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REVIEW

Studies on Biocontrol of Air-borne Plant Disease by a Filamentous Fungus Producing Antifungal Volatiles

Motoo KOITABASHI[1]* and Seiya TSUSHIMA[2]

[1] National Agricultural Research Center for Kyushu Okinawa Region (Kurume, Fukuoka 839–0851, Japan)
[2] National Institute for Agro-Environmental Sciences (Tsukuba, Ibaraki 305–8604, Japan)

Abstract

Microorganisms isolated from wheat leaf surfaces were screened for inhibition of wheat powdery mildew. A new screening method, in which wheat leaves were inoculated with *Blumeria graminis* f. sp. *tritici* and incubated with the cultured microorganisms under non-contact conditions, was developed in our study. Among these strains, a fungus designated as Kyu-W63 had an especially strong inhibitory effect. Kyu-W63 had a strong aromatic odor when being cultured. Nuclear magnetic resonance analysis revealed that Kyu-W63 produced two types of volatile substances, 5-pentyl-2-furaldehyde and 5-(4-pentenyl)-2-furaldehyde. Although the two are known to be nematicidal substances, the antifungal activity of 5-(4-pentenyl)-2-furaldehyde is first confirmed in our study. In contrast, 5-pentyl-2-furaldehyde was found to have strong suppression activities to various fungal species in this study, although it has been reported to suppress the growth of yeast, *Nematospora coryli*. Strain Kyu-W63 completely agreed with *Irpex lacteus* in the rDNA internal transcribed spacer (ITS) sequences, and strain Kyu-W63 was inferred to be *I. lacteus*. Biocontrol of parsley powdery mildew, caused by *Oidium* sp., was examined using a filamentous fungus, Kyu-W63 that produces antifungal volatiles, for 3 years under greenhouse conditions. Kyu-W63 treatment significantly inhibited disease severity compared to control plots. In addition, Kyu-W63 suppressed other harmful fungi such as *Penicillium* sp. and *Aspergillus* sp. which are plant pathogens or human allergens existing in the environment.

Discipline: Plant protection
Additional key words: *Irpex lacteus*, phylloplane fungi, powdery mildew

Introduction

Under natural conditions, the exposed plant surface is usually covered with a large number of various microorganisms, including phylloplane fungi[3,5,15]. Among these microorganisms are pathogenic fungi that infect and damage the host plant. Antagonistic microorganisms that suppress the activity of pathogenic fungi are also present[1,2]. Powdery mildews are one of the most conspicuous and extensively studied groups of epiphytic pathogens. *Blumeria graminis* (de Candolle) Speer f. sp. *tritici* Marchal (*B. graminis*), causing powdery mildew a major pathogen of wheat, grows mainly on the surface of leaves. As a consequence, phylloplane fungi are presumed to have a greater influence on *B. graminis* than they do on other fungi that grow in plant tissue. Because *B. graminis* is an obligate parasite which can grow only on live plants, antagonistic organisms can not be screened against powdery mildew grown on culture media. We developed a new method for selecting filamentous fungi that have a non-contact inhibitory effect on wheat powdery mildew using wheat leaves infected by *B. graminis*. In this study we selected filamentous fungi from the phylloplane of wheat. Antagonistic substances produced by an isolate that strongly inhibited powdery mildew were also investigated.

A sterile fungus strain, Kyu-W63, isolated from wheat leaves in Fukuoka, Japan, inhibited growth of the wheat powdery mildew fungus and other plant pathogenic fungi[12]. Our analysis using gas chromatography-mass spectrometry (GC–MS) revealed that its inhibitory activ-

Present address:
[1] National Institute for Agro-Environmental Sciences (Tsukuba, Ibaraki 305–8604, Japan)
*Corresponding author: e-mail koita@affrc.go.jp
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ity was attributed to the production of volatile substances and that this strain produced two types of volatile substances. The objectives of this research were to determine the structure of the antifungal substances from the fungal strain Kyu-W63 in order to analyze their antifungal activity against plant pathogens and to clarify the taxonomic position of Kyu-W63. An additional objective of our research was to develop a new biocontrol technique that would reduce labor compared with spraying an aqueous chemical to control the disease.

