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Streptomyces with Antifungal Activity Against Rice Blast Causing Fungus, *Magnaporthe grisea*

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Abstract Screening tests against fungus causing rice blast, *Magnaporthe grisea*, were performed in order to develop biopesticides. More than 400 actinomycetes collected at several sites near Hanla Mountain on Jeju Island, Korea were tested, and strain BG2-53 showed potent antifungal activity. The *in vivo* screening was performed with fermentation broth, and the strain taxon was identified.

Key words: *In vivo* antifungal activities, *Magnaporthe grisea*, rice blast, *Streptomyces*

Rice blast is the most important disease concerning rice, because it is distributed worldwide and is very destructive. Its severity is the loss of up to 50% of yield. The disease destroys rice that can feed more than sixty million people worldwide. Its occurrence is reported in over eighty-five countries. The fungus causing rice blast is *Magnaporthe grisea*, whose strain attacks rice as well as other cereals, such as wheat and barley. To control the disease, fungicides and preventive measures have been applied, however, it is difficult to predict the disease, and fungicide treatments are also expensive. In addition, due to consumer rejection of synthetic chemical pesticides, development of biopesticides is required. Since blast spores can overwinter, microbial fungicides may solve the problem. Therefore, we undertook to find a potent strain against *Magnaporthe grisea*.

More than four hundred actinomycetes were screened, which were isolated from the soil samples from Jeju Island [2]. The broth of each strain was used for screening instead of the microorganisms themselves [11, 12]. Therefore, secondary metabolites exhibited antifungal activities. Soil samples were collected during 2001 at several sites near Hanla Mountain, on Jeju Island, Korea. Samples were used as substrates for isolation of actinomycetes that exhibited antifungal activities. The isolation medium was starch-casein agar. Autoclaved cyclohexamide was added to the isolation medium (50 μg/ml) for inhibition of fungal growth, and sterilized heat-labile nystatin (50 μg/ml) was also added. The final pH of the medium for isolation of actinomycetes was adjusted to 7.0–7.2. Approximately 1 g of soil was ground in a petri dish and heated at 60°C for 90 min in a drying oven. Ten ml of sterile distilled water were added and the samples were transferred to sterilized bottles. Soil suspensions after vigorous mixing were left to stand for 30 min. Then, one-tenth ml portions were innoculated into starch-casein agar, and the plates were incubated at 28°C for 14 days. Colonies showing the typical characteristics of actinomycetes were selected from the plates, and then transferred to Bennet agar medium adjusted to pH 7.2. Colonies were cultured for 10 days at 28°C. Samples of each colony were inoculated into 30 ml of Bennet broth and cultured in a shaking incubator at 28°C for 30 days. Fermentation broths were mixed with isopropanol (1:1, v/v) and centrifuged. Supernatants were concentrated under reduced pressure and dissolved in dimethyl sulfoxide, and final concentrations were adjusted to 50 μg/ml [4].

Samples were tested for *in vivo* antifungal activities against rice blast (*Magnaporthe grisea* (Hebert) Barr). The crop used for testing was rice (*Oryza saliva* L, cv Nakdong), which was grown in vinyl pots in a greenhouse at 25(±5)°C for 1–4 weeks. Potted crop seedlings were sprayed with broth dissolved in water+methanol (95:5, v/v) containing Tween 20 (250 mg/l) as a wetting agent, and allowed to stand for 24 h [4].

In order to develop pathogenic fungi, treated crop seedlings were inoculated by spraying with fungal spore

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suspensions (10^7–10^9 spores/l). After incubation of the seedlings in the dark for one day at 25(±2)°C and 100% RH, the seedlings were transferred to a growth chamber and maintained at 25(±2)°C and 70–80% RH with 12 h of light per day. Disease severity was determined as a percentage of the infected leaf area.

Pots were arranged as a randomized complete block with three replicates per treatment. Each pot was assayed for extent of infection by visual estimation of the percentage of leaf area covered by sporulating lesions, or the percentage of chlorotic present with necrotic symptoms on the inoculated foliage or sheaths. Data are the result of two trials. The mean value of six estimates for each treatment was converted into a percentage of fungal control using an equation:

$$\% \text{ control} = 100\ [(A - B)/A]$$

where A=area of infection (%) on leaves or stems sprayed with Tween 20 solution alone and B=area of infection (%) on treated leaves or sheaths [6].

