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An Antifungal Property of *Burkholderia ambifaria* Against Phytopathogenic Fungi

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Abstract Even though many pesticides are known for barley powdery mildew and wheat leaf rust, alternative controls are necessary, because of consumer rejection of chemical pesticides and the appearance of fungi resistant to fungicides. To discover biopesticides, many broths of microorganisms were screened. Of those, a culture broth of *Burkholderia ambifaria* showed an excellent antifungal activity against both *Erysiphe graminis* and *Puccinia recondita*, which cause barley powdery mildew and wheat leaf rust, respectively.

Key words: *Burkholderia ambifaria*, barley powdery mildew, wheat leaf rust, *Erysiphe graminis*, *Puccinia recondita*

Barley powdery mildew (BPM) is a serious barley disease [15, 16], and caused by *Erysiphe graminis*. This disease starts from the lower leaves of barley and progresses to the upper leaves where white patches appear, and the patches become a gray-brown color in the fruiting stage. *E. graminis* is a fungus that can overwinter as mycelium on barley. The disease is developed at 15°C to 25°C and favors 80% humidity. The management for this disease includes crop rotation and elimination of barley residues. For chemical control, flusilazole and benomyl are used, which are 1-[[bis(4-fluorophenyl)methylsilyl]methyl]-1*H*-1,2,4-triazole and methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate, respectively.

Wheat leaf rust (WLR) is caused by fungus *Puccinia triticina*, and reduces wheat yields. When the spore masses of the fungus break through the surface of the leaf, the color of the leaf shows reddish orange like rust, so that the disease is called leaf rust. Since the leaf rust spores are carried by wind, the disease can spread quickly. 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 rapidly intensify [8, 11]. Fungicides to control WLR include mancozeb, flusilazole, and propiconazole; the chemical names of mancozeb and propiconazole are manganese ethylenebis(dithiocarbamate) and 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1*H*-1,2,4-triazole, respectively.

Even though chemical pesticides exist for BPM and WLR, we need to find alternative controls, because of consumer rejection of chemical pesticides and the appearance of fungi resistant to fungicides. In order to discover biopesticides, many culture broths of microorganisms were screened. Among those, a broth produced by *Burkholderia ambifaria* showed excellent antifungal activities against both BPM and WRL. *B. ambifaria* is a novel member of the *Burkholderia cepacia* complex [10]. The *B. cepacia* found by Walter Burkholderia in 1950 was first called *Pseudomonas cepacia* [2]. However, further taxonomic analysis determined that *B. cepacia* is the same as *P. cepacia* [1]. Therefore, the genus Burkholderia belongs to the subdivision of Proteobacteria and includes 26 species [17–19]. One of them, the *B. cepacia* complex, consists of at least 9 genomovar strains, and *B. ambifaria* is *B. cepacia* genomovar VII [3]. It is ubiquitous in soil, plants, animals as well as humans. In the view of a plant pathogen, it can be a foe; however, in the view of plant protection, it can be considered as a friend. Even though its notorious role, called cepacia syndrome, has been known [4], like

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other microorganisms, *B. ambifaria* produces antibiotics as its secondary metabolites, so that its antifungal activity test is valuable [14].

B. ambifaria strain (ATCC BAA-244) was purchased from American Type Culture Collection (ATCC). The strain was cultured in modified Brain Heart Infusion medium (per 1 l, brain extract 12.5 g, heart extract 5.0 g, peptone 10.0 g, dextrose 2.0 g, sodium chloride 5.0 g, disodiumphosphate 2.5 g, pH 7.4). The growth temperature was 28–30°C and the speed of the shaking incubator was 150–180 rpm. After three days, the cultured broth was filtered, and the filtrate was tested for *in vivo* antifungal activity against wheat leaf rust (*Puccinia recondita* Rob ex Desm) and barley powdery mildew (*Erysiphe graminis* f sp hordei Marchal). Wheat (Triticum aestivum L, cv Chokwang) and barley (Hordeum sativum Jessen, cv Dongbori) plants were grown in vinyl pots (4.5-cm diameter) in a glasshouse at 25 (±5)°C for 1–4 weeks [6].

For WLR caused by *P. recondita*, wheat plants at the first leaf stage (four plants/pot) were sprayed with each test solution. The solution was the mixture of 1.5 ml of the broth and 28.5 ml of Tween 20. The plants were sprayed with a suspension (60 mg/100 ml of 250 ppm Tween 20) of uredospores collected from the second leaf of wheat and then placed in a moist chamber. One day after inoculation, the plants were held in a growth chamber (20°C and 70% humidity), and the fungicidal activities of the test samples were tested 10 days after the inoculation. For BPM caused by *E. graminis*, barley plants with a fully expended first leaf were sprayed with a suspension of the test material. The treated plants were dusted with *E. graminis* conidia formed on leaves of barley.

Data are the result of three trials. The mean value for each treatment was converted into a percentage of fungal control using the following 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. Analysis of variance was performed on the data with the PROC GLM procedure (SAS Institute, Cary, NC, U.S.A.). If P>F was less than 0.01, means were separated with the least significant different (LSD) test at the P=0.05 level [6].

