Mode of Action of Ferimzone, a Novel Systemic Fungicide for Rice Diseases: Biological Properties Against *Pyricularia oryzae* in Vitro

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ABSTRACT

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Ferimzone (TF-164), (Z)-o-methylacetophenone 4, 6-dimethyl-2-pyrimidinyl-hydrazone, inhibited the mycelial growth of *Pyricularia oryzae* by more than 96% at 5 μg/ml; the inhibition was observed about 4 hr after addition of ferimzone. Ferimzone at 20 μg/ml did not inhibit spore germination, and germ tubes grew to several cells with an apparently normal nucleus in each cell. However, the cytoplasm of the spores and hyphae were granulated and localized. Application of fluorescent probes such as calcofluor white, aniline blue, and FITC-conjugated lectins did not reveal any effect of ferimzone in the cell wall architecture of mycelia. The activity of ferimzone was nullified by incubating the treated spores or mycelia in the toxicant-free medium. This suggests that the action of ferimzone is fungistatic and that the binding of ferimzone to cellular target components are rather loose. Ferimzone did not affect the respiratory activity of the mycelia of *P. oryzae*. Ferimzone caused the leakage of some electrolytes from mycelia, which decreased the pH of the medium, suggesting that ferimzone disrupted membrane function.

Additional keywords: antifungal activity, membrane function, rice blast.

Ferimzone (TF-164), (Z)-o-methylacetophenone 4, 6-dimethyl-2-pyrimidinyl-hydrazone (Fig. 1), is a novel systemic fungicide developed for the control of rice blast and brown spot diseases caused by *Pyricularia oryzae* and *Bipolaris oryzae*, respectively (10). Many fungicides have been developed and used over the last two decades for the control of rice blast disease. Most of these fungicides are protectants; few exhibit curative activity. Ferimzone has exhibited excellent curative activity against rice blast and brown spot diseases. Repetitive application of systemic fungicides has given rise to the emergence of strains resistant to the fungicides and this became a problem in practical agriculture (9,15). As for antiblast fungicides in Japan, development of resistance to the fungicides has become evident in *P. oryzae* (3,5,18). Ferimzone exhibited excellent antifungal and disease control activity against strains of *P. oryzae* resistant to organophosphorous fungicides and several antiblast antibiotics such as kasugamycin and blasticidin S (T. Okuno and K. Matsuura, *unpublished data*). This implies that primary site of action of ferimzone may be different from that of those fungicides.

It is important to know the mode of action for systemic fungicides, especially before they are introduced into the field, in order to evade or minimize the occurrence of resistant strains of pathogens. For instance, combined application with other fungicides that have different mode of action may discourage resistance of the pathogens.

In this paper some biological properties of ferimzone against *P. oryzae* were studied in vitro and the mode of action is discussed in comparison with those of other systemic fungicides.

MATERIALS AND METHODS

Chemicals. Ferimzone (technical compound containing Z-form of more than 97%, Takeda Chemical Industries Ltd.) was used throughout all experiments. Ferimzone was dissolved in ethanol and diluted with water to obtain a stock solution containing 200 μg/ml of ferimzone and less than 0.4% ethanol. Fluorescein isothiocyanate (FITC)-conjugated lectins were purchased from Maruzen Oil Biochemicals (Tokyo, Japan). Calcofluor white (CFW) (4, 4-bis [4-anilino-6-bis (2-hydroxymethyl) amino-s-triazin-2-ylamino] 2, 2′-stilbene disulfonic acid) was kindly supplied from Nihon Kayaku Co. Ltd. (Tokyo, Japan). DAPI (4, 6-di-8 amidino-2-phenylindole) was purchased from Nacalai Fesque Co. Ltd. (Kyoto, Japan). Oligomycin was purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of spores and mycelia. *Pyricularia oryzae* Cav., P-2 was cultured at 28 C for 5–7 days on a V-8 juice agar medium containing 100 ml of V-8 juice, 20 g of agar, 1.8 g of $CaCO_3$ and 900 ml of deionized water. Spores were obtained as described (13) and approximately 2×10^5 spores per milliliter were incubated in 100-ml flasks containing 20 ml of Czapeck-sucrose-yeast medium (CSY) (12) without shaking in the dark at 28 C for 16–20 hr. Mycelia were collected in 10-ml test tubes by centrifugation at 400 *g* for 5 min, suspended in a sterile deionized water. Then mycelia were collected on a sheet of lens paper (Nikon Co. Ltd., Tokyo, Japan) to wash away ungerminated spores with a glass Pasteur pipette. Spore-free mycelia were collected into 10-ml test tubes, washed twice with water by centrifugation, and suspended in an appropriate incubation medium.