Screening of antagonistic microorganisms

1. Isolation of microorganisms

Phylloplane microorganisms were isolated from leaves of wheat (*Triticum aestivum* cv. Gogatsu-komugi) grown in a field at Chikugo, Fukuoka, Japan. Leaves were collected once every 2 weeks in the spring (March–May) from 1989 to 1991. Isolation was performed as described by Koitabashi et al. using the leaf washing method[12]. A total of 1,845 strains of filamentous fungi and yeast were isolated over a 3-year period. The fungal strains were identified based on their morphology. The most predominant isolates, isolated every year, were *Cladosporium* spp., with a relative frequency of 19.8%. Other genera isolated were *Alternaria* (2.5%), *Epicoccum* (2%), *Fusarium* (1.2%), and *Botrytis* (1%). Among these, 408 isolates, 371 strains of filamentous fungi and 37 strains of yeast of various colony types were screened for fungicidal activity in this study.

2. Screening of antagonists

Leaf segments (5–6 cm) were obtained from 7-day-old wheat (cv. Minamino-komugi) seedlings that were grown under artificially controlled climate condition and free from *B. graminis*. A lid of a Petri dish was placed upside down, and wet filter paper was spread inside the lid. Five leaf segments were placed with abaxial side up and were inoculated with *B. graminis* by sprinkling conidia over the leaf surface. The 371 strains of filamentous fungi or 37 strains of yeast were cultured on potato dextrose agar (PDA) for 10 days. The lids of Petri dishes used to culture the strains were removed and the dishes were inverted over the previously described lids containing the filter paper and inoculated leaf segments (Fig. 1). The leaf segments covered with the inverted Petri dishes were incubated at 20ºC for 8 days. The growth of *B. graminis* on the leaf segments was evaluated using the 0 to 4 scoring index of Finkner[6] in which 4 represents the most severe occurrence of the disease. Of the 408 isolates screened from wheat leaf surfaces, 223 microorganisms had scores greater than 3.1, indicating that the growth of powdery mildew was unaffected. Ten strains of filamentous fungi inhibited the growth of powdery mildew moderately or strongly (growth scores < 1.5). A fungus designated as Kyu-W63 had the strongest inhibitory effect, with a growth score of 0.2. After 10 days incubation, there was little or no development of powdery mildew colonies (Fig. 2). Kyu-W63 formed white colonies and produced a characteristic odor.

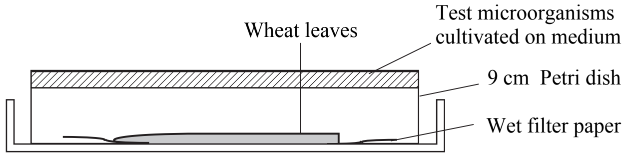


Fig. 1. Screening method for microorganisms antagonistic to powdery mildew
Blumeria graminis-inoculated wheat leaf segments were placed under the colony of a tested microorganism that had been previously cultured on PDA for 10 days.

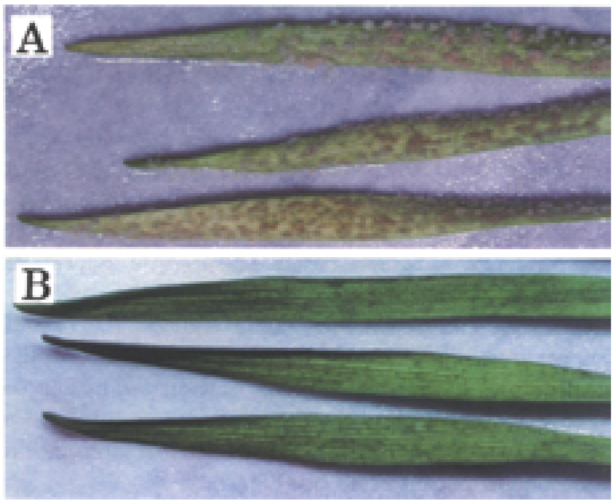


Fig. 2. Inhibitory effect of Kyu-W63 against wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) on wheat leaves
A: Untreated wheat leaves; powdery mildew symptom appeared 10 days after inoculation.
B: Wheat leaves treated with Kyu-W63; the symptoms were not conspicuous 10 days after inoculation.

Mechanism of Kyu-W63 inhibitory effect on the growth of *B. graminis*

The growth of wheat powdery mildew was suppressed even with the placement of a membrane filter with a pore size of 0.45 µm between the mycelial colony of Kyu-W63 and the wheat leaf segment. However, when activated charcoal was placed between the leaf segments and Kyu-W63, the inhibitory effect disappeared. In addition, when Kyu-W63 was cultured in PDA medium for 30 days, it lost both its odor and its inhibitory effect. We concluded that some volatile substances were involved in the inhibitory effect of Kyu-W63. When Kyu-W63 mycelium that had lost its odor was cultured in new medium, it regained not only the odor but also its inhibitory effect.

