitro* bioassay results of *Streptomyces sindeneusis* isolate 263 against *Magnaporthe oryzae* in rotary cultures indicative of production time versus inhibition zones

Monitoring activity and growth curve: Activity reached maximum after 5 days in rotary cultures. In shaken 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. 3.

Taxonomy of streptomyces isolate 263: Isolate 263 was identified as *Streptomyces sindeneusis* isolate 263 which is a new record from Iran. Table 1 shows the complete identification of this isolate based on morphological and biochemical characterization. Figure 4 shows scanning electron micrograph of spore chains of *S. sindeneusis* isolate 263.

Determination of MIC: In well diffusion-method, MIC of the crude was determined as 3.125 mg mL⁻¹ against *M. oryzae*.

Solubility active crude in organic solvents: Solubility results are indicated in Table 2. As the results show, apparently there is more than one active principle involved since activity is traceable both in polar and nonpolar solvents of H₂O, methanol and chloroform.

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

Table 2: Bioassay results of solubility tests of the antifungal principle(s) of *Streptomyces sindeneusis* isolate 263 against *Magnaporthe oryzae* in fractions of different solvents indicated by well diffusion-method at 20 mg mL⁻¹ of dry crude

Solvent	Fraction*	Activity	Inhibition zone (mm)
H ₂ O	SP	++	18 20
Methanol	SP	++	21 12
chloroform	SP	++	15 22

*S: Supernatant, P: Pellet

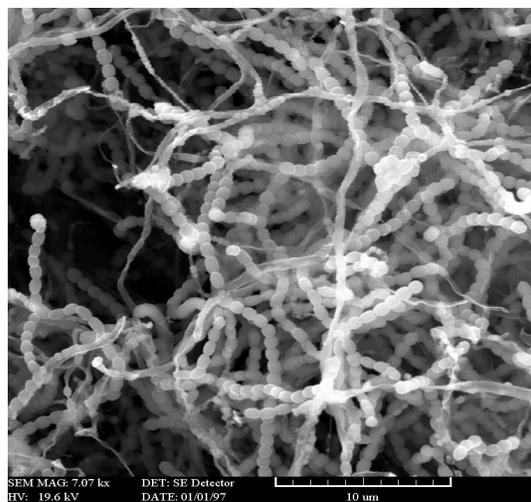


Fig. 4: Scanning electron micrograph of spore chains of *Streptomyces sindeneusis* isolate 263

Determination of TIP: Bioactivity of active isolate diminished to zero at 170°C.

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 *S. sindeneusis*.

In vivo greenhouse studies: The results of biological control of *Streptomyces sindeneusis* isolate 263 against *M. oryzae* the causal agent of rice blast are indicated in Fig. 5. Treatment of plants with pathogen only resulted typical blast symptoms and percent of diseased leaf area evaluated 8% and in treatment of plants with pathogen plus *Streptomyces* evaluated 0.5% according to the method developed by IRRI. That is indicative of significant reduction in the number of lesions in pots

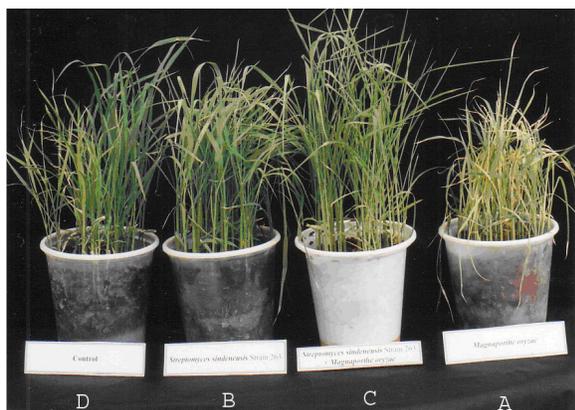


Fig. 5: *In vivo* greenhouse results in rice plants indicative blast disease (A) in plants inoculated with the pathogen alone and (B) plants inoculated with the *Streptomyces sindeneusis* isolate 263 alone, (C) plants inoculated with both pathogen and the antagonist, *S. sindeneusis* isolate 263 and (D) untreated control plants



Fig. 6: Comparative result of Biological activity of *Streptomyces sindeneusis* isolate 263 against *Magnaporthe oryzae* on rice seedlings. (A, B): indicate leaf symptoms resulted from infections by *M. oryzae* and (C-F): show the effect of mixed spore sprays of leaves with both *S. sindeneusis* isolate 263 and the pathogen which indicates strong inhibition in blast development

which received the antagonist. Treatment of rice plants with antagonist in B and C groups had no significant effect on dry weight of rice plants. Figure 6 indicates comparative result of biological activity of *S. sindeneusis* isolate 263 against *M. oryzae* on rice seedlings. A and B indicate leaf symptoms resulted from infections by *M. oryzae* and C-F show the effect of mixed spore sprays of leaves with both *S. sindeneusis* isolate 263 and the pathogen which indicates high degree of inhibition in blast development.

DISCUSSION

In sustainable agriculture natural biofungicides are safe and pro environment. Since most of synthetic fungicides do harm the ecosystem to some extent, their usage should be banned and switched to safer strategies as biological control techniques. Our findings represent the presence of potential antifungal metabolite (s) in *S. sindeneusis* isolate 263 against *M. oryzae*. Antifungal activity of the isolate found in this study highlights its importance as a candidate for further investigation in biological control of the world-wide destructive rice blast disease. 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. 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 *Streptomyces* isolates which would lead to environmentally safer measures in plant-pest management.

ACKNOWLEDGMENTS

This research is dedicated to Mr. A. Afzalipour and Mrs. Fakhereh Saba the founders of Universities in Kerman. This paper is part of M. Ebrahimi Zarandi dissertation fulfilled for degree of Master of Science in Dept. of Plant Pathology, College of Agriculture, Kerman University of Shahid Bahonar, Kerman, Iran.

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