Measurement of dry weight. For a dosage response of ferimzone in inhibition of mycelial growth of *P. oryzae* (mycelia approximately 1.5 mg in dry weight) or 2×10^5 spores per milliliter were incubated in 50-ml flasks containing 10 ml of CSY at various concentrations of fermizone. After 3 days of incubation at 30 C in a reciprocal shaker, the mycelia of triplicate flasks for each concentration were collected on a glassfiber filter (GC-50, Toyoroshi Co. Ltd., Tokyo, Japan) and washed with water in vacuum filtration. The filter paper was dried at 120 C for 60 min and weighed. For a time course study, mycelia (2.5 mg in

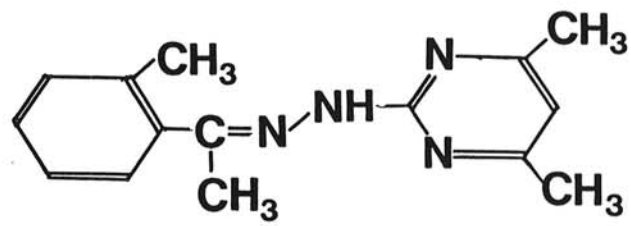

Fig. 1. Chemical structure of ferimzone.

dry weight) were incubated in CSY at 30 C in the absence and the presence of 20 μg/ml of ferimzone and the mycelia of triplicate flasks for each treatment were collected after 0, 1, 2, 3, 4.5, 6.0, 7.5, 9.0, and 10.5 hr of incubation and weighed as described above.

Observation of morphology. Spores and mycelia were incubated at 30 C in 20-ml flasks containing 10 ml of CYS in the absence and the presence of 20 μg/ml of ferimzone and after 0, 2, 4, 6, 8, 16, 24, and 48 hr of incubation, specimens were taken on a slide glass and observed with a Nikon inverted interference contrast microscope. Some specimens were stained with 0.1% cotton blue solution containing lactic acid, phenol, and deionized water (1:1:1, v/v/v) for 1 min and then observed.

Application of fluorescent probes. Approximately 2×10^5 spores per milliliter were incubated at 28 C in 20-ml flasks containing 5 ml of CYS in the absence and the presence of 20 μg/ml of ferimzone in a reciprocal shaker. After 16–24 hr of incubation, mycelia and germinated spores were collected by centrifugation, washed twice with water. Specimens (30 μl) were placed on a slide glass, and an equal volume of fluorescent probes was applied for staining. CFW was applied at 0.5 μg/ml in an aqueous solution. Aniline blue was applied at 0.05% in 0.067 M KH_2PO_4, pH 8.7, after decolorization with K_3PO_4 (17). The FITC-labeled lectins, concanavalin A (Con A), wheat germ agglutinin (WGA), and *Ricinus communis* agglutinin (RCA) were applied at 20 μg/ml in 0.01 M Tris-HCl buffer, pH 7.5, containing 0.001 M $CaCl_2$ for Con A and in 0.01 M phosphate buffer, pH 7.5, for WGA and RCA, respectively. DAPI was applied at 0.5 μg/ml in ethanol solution. Specimens were allowed to react for 1 min and examined with a Nikon microscope equipped with an epifluorescent attachment. For CFW, analine blue, and DAPI-induced fluorescence, a filter block (UV) with an excitation filter UV330-380, a dichroic mirror DM400, and a barrier filter 420K and for FITC-fluorescence, the corresponding filter block (B2) with filters IF460-485, DM510, and 520W were used, respectively.

Observation of spore germination, appressorial formation, and germination. Spores were incubated on nitrocellulose membranes in the absence and the presence of 0.8, 1.6, 3.1, 6.3, 12.5, 25, and 50 μg/ml of ferimzone as described (13). Approximately 200 spores were observed for spore germination and appressorial formation and approximately 200 appressoria were observed for appressorial germination as described (13).

