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Control of *Bipolaris Oryzae* the Causal Agent of Rice Brown Spot Disease Via Soil *Streptomyces* Sp. Isolate G

Meysam Soltani Nejad[*1], Gholam Hosein Shahidi Bonjar[1], Fereydoon Padasht Dehkaei[2]

[1]*Department of Plant Pathology, College of Agriculture, Shahid Bahonar University of Kerman.*
[2]*Rice Research Institute, Rasht, Guilan, Iran*

Abstract

Bipolaris oryzae causes rice brown spot disease which reduces rice yield with substantial global impact. Streptomycetes isolated from rice fields of Guilan Province, Iran, showed antifungal activity against the tested pathogen. From five active *Streptomyces* spp. isolates, isolate G showed high antagonistic activity. To determine its taxonomical identity, its colonies characterized morphologically by scanning electron microscope. Some of the physio-biological properties of antifungal principle (s) also determined. The PCR molecular analysis of active isolate represented its identity partially. In this regard, 16S rDNA of isolate G was amplified using universal bacterial primers FD1 and RP2. The PCR products were purified and sequenced. Sequence analysis of 16S rDNA was then conducted using NCBI BLAST method. Our findings are early steps in characterization of this isolate. We hope to determine its precise physiological criteria including its in-depth biological activity for its future In Vivo evaluation under field condition.

Key Words: *Bipolaris oryzae***,** Rice Brown Spot, *Streptomyces* sp**,** 16S rDNA.

Introduction

Rice brown spot caused by *Cochliobolus miyabeanus* (Ito and Kuribayashi) Drechs. ex Dastur. (Anamorph: *Bipolaris oryzae* (Breda de Haan) Shoemaker), is an orphan disease of rice, despite the fact that the disease chronically affects millions of hectares worldwide every year (Chakrabarti 2001; Padmanabhan 1973; Savary et al. 2000). Brown spot is still widely reported across India (Reddy et al. 2010) and more generally in the South and South-East Asian countries (Savary et al. 2000). Biocontrol with beneficial bacteria is one promising alternative to fungicides (Li et al., 2008). The antibiotic metabolites of microbial origin have been included in the biological control as supplement or an alternative to chemical control for the management of plant diseases (Fravel, 1988; Shimizu et al., 2000). Agricultural antibiotics produced by different species of actinomyces, are biological products from natural resources. They have been attracting

Corresponding Author E-mail: meysamsoltaninejad@gmail.com

growing interest with the development of environmentally friendly and safe integrated crop management (Marten et al, 2001). Actinomycetes are unparalleled sources of bio-active metabolites including antibiotics, plant growth factors, and other substances (Keiser et al., 2000; Omura, 1986; Shahidi et al., 2004). Actinomycetes play a pivotal role in maintaining a satisfactory biological balance in soil, largely because of their ability to produce antibiotics and other secondary metabolites (Strohl, 2004). *Streptomyces* are major contributors to biological buffering of soils and have roles in organic matter decomposition conducive to crop production (Dhingra and Sinclair, 1995). The goal of the present research was to study diverse actinomyces population of rice fields at Guilan Province for evaluating their antagonistic effects against *Bipolaris oryzae* causing rice brown spot.

Materials and methods

Microorganisms and culture conditions

Pure culture of *Bipolaris oryzae* was obtained from Rice Research Institute of Iran (RRII), 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 ingredients as described by Kuster and Williams (1964) and used as Actinomycetes culture.

Soil sampling and isolation of Streptomycetes

Soil samples were collected from, Rice fields in different localities of Guilan Province in northern 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 10- 15 days and then passed through a 0.8 mm mesh sieve. 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 1ml of 10^{-3}-10^{-6} soil dilutions to autoclaved CGA (1, 25 mL^{-1} CGA) at 50 $^{\circ}$C before pouring the 9 cm Petri plates and solidification. Three replicates were considered for each. Plates were incubated at 28°C for up to 10 days. From day 4 on, *Streptomyces* colonies were isolated on CGA, incubated at 28°C for two week and stored refrigerated as pure cultures before use. Twenty strains of *Streptomyces* spp. isolated from herbal Rice fields of Guilan Province.

Screening of Streptomyces isolates against Bipolaris oryzae

To evaluate the antifungal activity of isolated *Streptomyces* spp. 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) and 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.