When the culture medium with conidia of *B. graminis* was covered with PDA without Kyu-W63 as a control, a maximum of 61.6% of the spores germinated during the observation period. On the contrary, in the presence of Kyu-W63, only 0.2% of the *B. graminis* conidia germinated over a period of 48 hr[12].

Inhibitory effect of Kyu-W63 on various fungi such as plant pathogenic fungi and allergenic fungi

Kyu-W63 strongly inhibited the growth of *Fusarium graminearum* (a pathogen of Fusarium head blight) on PDA medium (Fig. 3). Kyu-W63 had a similar affect on the growth of 10 plant pathogenic fungi belonging to the Ascomycetes, Basidiomycetes, Zygomycetes, and Deuteromycetes. The antifungal spectrum of Kyu-W63 appears to be rather broad. There was no lysis or morphological change in the mycelium of any of these fungi when observed with a light microscope. All the tested fungi resumed normal growth when Petri dishes containing Kyu-W63 were removed. Thus, the inhibitory effect of Kyu-W63 was fungistatic but not fungicidal. In addition to plant pathogens, Kyu-W63 also showed the inhibitory effect on allergenic fungi such as *Penicillium islandicum*, *Aspergillus fumigatus*, *Aspergillus restrictus*, etc. which are often detected in houses and other environments (Fig. 4)[16].

Identification of volatiles produced by Kyu-W63

GC-MS analysis was used to identify two substances produced by Kyu-W63 with molecular weights of 164 and 166 (Fig. 5). Nuclear magnetic resonance analysis revealed that Kyu-W63 produces two types of volatile

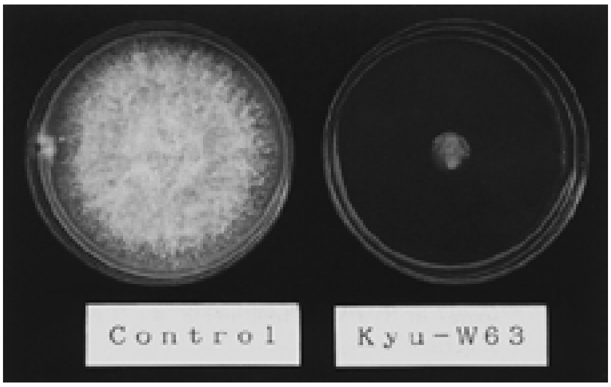


Fig. 3. Radial growth of *Fusarium graminearum* covered for 5 days with Petri dish containing either Kyu-W63 or the PDA control

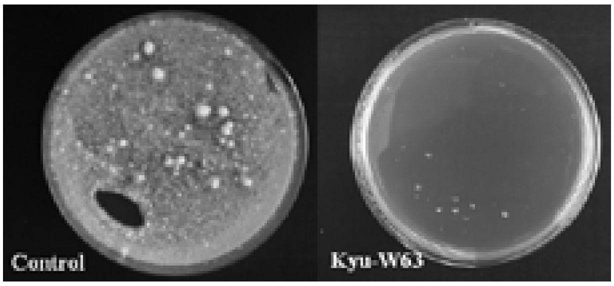


Fig. 4. Colony of *Penicillium islandicum* NBRC5234 (NBRC Biohazard Level 2) covered for 4 days with Petri dish containing either Kyu-W63 or the PDA control

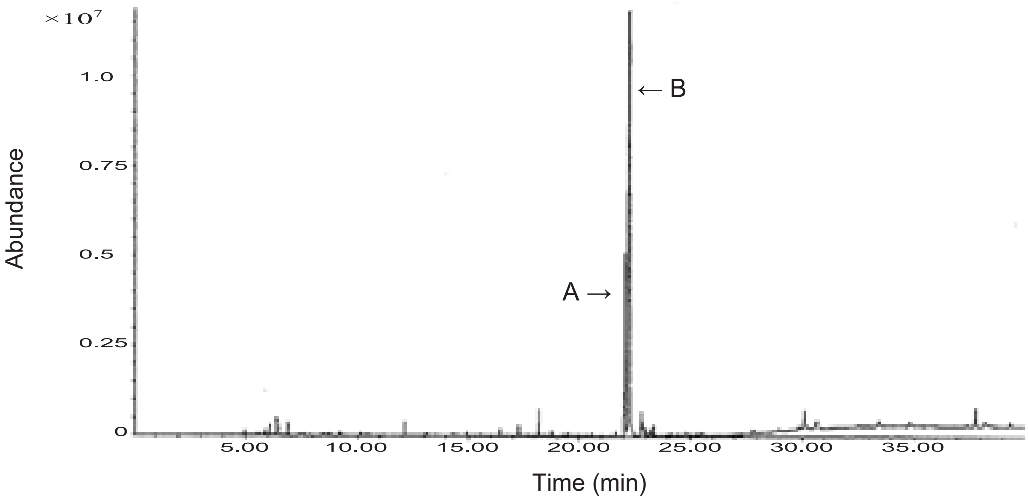
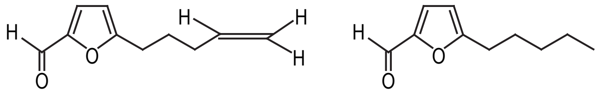


