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Antifungal Activity of *Streptomyces* sp. Against *Puccinia recondita* Causing Wheat Leaf Rust

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Abstract To discover a potent strain against wheat leaf rust, soil samples collected from Ilgamho, Seoul, Korea were tested *in vivo* and a strain belonging to *Streptomyces* sp. was found to show good antifungal activity when fermented in a broth. The identification of the strain was carried out based on 16S rDNA analysis, and the active compound was separated from the fermented broth. Even though its structure was not determined completely, the authors report the results obtained so far indicate that the fermented broth of the strain showed activity against wheat leaf rust. Therefore, we propose that this may be a potential novel strain showing antifangal activity against *Puccinia recondita*.

Key words: *In vivo* antifungal activities, *Puccinia recondita*, wheat leaf rust, *Streptomyces*

Wheat leaf rust is caused by the fungus *Puccinia triticina*. It reduces wheat yields. When the spore masses of the fungus break through the surface of the leaf, the color of the leaf becomes reddish orange like hence the disease is called leaf rust. Since the leaf rust spores are carried by wind, the disease can spread rapidly. Under favorable moisture and temperatures of 15°C to 25°C, leaf rust spores germinate and penetrate into the leaf. After 7 to 10 days the fungus can produce more spores. As a result, the disease can increase rapidly. Yield losses of wheat by leaf rust depend on its severity and the duration of infection, but severe losses occur from the seedling stage to maturity. The severity of the disease can be determined based on the amount of leaf area covered by leaf rust [2, 5, 8].

Fungicides to control wheat leaf rust include mancozebs, propiconazole, and azoxystrobin. Since there has been a growing tendency in recent years for consumers to reject chemical synthetic pesticides, we have launched this study to discover naturally occurring biopesticides in microorganisms [3].

Soil samples were collected during 2002 at several sites of Ilgamho, Seoul, Korea. Actinomycetes were isolated by the method previously published by us [4]. Colonies showing the typical characteristics of actinomycetes were selected. They were transferred to Bennet agar medium, and the pH was adjusted to 7.2. They were then cultured for ten days at 28°C. Colonies selected from the agar were inoculated into Bennet's medium and cultured in a shaking incubator at 28°C for an additional ten days. Fermented broth was used to test antifungal activity against wheat leaf rust (*Puccinia triticina* recondita). Furthermore, 10 l of the cultured broth was prepared to perform the isolation and separation of active compounds.

The crop used for the activity assay was wheat (*Triticum aestivum* L, cv Chokwang). It was grown in vinyl pots placed in a greenhouse at 25 (±5)°C for 1-4 weeks. Potted crop seedlings were sprayed with broth dissolved in a mixture of water and methanol (95:5, v/v) containing Tween 20 (250 mg/l) as a wetting agent, and allowed to stand for 24 h [6]. Treated crop seedlings were inoculated to develop pathogenic fungi by spraying with fungal spore suspensions (10^7-10^9 spores/l). After incubation of the crop seedlings in the dark, they were transferred to a growth chamber for one day at 25 (±2)°C and 100% RH, and were maintained at 25 (±2)°C and 70-80% RH with 12 h of light per day. The severity of the disease was determined based on a percentage of the infected leaf area. The experiments were arranged as a randomized complete block with three replicates per treatment. An assay of each pot was performed so as to visually estimate the percentage of leaf area covered by sporulating lesions, or the percentage of chlorotic presence with necrotic symptoms

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on the inoculated foliage. Data are the result of three trials. The mean value of nine estimates for each treatment was converted into a percentage figure of fungal control using the equation:

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

where A=area of infection (%) on leaves sprayed with Tween 20 solution alone, and B=area of infection (%) on treated leaves.

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S  AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAAC 60
K  AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAAC 60

S  GATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCC 120
K  GATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCC 120

S  CTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACGACGCGCTCGGGCA 180
K  CTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAACACCTGCCCGGGCA 180

S  TCCGATGTGCGTGGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTG 240
K  ------GTGGGTTAAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTG 234

S  GTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC 300
K  GTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC 294

S  ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA 360
K  ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA 354

S  TGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC 420
K  TGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC 414

S  TCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTAC 480
K  TCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTAC 474

S  GTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAA 540
K  GTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAA 534

S  GAGCTCGTAGGCGGTCTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGC 600
K  GAGCTCGTAGGCGGCTTGTCACGTCGGGTGTGAAAGCCCGGGGCTTA-CCCCGGGTCTGC 593

S  ATTCGATACGGGCAGACTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGA 660
K  ATTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGA 653

S  AATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGAC 720
K  AATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGAC 713

S  GCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTA 780
K  GCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCCGGTAGTCCACGCCGTA 773

S  AACGGTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTA 840
K  AACGGTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTA 833

S  AGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC 900
K  AGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC 893

S  GCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTT 960
K  GCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTT 953

S  GACATACGCCGGAAACACCCAGAGATGGGTGCCCCCTTGTGGTCGGTGTACAGGTGGTGC 1020
K  GACATACACCGGAAACGGCCAGAGATGGTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGC 1013