Measurement of respiration. Respiratory activity of mycelia was measured with a Clark oxygen electrode (Yellow Springs Instrument Co. Inc.) in the relative rate of oxygen consumption. Mycelia, washed with 0.15 M phosphate buffer, pH 6.4, were incubated in the buffer at 30 C for 4 hr for starvation. The mycelia were washed twice with phosphate buffer by centrifugation, suspended in the buffer containing 0.1 M glucose, and aliquots (10 ml) were taken into 20-ml flasks. Ferimzone, oligomycin, and sodium azide were added to the flasks to give final concentrations of 25, 10, and 6.5 μg/ml, respectively. As a control 0.4% ethanol solution was added. Mycelia were incubated at 30 C in a reciprocal shaker, and every 60 min aliquots (0.5 ml) were taken into reaction vessels. The oxygen consumption rate of mycelia was determined during a 5-min incubation at 30 C in the vessel. The experiments were repeated three times.

Leakage of electrolytes. Mycelia were suspended in 200 ml of deionized water and 90-ml portions were taken into 100-ml flasks. After the addition of 10 ml of 200 μg/ml ferimzone or 0.4% ethanol, aliquots (5 ml) were taken into 20-ml flasks and incubated at 30 C in a reciprocal shaker. After 0, 1.5, 3.0, 4.5, 6.0, 7.5, 22, and 30 hr of incubation, the contents of duplicate flasks for each treatment were taken into 10-ml test tubes and centrifuged at 500 *g* for 10 min to obtain supernatants. After filtration with a filter paper (No. 2, Toyoroshi Co. Ltd.), aliquots (3.5 ml) of the clarified solution were used to measure pH with a pH meter (Horiba Seisakusho Co. Ltd., Kyoto, Japan). The same solution was used to measure conductivity with an electric conductivity meter (Shibata Scientific Technology Ltd., Tokyo, Japan). Other aliquots (0.5 ml) of the clarified solution were used for the detection of ninhydrin reactive substances according to the method of Yemm and Cocking (20). Sample solution (0.5 ml) was mixed with 0.85 ml of 0.6% ninhydrin solution containing 0.07 M citric acid, 0.12 M NaOH, 0.12 mM KCN, and 8.8 M methylcellosolve and boiled at 100 C for 15 min. After cooling with water, 2.5 ml of 60% ethanol was added, and absorbance at 570 nm was measured using a Hitachi 200-20 spectrophotometer. The experiments were repeated three times.

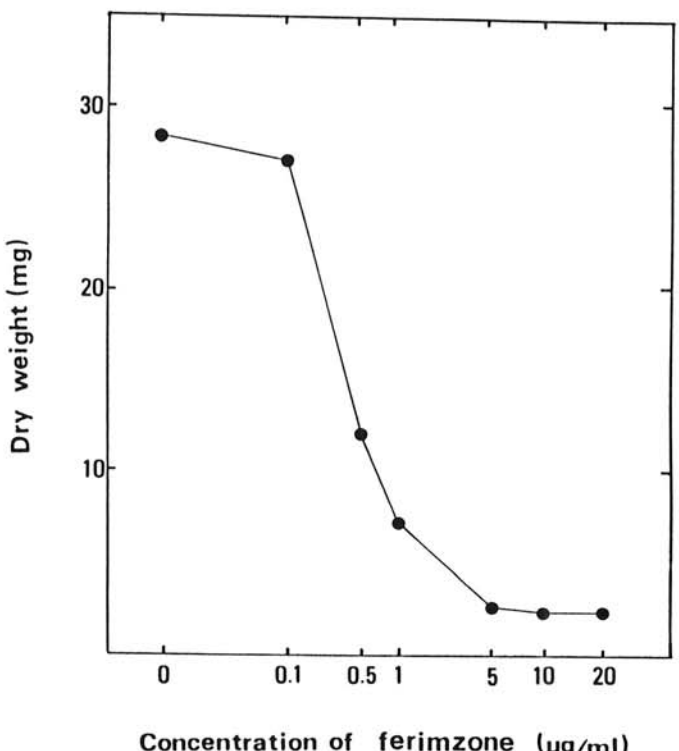

Fig. 2. Dosage response curve of ferimzone in mycelial growth of *Pyricularia oryzae*. Mycelia were incubated at 30 C for 3 days and the dry weight of mycelia was measured. Each point is an average of three replicates. The X represents the initial value of mycelial dry weight.

