Biological Mode of Action of Flutolanil in Its Systemic Control of Rice Sheath Blight

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ABSTRACT

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The systemic fungicide flutolanil is used to control rice sheath blight caused by *Rhizoctonia solani* with application of granules to the paddy in Japan. We studied the effect of flutolanil on the infection process of *R. solani* by this application method. In a greenhouse study, a linear correlation ($P < 0.001$; $r = 0.944$) was obtained between the log of flutolanil concentration in rice leaf sheaths and disease suppression, with approximately 1.8 μg/g of flutolanil in leaf sheaths contributing to about 80% suppression. Percent inhibition of hyphal growth and infection-cushion formation of *R. solani* on leaf sheaths increased as the amount of active ingredient increased, with 2 μg/g of flutolanil in the leaf sheaths causing about 80% inhibition. Micromanipulation in a scanning electron microscope revealed that penetration from infection cushions was completely inhibited on leaf sheaths containing approximately 2 μg/g of flutolanil. These results suggest that the inhibitory effects of flutoalanil on fungal growth and penetration depend on flutolanil concentration in leaf sheaths.

Flutolanil is a systemic fungicide used to control rice sheath blight caused by *Rhizoctonia solani* Kühn (teleomorph = *Thanatephorus cucumeris* (Frank) Donk) and other diseases caused by *Rhizoctonia* spp., *Corticium* spp., *Typhula* spp., and several genera of rust fungi (1,2). Earlier studies in the greenhouse and field showed that flutolanil was highly effective against rice sheath blight when applied as a foliar spray or when applied directly to paddy water (12). The biochemical mode of action of flutolanil seems to be inhibition of a succinate dehydrogenase complex (SDC), an important enzyme complex in the respiratory chain of basidiomycetous fungi but not of fungi in other classes (13).

R. solani mainly grows on the surface of the inner epidermis of rice leaf sheaths, forming infection cushions and penetrating the inner and later the outer epidermal cells of leaf sheaths (6,10,11). To study the effects of flutolanil on the infection process of *R. solani*, we have focused on fungal growth on rice leaf sheaths.

When flutolanil was used as a foliar spray, inhibition of SDC activity resulted in inhibition of fungal growth on the leaf sheaths, followed by collapse of hyphae and infection cushions probably due to a decrease in adenosine 5′-triphosphate (3). However, little is known about how flutolanil affects the infection process of the fungus and the control of rice sheath blight when the chemical is applied to the paddy water. Thus, our objective was to study the infection process of *R. solani* when flutolanil was applied to the paddy water. A preliminary report has been presented (4).

MATERIALS AND METHODS

Inoculum. Isolate A-7 of *R. solani* anastomosis group (AG)-1A (isolated from naturally infected rice from Chugoku National Agricultural Experiment Station, Japan) was used in all experiments. For the greenhouse study, hyphal inoculum of *R. solani* was prepared from 1-wk-old rice husk-wheat bran cultures grown at 25 C after transfer from 1-wk-old potato-dextrose agar (PDA) cultures (12). For the laboratory study, sclerotial inoculum was obtained from 3- to 4-day-old PDA cultures grown at 25 C.

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Plants. Three rice plants (*Oryza sativa* L. 'Nipponbare') were grown in each 9-cm-diameter pot containing soil and sand in a greenhouse. Plants at the eight-leaf to 10-leaf stage were used for experiments.

Fungicide and application. Flutolanil 25% WP (Nihon Nohyaku Co., Ltd., Japan) was applied at various concentrations into paddy water in pots with rice plants grown in the greenhouse. Application of 12.7 ml at 100 μg a.i./ml in a 9-cm-diameter pot was equal to 2 kg a.i./ha. Paddy water in pots was kept 2 cm deep after application. Plants were used for greenhouse and laboratory studies 7 days after application.