Among more than 400 actinomycetes screened against *Magnaporthe grisea*, the strain BG2-53 showed 98% of the fungal control at 50 μg/ml (Fig. 1). The activity was compared with that of fungicides used in the field, Blasticidin-S and Tricyclazole, whose control percentages are 86 (at 1 μg/ml) and 96 (at 0.5 μg/ml), respectively. Since the concentrations of pesticides used as references were different from that of the strain BG2-53, its activity could not be compared directly. But the identification of the strain indicated that it was novel.

16S rDNA analysis was performed for the identification of the strain BG2-53. The partial nucleotide sequences containing the hypervariable α region of 16S rDNA from DNAs of the strain were analyzed [1, 3]. DNAs were prepared by the modified bead beater-phenol method. A loopful of the culture was suspended in 200 μl of TEN buffer (100 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl; pH 8.0), placed in a 2.0-ml screw capped tube filled with 100 μl (packed volume) of glass beads (ϕ0.1 mm; Biospec products, Bartlesville, OH, U.S.A.) and 100 μl of phenol-chloroform-isoamyl alcohol (50:49:1). The tube was oscillated on a Mini-Bead Beater (Biospec Products) for 1 min to disrupt the fungus. Then, the tube was centrifuged (12,000 ×g, 5 min) to separate the aqueous phase. After the aqueous phase was transferred to a clean tube, 250 μl of ice-cold ethanol was added to allow the DNA to precipitate, and the mixture was maintained at –20°C for 10 min. The DNA pellet was washed in 70% ethanol, dissolved in 60 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0), and stored. PCR analysis of 500-bp 16S rDNA was performed according to the method of Ueda *et al*. [13]. The sequences of synthesized oligonucleotides used were: sense primer (16Sf), 5'-TCACGGAGAGTTTGATCCTG-3'; and antisense primer (16Sr), 5'-GCGGCTGCTGGCACGTAGTT-3'. The sequence of each primer was selected from the conserved region and corresponded to nucleotide positions 1 to 20 for the sense primer and 481 to 500 of the antisense primer of the *S. ambofaciens* rDNA sequences [4, 5, 13].

PCR was performed in a 20 μl reaction mixture tube (Accupower PCR PreMix; Bioneer, Chungbuk, Korea). The reaction mixture was subjected to 30 cycles of amplification (60 sec at 95°C, 45 sec at 60°C, and 80 sec at 72°C) followed by a 5 min extension at 72°C (model 9600 Thermocycler; Perkin-Elmer, Foster City, CA, U.S.A.). PCR products were electrophoresed on 3% agarose gel and purified using a QIAEX II gel extraction kit (Qiagen, Hilden, Germany). The nucleotide sequences (459-bp) of purified PCR products were directly determined with sense and antisense

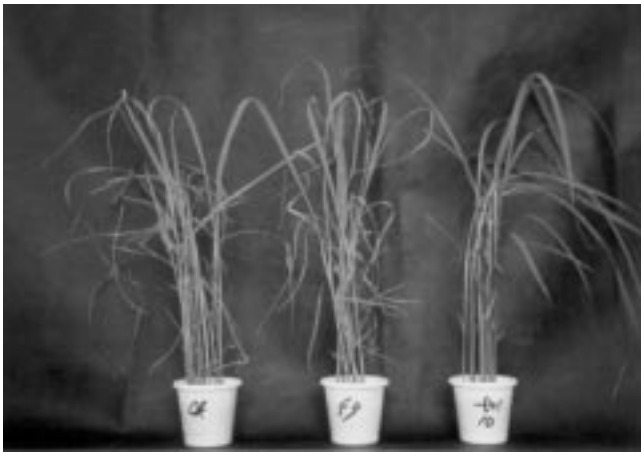

Fig. 1. Activity of BG2-53 against rice blast (left: control; center: BG2-53; right: Tricyclazole at 0.5 μg/ml).