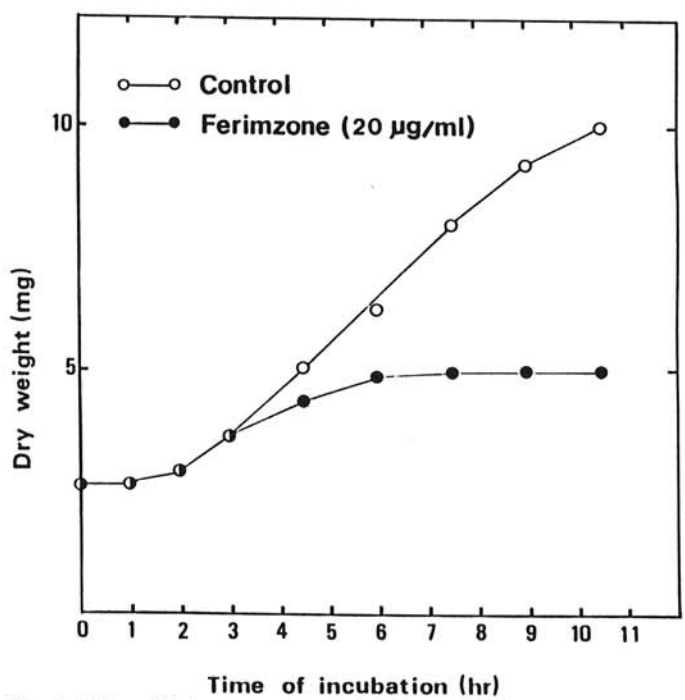

Fig. 3. Effect of ferimzone on mycelial growth of *Pyricularia oryzae* in time course. Mycelia were incubated at 30 C in the absence and the presence of 20 μg/ml of ferimzone for indicated times and the dry weight of mycelia was measured. Each point is an average of three replicates.

RESULTS

Effects on mycelial growth. A dosage response of ferimzone in inhibition of the mycelial growth of *P. oryzae* is shown in Figure 2. Mycelial growth was inhibited by more than 96% at concentrations of 5 μg/ml and above (Fig. 2). Similar results were obtained when spores were used as an initial material. The effect of 20 μg/ml of ferimzone on time course increase of mycelial growth showed that inhibition occurred about 4 hr after addition of ferimzone (Fig. 3). The sensitivity of mycelia to ferimzone was not influenced by the age of mycelia at least up to 3 days after incubation.

Effects on morphology. When spores of *P. oryzae* were incubated in CSY containing ferimzone at 10 μg/ml or higher concentrations, they germinated normally and hyphae grew to 20–40 μm and then ceased growth (Fig. 4). The hyphae consisted of two to three cells with septa (Fig. 4F), and each cell contained one nucleus apparently indistinguishable from that of the untreated hyphae stained with DAPI (Fig. 4C and D). The cytoplasm of hyphae elongated in the presence of ferimzone was