Greenhouse study. Hyphal inoculum (0.2 g) was placed in each leaf sheath of both treated and untreated rice plants 2 cm above the water line and covered with aluminum foil. Inoculated plants were incubated at 25 C in a moist chamber at 100% relative humidity, and average disease severity was determined by measuring the distance between the inoculation point and the top margin of the uppermost lesion on each leaf sheath. Percent disease suppression was assessed as follows: disease suppression (%) = [1 − (disease severity of treated plants)/(disease severity of untreated plants)] × 100. A randomized complete block design with eight replicates was used to test flutolanil rates of 0.25, 0.5, and 1 kg a.i./ha; three trials were conducted. In another test, rates of 0.75, 1.5, and 3 kg a.i./ha were used. At inoculation, the two outermost leaf sheaths of one plant were sampled from each pot and assayed for the concentration of flutolanil in sheath tissues as described previously (12).

Inoculation with sclerotia. Leaf sheaths about 15 cm long, detached from treated and untreated plants, were split longitudinally into two strips. Half of each strip was inoculated and the other half was assayed for flutolanil concentration. Six strips of leaf sheaths were inoculated in each treatment. For inoculation, a sclerotium was placed on the inner epidermis of the sheath strip. Inoculated strips were incubated in a moist box at 25 C and hyphal growth and infection-cushion formation were evaluated. Average hyphal growth was determined by measuring the distance between the inoculation point and the hyphal tip farthest from that point on each strip. Average infection-cushion formation was determined by measuring the distance between the inoculation point and the farthest edge of the infection cushion formed farthest from that point on each strip. A randomized complete block design with three replicates of three flutolanil

treatments was used; the experiment was repeated twice.

Scanning electron microscopy. Leaf sheath strips inoculated with sclerotia were cut into small pieces (1.0 × 0.5 cm) 48 hr after inoculation and treated by the tannic acid fixation method (8) for observation in a scanning electron microscope (SEM) as described previously (5). Infection cushions were removed by micromanipulation in an SEM to expose their impressions on leaf sheaths. Fifty infection cushions on six different leaf sheaths were observed for each treatment.

RESULTS

Greenhouse study. Flutolanil effectively inhibited disease development of sheath blight when applied to paddy water in pots 7 days before inoculation with *R. solani*. A highly significant ($P < 0.001$) linear relationship was obtained between the log of flutolanil concentration in rice leaf sheaths and disease suppression expressed as arcsins (Fig. 1). Flutolanil at approximately 1.8 μg/g in leaf sheaths contributed to about 80% suppression of sheath blight.

Inoculation with sclerotia. On detached leaf sheaths of untreated plants, hyphae grew from the inoculation point at the rate of 0.86 mm/hr (Fig. 2), and branching hyphae about 10 mm behind the hyphal tips coalesced and formed infection cushions. Sites where infection cushions were formed farthest from the inoculation points migrated at the rate of 0.88 mm/hr. On leaf sheaths of plants grown in water treated with 0.5 or 1 kg a.i./ha, hyphae grew at the rate of about 0.41 and 0.45 mm/hr, and sites where infection cushions were formed farthest from the inoculation points migrated at the rate of 0.49 and 0.29 mm/hr, respectively. Although hyphal tips 18 mm from the inoculation point 24 hr after inoculation formed infection cushions about 12 hr later on untreated plants, hyphal tips on plants treated with flutolanil at 0.5 and 1 kg a.i./ha took 36 and 56 hr, respectively, to reach the same length and 22 to more than 24 hr to form infection cushions, respectively.

Hyphal growth and infection-cushion formation were completely inhibited until 48 hr after inoculation on plants grown in water treated with flutolanil at 2 kg a.i./ha. The relationship between flutolanil concentration in leaf sheaths and inhibition of fungal growth is shown in Table 1. As flutolanil concentration in leaf sheaths increased, inhibition of hyphal growth and infection-cushion formation increased. Both hyphal growth and infection-cushion formation were reduced about 80% on leaf sheaths containing 1.95 μg/g of flutolanil as compared with the untreated control.

Scanning electron microscopy. The infection process on untreated leaf sheaths was observed in an SEM. Infection cushions of *R. solani* (Fig. 3) on the surfaces of the inner epidermis of leaf sheaths were similar to those reported by Matsuura (11). To observe penetration sites, infection cushions on leaf sheaths were removed with a microneedle 48 hr after inoculation. Wall debris of infection cushions was observed on the sheath surface, and penetration pores were clearly revealed in the impressions of the infection cushions (Fig. 4a and b). Penetration pores were approximately 1 μm in diameter (Fig. 4b). Infection cushions appeared to be attached tightly to the surface of the epidermis because they were not readily lifted and left much wall debris on the surface after they were removed. When the epidermal cell wall (Fig. 4a) was inverted by a microneedle, infection hyphae were observed in the cell lumen (Fig. 5), suggesting that penetration was completed in untreated leaf sheaths by 48 hr after inoculation.