Fig. 5. GC–MS chromatogram of Kyu-W63 after 1-week incubation on PDA
A: 5-(4-pentenyl)-2-furaldehyde, B: 5-pentyl-2-furaldehyde.



A B

Fig. 6. Structures of 5-(4-pentenyl)-2-furaldehyde (A) and 5-pentyl-2-furaldehyde (B)

substances: 5-pentyl-2-furaldehyde (PTF) and 5-(4-pentenyl)-2-furaldehyde (PNF) (Fig. 6). Although these compounds are known to be nematicidal[8], this is the first report of antifungal activity for 5-(4-pentenyl)-2-furaldehyde[13]. The antifungal activity of PTF produced by filamentous fungi has not been known with the exception of antifungal activity against *Nematospora coryli*[14]. Culture experiments revealed that synthesized PTF had antifungal activity against three other pathogens: *Fusarium oxysporum* f. sp. *lycopersici*, *Glomerela cingulata*, and *Botrytis cinerea*, with a minimum inhibitory concentration of 2–4 μg/ml. The two substances (PTF and PNF), which were fractionated from the extract of Kyu-W63 grown on PDA plates, also showed inhibitory activity toward *G. cingulata*.

Herrington et al.[9] reported that volatile substances, including methyl vinyl ketone and methyl ethyl ketone in *Streptomyces griseoruber*, inhibited spore germination of *Cladosporium cladosporioides*. Volatile antimicrobials such as monoterpene and polycyclic aromatic hydrocarbons are also produced by a variety of plants[4,7]. However, no volatile substances produced by filamentous fungi have been reported to inhibit the growth of *B*. *graminis* and other plant pathogenic fungi.

Characteristics of Kyu-W63

Because the absence of spores in the strain Kyu-W63 made its identification difficult, the rDNA internal transcribed spacer (ITS) region of Kyu-W63 was sequenced and compared with that of *Irpex lacteus*, which produces 5-pentyl-2-furaldehyde and 5-(4-pentenyl)-2-furaldehyde. The ITS sequences of Kyu-W63 completely agreed with those of *I. lacteus*, and the strain Kyu-W63 was therefore inferred to be *I. lacteus*[13].

Two strains of *I. lacteus* (NBRC 5367 and MAFF 420021) tested also produced the two furaldehydes and suppressed the growth of *F. oxysporum* f. sp. *lycopersici*. Both Kyu-W63 and the above strains produced the two furaldehydes on PDA but not on water agar. The productivity of the antifungal substances from the *I. lacteus* and Kyu-W63 strains and their antifungal activities were compared. Among these three strains, Kyu-W63 had the strongest inhibitory activity.

Biocontrol of parsley powdery mildew

The incidence of parsley powdery mildew caused by *Oidium* sp. has been increasing recently in Japan, but the limited number of registered fungicides and the absence of disease-resistant parsley cultivars make it difficult to control the disease[10]. Because powdery mildews have mostly an ectotrophic life cycle, one can assume that they are easy targets for antagonists. Research on biological control of powdery mildew has mainly focused on studies of its hyperparasites[1,2], but little is known about the usefulness of filamentous fungi that produce antifungal volatiles.

Biocontrol of parsley powdery mildew was examined using Kyu-W63 for 3 years in a greenhouse (17 m long and 5.4 m wide with top height of 3.2 m) under conditions similar to growers' conditions in Kyushu. Ten parsley plants (cv. Kitano No. 1) per row were transplanted at 35-cm intervals in ridges 2 months after sowing. Each ridge had four rows that were 20 cm apart. Kyu-W63, cultured on PDA medium in sterile polycarbonate pots (100 × 100 × 100 mm), was placed at 30-cm intervals in the center of each ridge (4.5 m long and 1.2 m wide) in 1998, 1999 and 2000[11].