S  ATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC 1080
K  ATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC 1073

S  TTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGG 1140
K  TTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGG 1133

S  TCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGC 1200
K  TCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGC 1193

S  ACACGTGCTACAATGGCAGGTACAATGAGCTGCGAAGCCGTGAGGCGGAGCGAATCTCAA 1260
K  ACACGTGCTACAATGGCAGGTACAATGAGCTGCGAAGCCGTGAGGCGGAGCGAATCTCAA 1253

S  AAAGCCTGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTA 1320
K  AAAGCCTGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTA 1313

S  GTAATCGCAGATCAGCAGTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG 1380
K  GTAATCGCAGATCAGCACTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG 1373

S  TCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGC 1440
K  TCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGC 1433

S  TGTCGAAGGTGGGACTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTA 1491
K  TGTCGAAGGTGGGACTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTA 1484
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Fig. 1. The result obtained by BLASTN.
K: KACC91021; S: *Streptomyces neyagawaensis.*

In order to identify the strain, a 16S rDNA sequence analysis was carried out. To extract DNA, the strain was fermented in Bennet's medium for 7 days and the fermented broth was centrifuged. The extraction of DNA was carried out using a Genomic DNA Extraction Kit (Intronbiotechnology, Kyungkido, Korea). Amplification of the DNA was performed using a PCR kit using primers (fD1 5'-AGA GTT TGA TCC TGG CTC AG-3', rP2 5'-ACG GCT ACC TTG TTA CGA CTT-3'). The conditions used for the thermal cycles were as follows: denaturation at 94°C for 5 min prior to cycling; 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min, further extension at 72°C for 10 min, and cooled to 4°C. The PCR products were cloned using a pGEM-T cloning kit (Promega, Madison, WI, U.S.A.) according to the manufacture's instructions. The sequences of the PCR products were determined by four internal primers which were : p510r 5'-TAT TAC CGC GGC TGC TG-3', p364f 5'-GGC AGC AGT GGG GAA TAT TG-3', p783f 5'-TAG ATA CCC TGG TAG TCC AC-3', and p1037f 5'-TCG TCA GCT CGT GTC GTG AG-3'. The determined sequences were analyzed by a BLAST program at the National Center for Biotechnology Information (Fig. 1). The analysis shows that the strain has a 98% homology (1484/1491) with *Streptomyces neyagawaensis.* The strain was deposited in the Korean Agricultural Culture Collection (KACC) at Suwon, Korea. Its registration number is KACC91021. The evolutional distance calculated by the Jukes and Cantor method [1], and a phylogenic tree created using the neighbor-joining method are shown in Fig. 2.

Morphological observations of strain KACC91021 were made using scanning electron microscopy (SEM, JSM 5410LV, JEOL, Peabody, U.S.A.). Strain KACC91021 for the SEM observation was cultured in a small Petri dish containing Bennet's medium supplemented with agar at 28°C for seven days. Samples were fixed in an osmotically balanced solution of 2% gluteraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) at 25°C for 3 h and were then washed three times at 4°C for ten min in 0.05 M sodium

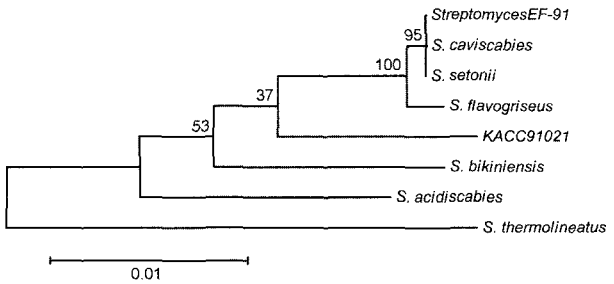

Fig. 2. The evolutional distance calculated by the Jukes and Cantor method and a phylogenic tree created using the neighbor-joining method.

Fig. 3. Morphological observation of strain KACC91021 obtained using scanning electron microscopy after growth on Bennet's medium at 28°C for 7 days.

cacodylate buffer (pH 7.2). Samples were post-fixed in 1% aqueous osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) for 2 h at 4°C, and were then washed twice with distilled water. Samples were dehydrated in a graded ethanol series (30, 50, 70, 80, 90, 100, 100, and 100), and were dried in liquid CO_2 using the Balzers CPD 010 (Balzers Instruments, Liechtenstein) mounted on aluminum stubs, and spatter-coated with gold/palladium using the Polaron SEM Coating Unit E5100 (Thermo VG Scientific, Beverly, MA, U.S.A.). The image obtained by SEM is shown in Fig. 3.

Figure 4 shows antifungal effects of the broth produced by strain KACC91021 against *Puccinia triticina*. Ten ml of the broth showed 80% of control. The broth was concentrated using a rotary evaporator under reduced pressure. Solvents such as ethylacetate, n-hexane, and buthanol were used for extraction employing a separation funnel. The fraction separated from the aqueous layer showed the best activity. Column chromatography with

Fig. 4. Effects of strain KACC91021 against *Puccinia triticina* recondita.
Left: wheat leaves infected by *Puccinia triticina* recondite; center: wheat leaves with the treatment of Flusilazole; right: wheat leaves with the treatment of the broth of the strain.