There was no difference between the features of infection cushions formed on untreated leaf sheaths (Fig. 3) and those on leaf sheaths containing 1.95 μg/g of flutolanil (Fig. 6). When the infection cushion shown in Figure 6 was removed 48 hr after inoculation, no penetration pores and no wall debris of the infection cushion were observed on the treated leaf sheath (Fig. 7). Similar observations were made when infection cushions on the leaf sheaths containing 1.95 μg/g of flutolanil were removed at 5 days after inoculation. When infection cushions on leaf sheaths containing 0.6 μg/g of flutolanil were removed 48 hr after inoculation, tiny penetration pores (0.3 μm diameter) were exposed (Fig. 8). However, penetration apparently had not been completed by 48 hr, as evidenced by comparison of penetration

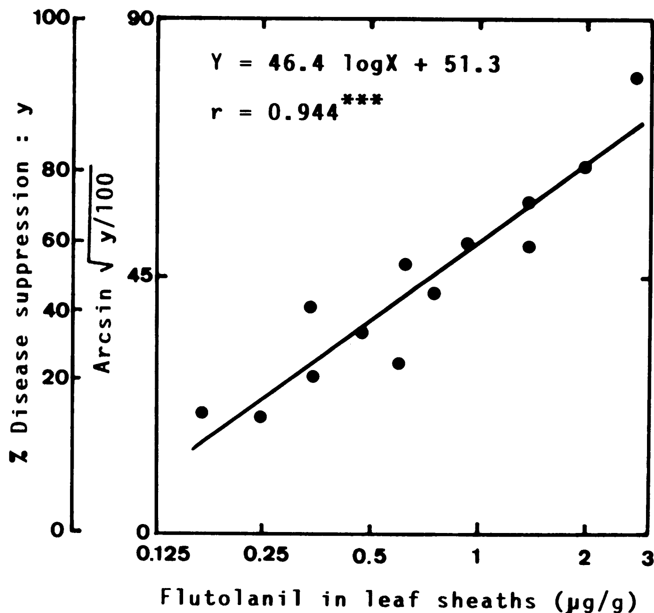


Fig. 1. Relationship between disease suppression of rice sheath blight by flutolanil and the log of flutolanil concentration in rice leaf sheaths. Flutolanil at various concentrations was applied to paddy water in pots 7 days before inoculation, and leaf sheaths were assayed just before inoculation. Asterisks denote significance at $P < 0.001$. Data points represent means of eight replicates.