In order to test the activity of the cultured broth of *B. ambifaria* against *E. graminis* f sp hordei, the mixture of 1.5 ml of the cultured broth and 28.5 ml of aqueous Tween 20 solution was sprayed on the potted crop seedlings. To compare the activity of the broth with the chemical pesticides, flusilazole (0.5 µg/ml) and benomyl (1 µg/ml) were used. Whereas flusilazole and benomyl showed 82 and 72 control %, respectively, the cultured broth showed 83%. To examine the activity against *P. recondita*, the same procedure was carried out. Similar to BPM, flusilazole (10 µg/ml) and mancozeb (10 µg/ml) were used as reference chemical pesticides, which showed 99 and 93 control %, respectively. The mixture of the broth and Tween 20 solution showed 93%.

The antifungal activities of the broth produced by *B. ambifaria* against *E. graminis* and *P. condita* can be compared with those of the chemical pesticides. Obviously, the active ingredient contained in the broth is not yet known. If a single compound with the activity is isolated, it could be one of the chemical pesticides. Since the goal of this experiment was to find a biopesticide instead of a chemical pesticide, the isolation of the active compound may not be required. However, because the different cultured broth may not contain similar amount of the active compound, the nature of the compound should be identified in order to keep the same activity. One liter of the fermented broth was mixed with the same volume of 100% isopropanol and the mixture was centrifuged. The supernatant concentrated with a rotary evaporator was filtered. The remnant was washed with 50% isopropanol and extracted with isobutanol. The extract was fractionated on an open column chromatography (CC) packed with Diaion HP-20. The major fraction was fractionated again on an Alumina CC. The major fraction was chromatographed on a prep-LC (Vydak C18 column, 4 mm×250 mm, Waters prep-LC system, Oregon, U.S.A.; 2996 Photodiode Array detector; eluent, acetonitril-water mixture) [9]. Based on the chromatogram obtained from the photodiode array detector, this fraction was a single compound. It did not show a strong activity comparing with the culture broth; however, it was a major fraction contained in the broth. In order to identify the compound, nuclear magnetic resonance (NMR) experiments were carried out on a Bruker Avance 400 (Karlsruhe, Germany) [7, 13]. As shown in Fig. 1, the ^{13}C NMR spectrum of the isolated compound consisted of 40 carbon signals as follows: 13.9 (q), 22.0 (t), 25.6 (t), 28.6 (t), 28.9 (t), 29.0 (t), 29.01 (t), 29.1 (t), 29.2 (t), 31.2 (t), 33.4 (t), 35.9 (t),

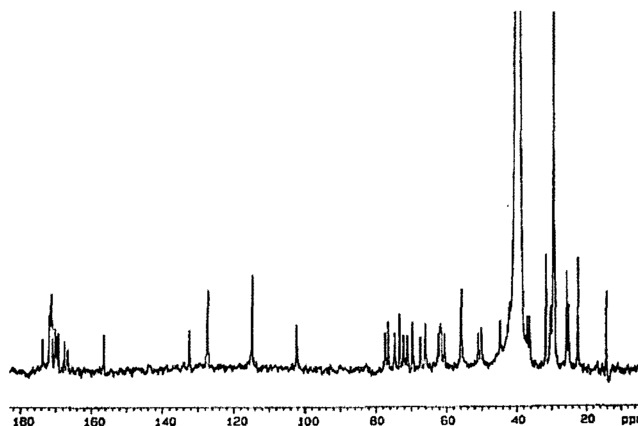

Fig. 1. The ^{13}C NMR spectrum of the compound isolated from the culture broth of *B. ambifaria*.

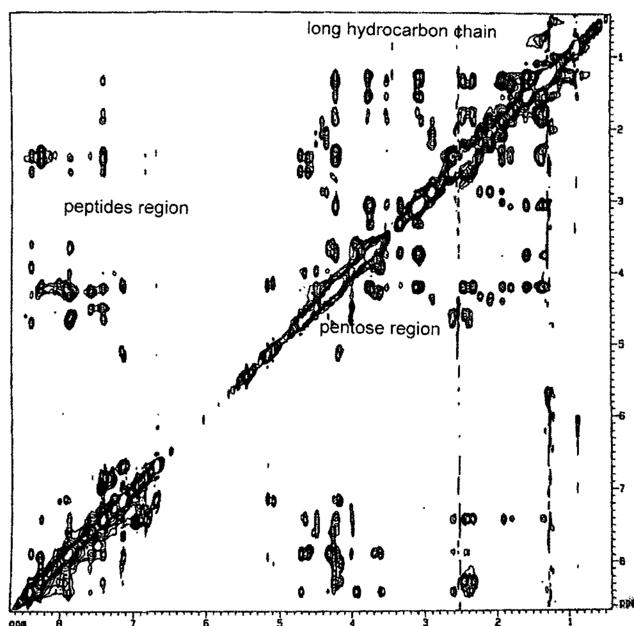

Fig. 2. The correlated spectroscopy of the compound.

36.3 (t), 42.7 (t), 44.4 (d), 48.5 (d), 50.9 (d), 54.5 (d), 57.1 (d), 59.1 (d), 60.5 (t), 61.9 (t), 67.5 (d), 69.0 (d), 70.2 (d), 75.7 (d), 114.6 (d), 126.6 (d), 132.9 (s), 156.2 (s), 169.2 (s), 169.9 (s), 170.3 (s), 170.4 (s), 171.1 (s), 171.3 (s), 171.9 (s), 172.3 (s), 174.9 (s), 175.1 (s), where q, t, d, and s denote CH_3, CH_2, CH, and carbonyl carbon, respectively. According to the NMR data, the compound includes one pentose, eight peptides, and a long hydrocarbon chain [5]. These partial structures were confirmed based on the interpretation of the correlated spectroscopy as marked in Fig. 2. The complete identification of the compound remains for future work.

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