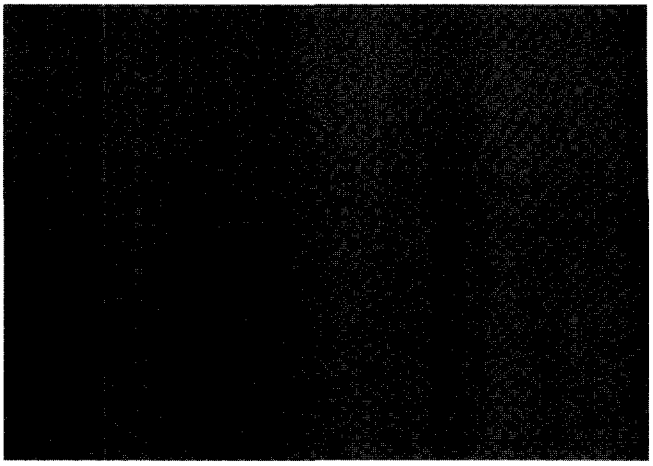

Fig. 5. The chromatogram of the fraction 21-4-1 obtained from HPLC with photodiode array detector.

Silica Gel was performed on a solvent system of methanol: chloroform (3:7, v/v). Seven fractions were collected using an auto-collector. They were named from 21-1 to 21-7. Among these, the fraction 21-4 showed the best activity. Fractions were separated again under a different solvent condition (methylenechloride:ethanol=10:1). They were named 21-4-1 to 21-4-6. Of the six fractions, 21-4-1 showed the best activity, and fortunately it did not need further separation. As shown in Fig. 5, the chromatogram obtained by HPLC (Waters, Milford, MA, U.S.A.) with a two-dimensional photodiode array detector gives a single spot. The HPLC analysis was performed on a Waters HPLC system with a flow rate of 1 ml/min, a Vydak C8 column of 4.5 mm×300 mm, and eluent of methylenechloride: ethanol= 10:1 [9]. The collected sample was freeze-dried. To identify the structure of 21-4-1, several NMR experiments were carried out on a Bruker DRX400 (Karlsruhe, Germany) [7].

The structural determination of the active compound requires further work. Even though the compound has not been identified, it is meaningful that the fermented broth of strain KACC91021 shows an activity against wheat leaf rust. Therefore, we propose that this may be a potential novel strain showing antifungal activity against *Puccinia recondita*.

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References

1. Jukes, T. H. and C. R. Cantor. 1969. Evolution of protein molecules, pp. 21-132. *In* H. N. Munro (ed.) *Mammalian protein metabolism*, Academic Press, New York, U.S.A.

2. Kang, J. G., J. Hur, S. Choi, G. Choi, K. Cho, L. Ten, K. Park, and K. Kang. 2002. Antifungal activities of *N*-arylbenzenesulfonamides against phytopathogens and control efficacy on wheat leaf rust and cabbage club root diseases. *Biosci. Biotechnol. Biochem.* **66:** 2677-2682.
3. Kim, B.-J., G. J. Choi, K. Y. Cho, H. Yang, C. Shin, C.-H. Lee, and Y. Lim. 2002. Antifungal activities against *Plasmodiophora brassicae* causing club root. *J. Microbiol. Biotechnol.* **12:** 1022-1025.
4. Kim, B.-J., M. Cho, J.-C. Kim, K. Y. Cho, G. J. Choi, C.-H. Lee, and Y. Lim. 2001. *Streptomyces* showing antifungal activities against six plant pathogenic fungi. *J. Microbiol. Biotechnol.* **11:** 1120-1122.
5. Kolmer, J. 2001. Molecular polymorphism and virulence phenotypes of the wheat leaf rust fungus *Puccinia triticina* in Canada. *Canadian J. Botany* **79:** 917-926.
6. Lee, C.-H., B.-J. Kim, G. J. Choi, K. Y. Cho, H. Yang, C. Shin, S. Min, and Y. Lim. 2002. *Streptomyces* with antifungal activity against rice blast causing fungus, *Magnaporthe grisea*. *J. Microbiol. Biotechnol.* **12:** 1026-1028.
7. Lee, J., Y. Lyoo, D. Park, J. Jung, C.-h. Lee, M. Kim, and Y. Lim. 2001. Therapeutic agents against bacteria causing porcine pneumonia. *J. Microbiol. Biotechnol.* **11:** 1102-1105.
8. Long, D., J. Kolmer, K. Leonard, and M. Hughes. 2002. Physiologic specialization of *Puccinia triticina* on wheat in the United States in 2000. *Plant Disease* **86:** 981-986.
9. Lyoo, Y., D. Park, S. Lee, Y. Choi, J. Jung, T. Jun, H. Ahn, C.-H. Lee, and Y. Lim. 2001. Antibacterial compound against pasturella multocida and actinobacillus pleuropneumoniae causing porcine pneumonia. *J. Microbiol. Biotechnol.* **11:** 350-353.