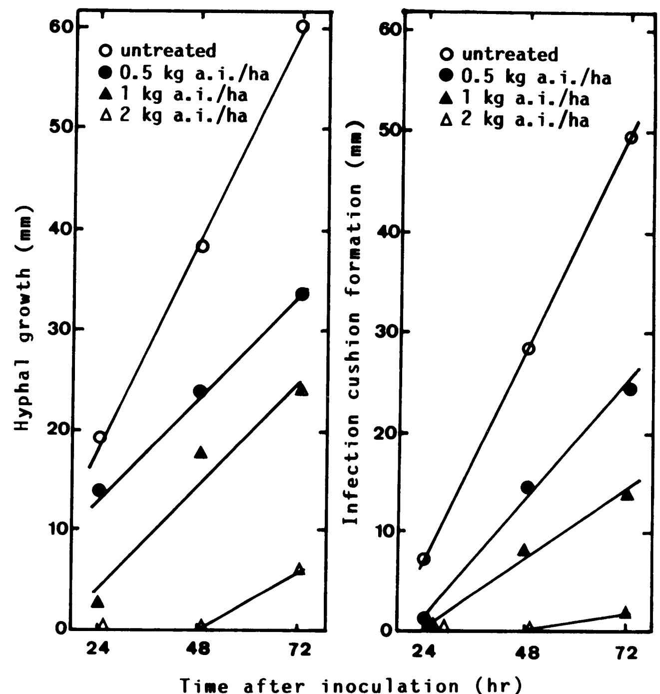
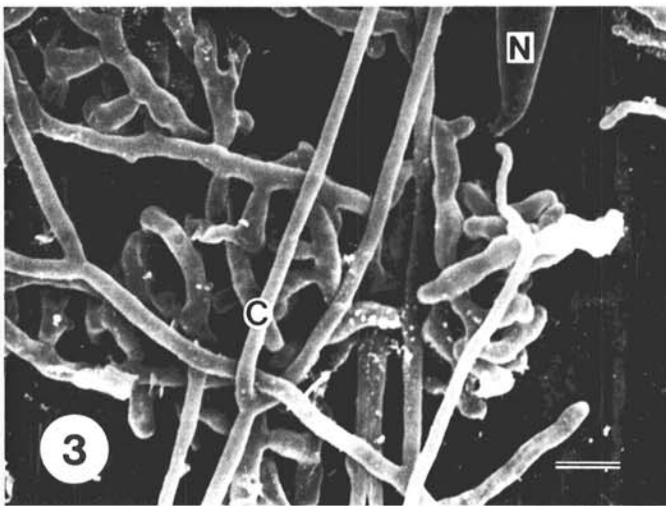
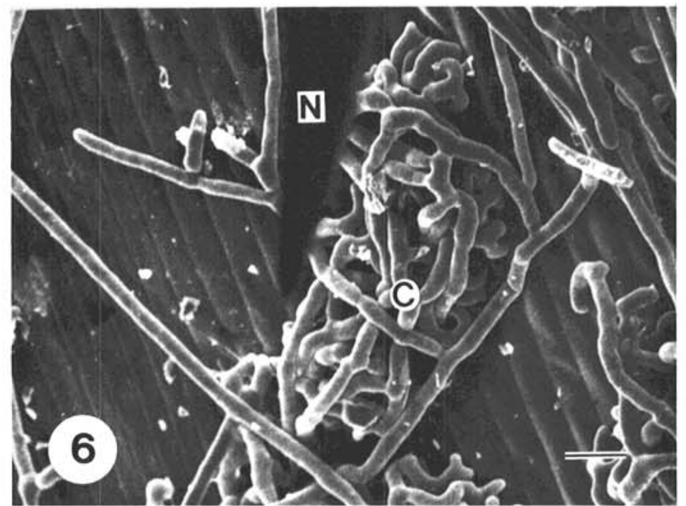
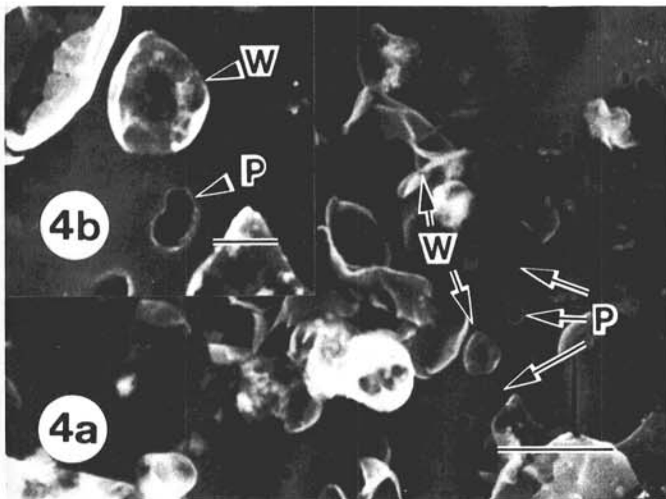
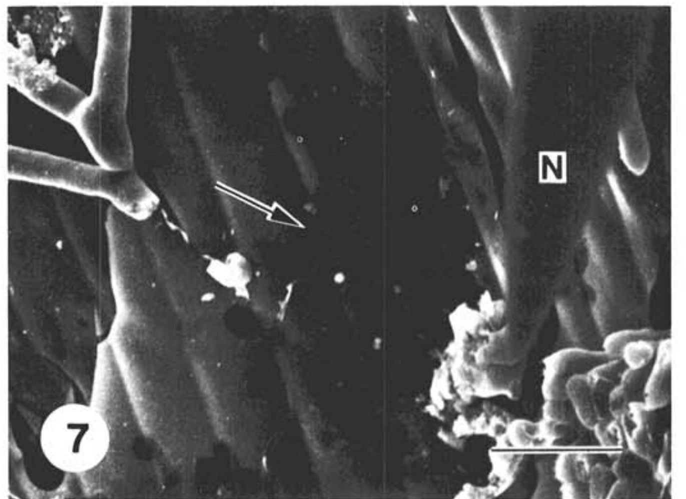