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BG2-53      1 CTGGCGGCGTGCTTAACACNTGCAANTCGAACGATGANNCCCTTCGGGGTNGATTAGTNG
S.lipmanii  1 CTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGG

BG2-53     61 CGAACGGGTGANTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACG
S.lipmanii 61 CGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACG

BG2-53     121 GGAGTCTAATACCAGGATACGACCTGCCGANGCATCTCGGCGGGTGGAAAGCTCCGGCGG
S.lipmanii 121 GG–GTCTAATACC–GGATACGACCTGCCGAGGCATCTCGGCGGGTGGAAAGCTCCGGCGG

BG2-53     181 TGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAAGGGCCCACCAAGGCGACG
Slipmanii  179 TGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACG

BG2-53     241 ACGGGTAGCCGGCCTGAGANGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTC
Slipmanii  239 ACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTC

BG2-53     301 CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCC
Slipmanii  299 CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCC

BG2-53     361 GCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTG
Slipmanii  359 GCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTG

BG2-53     421 ACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGT
Slipmanii  419 ACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGT
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Fig. 2. A comparison of BG2-53 gene for 16S rDNA with *Streptomyces lipmanii* gene (boxed characters denote mismatched gene).

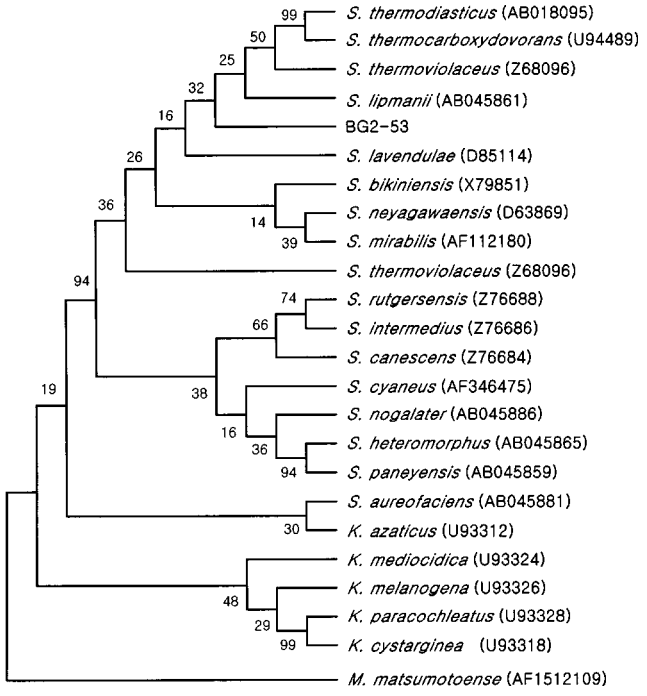

Fig. 3. Phylogenetic tree of strain BG2-53.

primers with an Applied Biosystems model 373A automatic sequencer and a BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems; part no. 4303153). The sequencing mixture was prepared according to the supplier's protocol. The mixture was directly sequenced using a PCR amplification of 30 cycles (10 sec at 95°C and 4 min at 60°C) [4, 7]. 16S rDNA sequences (459-bp) of the strain BG2-53 identified from GenBank by the BLAST program showed the highest homology value (96% identity) with *Streptomyces lipmanii*. A comparison of strain BG2-53 gene with *S. lipmanii* gene is shown in Fig. 2.

The evolutional distance was calculated by the Jukes and Cantor method, and a phylogenetic tree was constructed using the neighbor-joining method (Fig. 3). Strain BG2-53 was found to belong to *Streptomyces lipmanii* with a high bootstrap value based on an analysis of the phylogenetic tree [10]. Strain BG2-53 was also identified as being closely related to *S. lavendulae* in the phylogenetic tree [8, 9]. However, the highest homology was 96%, therefore, the strain must be new. In conclusion, the strain BG2-53 isolated from soil samples collected on Jeju Island, Korea has a potent activity against fungus causing rice blast, *Magnaporthe grisea*, and it appears to be a novel *Streptomyces* with 96% homology to *Streptomyces lipmanii*.

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