The symptoms of powdery mildew were investigated every week after the treatment with Kyu-W63. At harvest, disease severity and efficacy of disease inhibition were assessed for 300 leaves that were mature enough to harvest from each ridge. The completely randomized experimental designs included three repetitions (ridges) of 40 plants each. Of the 40 plants, 30 were randomly selected, and then a total of 300 leaves (10 leaves per plant) were assessed for disease severity.

For 3 years, Kyu-W63 kept in tandem in the center of each ridge in the greenhouse significantly inhibited disease severity compared to control plots. A significant difference in disease severity was observed between the control and the Kyu-W63 treated plot for 3 years (Figs. 7 & 8). Neither injury nor abnormal growth of parsley with the use of Kyu-W63 was observed during 3 years. The effectiveness of Kyu-W63 antifungal volatiles on disease suppression was first demonstrated under greenhouse conditions similar to growers' conditions in this experiment, though symptoms of powdery mildew were not completely suppressed. As a result of this experiment, we know that Kyu-W63 is more useful than other techniques because it is labor-saving and low cost.

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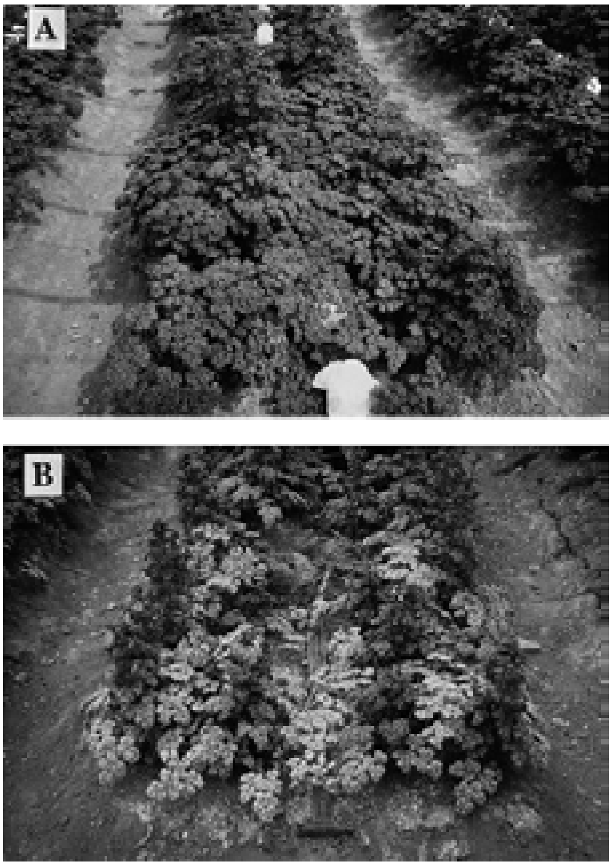

Fig. 7. Symptoms of greenhouse parsley plants infected with powdery mildew (April 24, 1998)
A: Kyu-W63 treated plot, B: Control (untreated).

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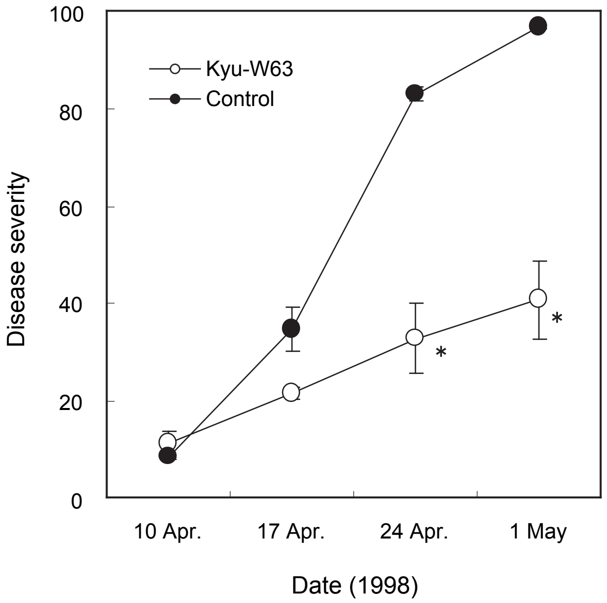

Fig. 8. Inhibition effect of Kyu-W63 treatment on the development of parsley powdery mildew in 1998
Inhibitory effects were compared among different treated plots in 1998. Kyu-W63 was kept in tandem in the center of each ridge (○) in the greenhouse in 1998. Untreated plot was used as control (●).
*: $P < 0.01$; significantly different from control according to Student's *t* test. Data represent means of three replicates. Vertical bars represent standard error.

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