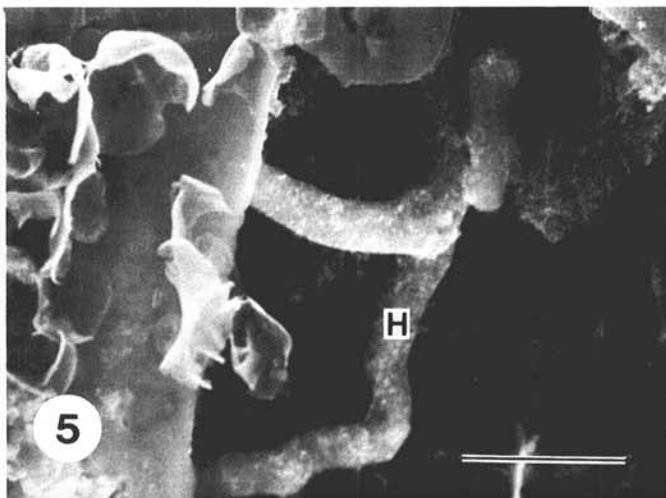
Fig. 2. Effect of flutolanil on hyphal growth and infection-cushion formation on untreated and treated rice leaf sheaths after inoculation with *Rhizoctonia solani* AG-1A. Flutolanil was applied to paddy water in pots at 0.5, 1, and 2 kg a.i./ha. Regression analyses of the results were performed, except for results on plants treated with 2 kg a.i./ha. Slopes of regression lines in hyphal growth and infection-cushion formation were 0.86, 0.88 (untreated), 0.41, 0.49 (0.5 kg a.i./ha), and 0.45, 0.29 (1 kg a.i./ha), respectively. Correlation coefficients (r) ranged from 0.970 to 0.999. Data points represent means of 12 replicates (six replicates pooled from two trials).