Detection of fungicidal and/or fungistatic activity

Small blocks of inhibition zones (1 mm^3) of *Streptomyces sp* isolate G against *Bipolaris oryzae* were transferred to fresh PDA plates and incubated for 10 days at 30° 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.

Evaluation of Streptomyces isolates for monitoring activity in shaked culture

Streptomyces sp. isolate G 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 20 days and the activity was evaluated by well diffusion-method (Dhingra and Sinclair, 1995; Acar and Goldstein, 2005).

Preparation of crude extracts

To prepare crude extract, the culture was harvested when it reached the maximum activity, 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 and kept refrigerated for further studies.

Determination of minimum inhibitory concentrations (MIC*)*

To measure the MIC values, two-fold serial dilutions of 10, 5, 1.25, 0.625, 0.312, and 0.156 mg ml^{-1} of the crude extract were prepared in Dimethyl sulphoxide: Methanol (DM) solvent and assayed by well diffusion-method as described by Shahidi Bonjar (2004). The MIC was defined as the lowest concentration able to inhibit any visible fungal growth. MIC was observed at least in duplicate as the lowest concentration that completely inhibited visible growth. All data represent average of three replicated experiments.

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).

Identification of active Streptomyces spp.

From five active *Streptomyces* spp. Isolates, isolate G showed high antagonistic activity. Colonies were characterized morphologically and physiologically and analyzed phylogeneticaly. The morphological characteristics of isolate G including surface ornamentation were assessed by scanning electron microscopy (SEM) of 10-day-old cultures grown on CGA. Genomic DNA was extracted from cultured cells with GeneAll® Exgene™ Cell Sv kit (http://www.geneall.com). The 16S rDNA of isolate G was then amplified by PCR, using universal bacterial primers FD1 and RP2 following Kim *et al*. (2006). The PCR products were purified and sequenced through Macrogen Company (Seol, Korea). Sequence analysis of 16S rDNA was then conducted using BLAST by NCBI (http://www.ncbi.nlm.nih.gov).

Results

Antifungal bioassays

From 20 tested Actinomycetes isolates, five isolates were active in dual culture methods from which *Streptomyces* isolate G showed the highest activity. Fig. 1 shows bioassay results of antifungal inhibitory effects of the *Streptomyces sp* isolate G against *Bipolaris oryzae* measured in agar disk-method.

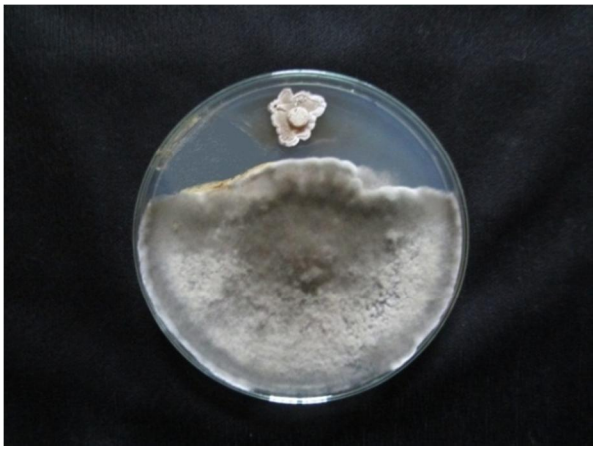

Figure 1: Inhibition effect of *Streptomyce sp* isolates G against *Bipolaris oryzae*

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 G.

Monitoring activity and growth curve

Maximum activity reached after 10 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.

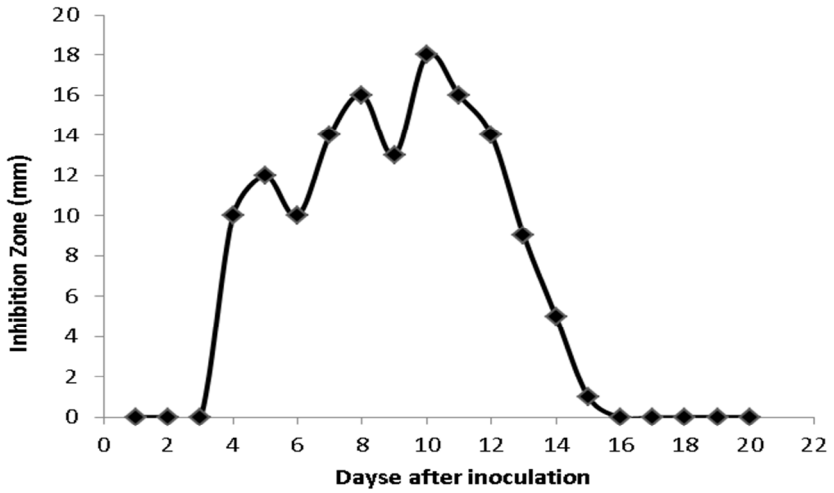

Fig. 2: In vitro bioassay results of *Streptomyces* sp isolate G against *Bipolaris oryzae* in rotary cultures indicative of production time versus inhibition zones

Determination of MIC

In well diffusion-method, MIC of the crude was determined as 1.25 mg/ mL against *Bipolaris oryzae*.

Determination of TIP

Bioactivity for *Streptomyces* sp isolate G diminished to zero at 100°C.

Identification of isolate G

Streptomyces sp isolate G was grown on CGA (29° C for 14 days) for microscopic observations. Spore chain morphology and spore ornamentation were observed by scanning electron microscopes. Fig. 3 shows scanning electron micrograph of spore chains of *Streptomyces* sp isolate G.

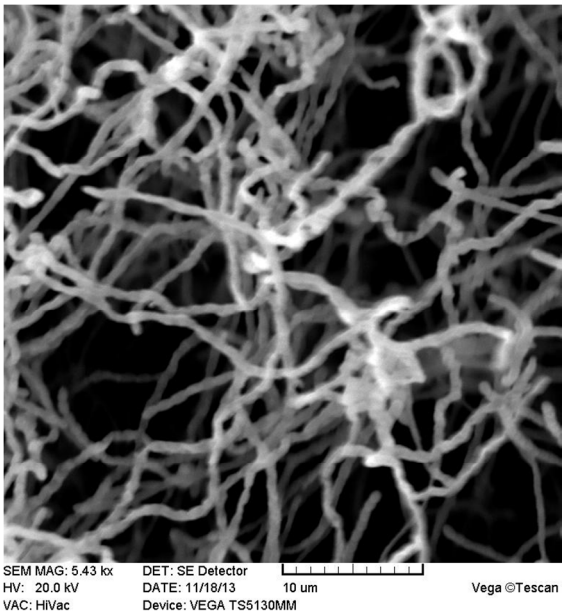

Fig. 3: Scanning electron micrograph of isolate G grown on CGA at 29° C for 14 days, showing spore chains.

The 16S rDNA of isolate G was then amplified by PCR as presented in Fig. 4. Comparison of the near full length 16S rDNA sequence of isolate G to GenBank sequences, showed that it was most similar to *Streptomyces* sp. (E-value = 0.0 and max. identity = 99 %).

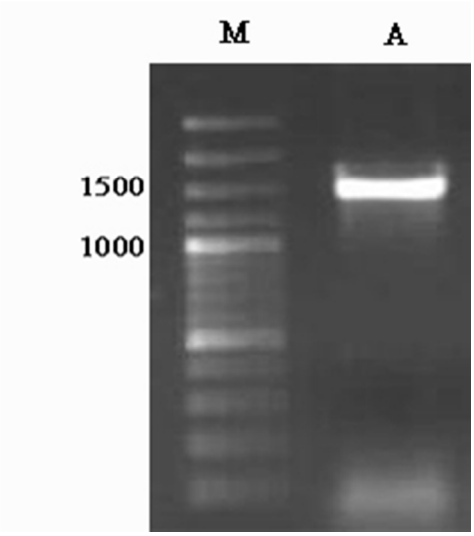

Fig. 4: Amplification of 16S rDNA of *Streptomyces* isolate G by PCR (A) and the ladder (M).

Discussion

In recent years the use of secondary metabolites of microbial origin is gaining great momentum in crop protection and these metabolites may be a supplement or an alternative to chemical control (Colins and Chafik, 1986; Fravel, 1988). Although several microbial secondary metabolites have been discovered as agrochemicals and many of them are commercially available at present, the first pesticide of microbial origin introduced in agriculture was originally developed for medicinal purpose (Yamaguchi, 1996). In sustainable agriculture natural biofungicides are safe. Since most of synthetic fungicides do harm the ecosystem to some extent, their usage should be banned someday and control strategies switched to safer methods as biological control techniques. Our findings represent the presence of potential antifungal metabolite in *Streptomyces* sp isolate G against *Bipolaris 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 brown spot disease. Further studies on these promising antagonists are needed to identify potential compounds produced and to determine possible mode of action related to the active principle.

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