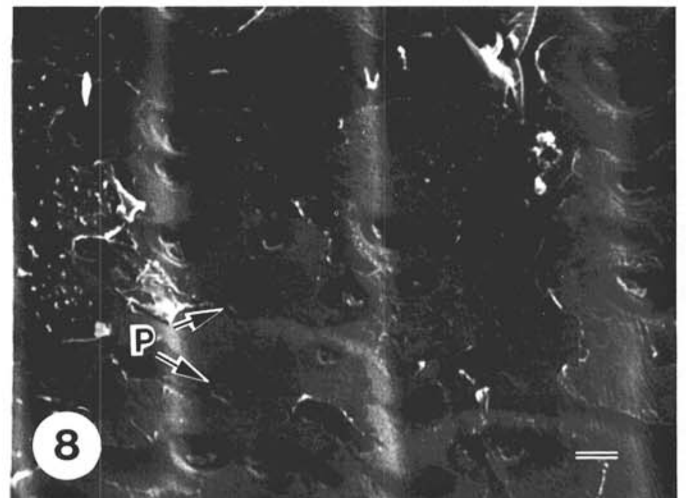












Figs. 3–8. Scanning electron micrographs of the infection process of *Rhizoctonia solani* on the inner epidermis of detached leaf sheaths from untreated and flutolanil-treated rice plants. C = infection cushion; H = hypha; N = microneedle; P = penetration pore; W = wall debris. **3,** Infection cushions on an untreated leaf sheath 48 hr after inoculation. Bar = 10 μm. **4a,** Wall debris and penetration pores observed in the impression of the infection cushion. The infection cushion (Fig. 3) was removed with a microneedle. The wall debris could not be removed. Bar = 5 μm. **4b,** An enlarged portion of the impression of the infection cushion shown in Figure 4a. A penetration pore and wall debris were visible on the flat surface, which was attached to the infection cushion. Bar = 1 μm. **5,** Infection hypha in an epidermal cell lumen. Bar = 5 μm. **6,** An infection cushion on a leaf sheath that contained 1.95 g/g of flutolanil 48 hr after inoculation. Bar = 10 μm. **7,** The impression (arrow) of the infection cushion shown in Figure 6. No wall debris and no penetration pores were visible. Bar = 10 μm. **8,** The impression of the infection cushion on a leaf sheath containing 0.6 μg/g of flutolanil 48 hr after inoculation. The infection cushion was removed with a microneedle. Tiny penetration pores are visible. Bar = 1 μm.

TABLE 1. Inhibitory effect of flutolanil on hyphal growth and infection-cushion formation of *Rhizoctonia solani* on rice leaf sheaths when flutolanil was applied to paddy water in pots

Flutolanil concentration in leaf sheaths[a] (μg/g)	Inhibition (%)[b]	
	Hyphal growth	Infection-cushion formation
1.95	77	87
0.64	53	67
0.34	22	29

[a] Flutolanil at various concentrations was applied 7 days before inoculation to water in pots in which rice plants were grown. The two outermost leaf sheaths were sampled just before inoculation and assayed for flutolanil concentration.

[b] Inhibition as a percent of growth and cushion formation on untreated leaf sheaths. The leaf sheaths were inoculated with sclerotia, and hyphal growth and infection-cushion formation were assessed 56 hr after inoculation. Each value represents the mean of six replicates.

pores on untreated and treated leaf sheaths (Figs. 4b and 8). Because infection cushions in leaf sheaths containing 0.6 and 1.9 μg/g were easily removed with a microneedle, they appeared to be less firmly attached than those on untreated sheaths.

DISCUSSION

In the greenhouse study, we showed that flutolanil was taken up from paddy water and translocated internally throughout the leaf sheaths. These results were consistent with previous studies reporting that flutolanil could be detected in leaf sheaths and blades after 6 days when applied to the paddy water (12). The acropetal translocation of the fungicide by this method of application also was established in experiments with ^{14}C-flutolanil (14). The correlation between flutolanil concentration in rice leaf sheaths and disease suppression (Fig. 1) indicates that disease suppression by flutolanil depends on its concentration in leaf sheaths. This conclusion is supported by both the laboratory study and scanning electron microscopy.

To verify how flutolanil affects the development of *R. solani* during the infection process, hyphal growth and infection-cushion formation were observed in our laboratory study, and we examined phenomena of penetration from infection cushions in an SEM. In the laboratory study, we demonstrated that flutolanil inhibited hyphal growth and infection-cushion formation on leaf sheaths, that the incident time of infection-cushion formation was slower on treated than on untreated plants (Fig. 2), and that about 2 μg/g of flutolanil in the leaf sheaths resulted in 80% inhibition (Table 1). Our observations in the SEM, coupled with micromanipulation, clearly showed that penetration from infection cushions was inhibited on leaf sheaths containing about 2 μg/g of flutolanil. Because multiple infections by *R. solani* occur from hyphae growing along rice leaf sheaths (6,10,11), we conclude that systemic control of rice sheath blight by flutolanil is due to its direct antifungal activity on fungal growth and penetration from infection cushions. Micromanipulation in an SEM is a useful technique to investigate penetration processes (5,7,9).

We reported previously that direct foliar application of flutolanil at 2 μg a.i./ml to the fungus growing on rice leaf sheaths resulted in inhibition of fungal growth and morphological changes in the fungus—that is, collapse of hyphae and infection cushions (3). There were no obvious morphological changes of the fungus growing on rice plants containing 2 μg/g of flutolanil when it was applied to paddy water in pots (Fig. 6); however, less than 1 μg/ml of flutolanil was detected in the ambient water droplets on leaf sheaths of these plants (H. Mochizuki, *unpublished*). Thus, the different morphological effect on the fungus probably was due to the varied concentrations of flutolanil directly in contact with the fungus.

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