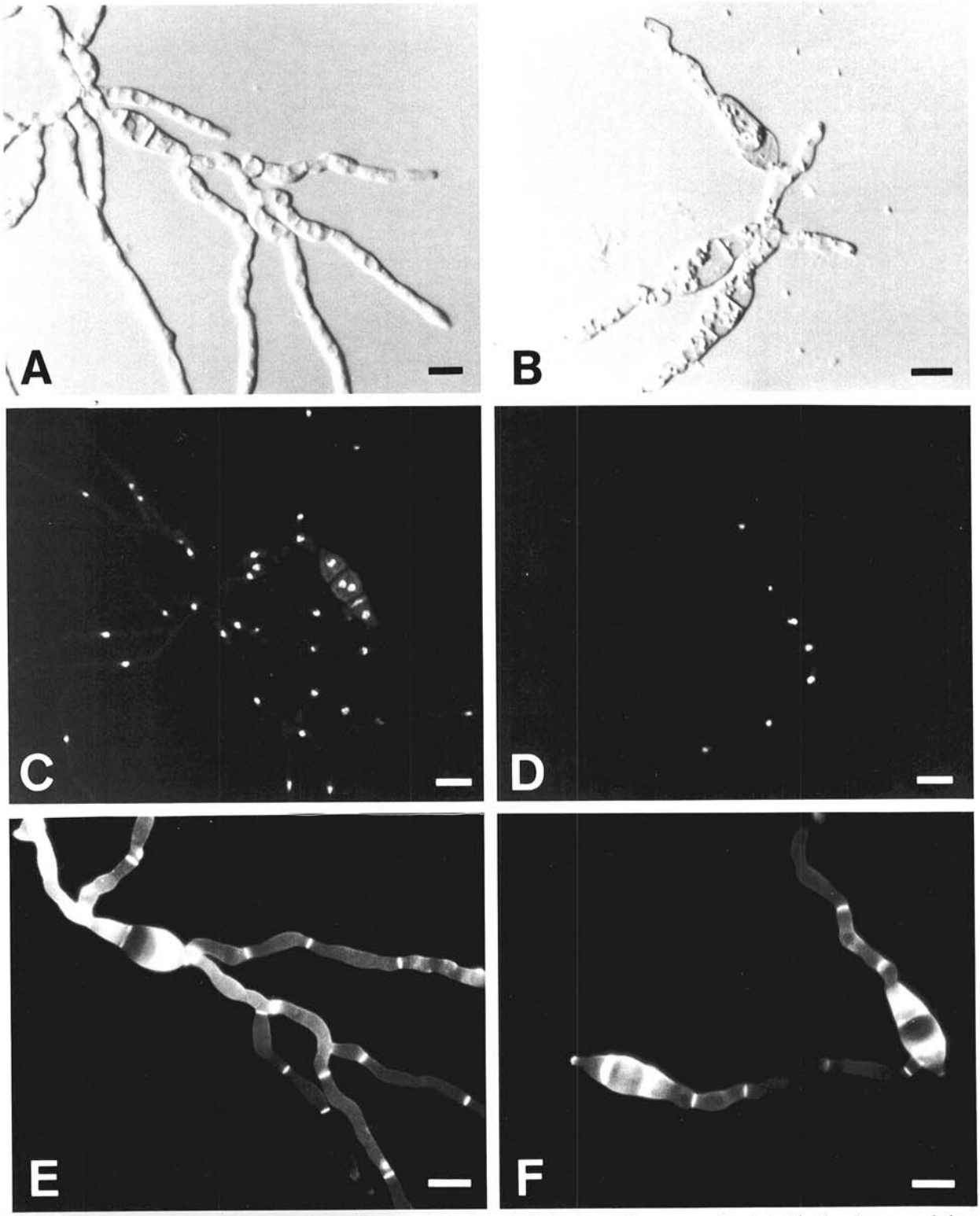

Fig. 4. Light and fluorescent photomicrographs of germinated spores of *Pyricularia oryzae*. Spores were incubated in the absence and the presence of 20 μg/ml of ferimzone at 28 C for 24 hr. **A, C and E,** control; **B, D and F,** treated with ferimzone. Specimens were stained with DAPI (C, D) and with calcofluor white (E, F). Bar represents 10 μm.

granulated and localized (Fig. 4B). When mycelia were incubated with 20 μg/ml of ferimzone, the granulation and localization of cytoplasm occurred irregularly in several cells after 6–7 hr of incubation and after 20 hr of incubation this was observed throughout whole mycelial cells (Fig. 5). No other morphological alterations such as swelling and distorted hyphal branching were induced by ferimzone. The antifungal activity of ferimzone and the effects on morphological appearance were nullified and decreased by incubating the treated spores or mycelia in ferimzone-free medium (Fig. 6), and the growth started again from the tips of hyphae (Fig. 6A). The restoration occurred even after 5 days of incubation with 20 μg/ml of ferimzone.

Responses to fluorescent probes for cell walls. Compared with untreated control, ferimzone did not induce any difference in response to all fluorescent probes used based on the observation of more than 100 germinated spores. Response to CFW was shown in Figure 4 (E and F).

Effects on appressorial formation and germination. More than 97% of spores of *P. oryzae* on nitrocellulose membrane germinated and about 70% of the spores formed dark-brown pigmented appressoria within 16 hr of incubation at 24 C. About 60% of appressoria germinated within an additional 48–96 hr of incubation at 28 C. When spores were incubated in the presence of 0.8, 1.6, 3.1, 6.3, 12.5, 25, and 50 μg/ml of ferimzone, apparently normal pigmented appressoria were formed at 1.6 μg/ml, but the germination of appressoria was completely inhibited at this concentration and the appressoria failed to penetrate the cellulose membrane. The pigmentation of appressoria was inhibited by about 95% at 3.1 μg/ml. The formation of unpigmented appressoria was not greatly influenced even at 12.5 μg/ml.

Effect on respiratory activity. The oxygen consumption rate of mycelia of *P. oryzae* was not influenced by 25 μg/ml of ferimzone up to 5 hr of incubation with the toxicant while oligomycin (10 μg/ml) and sodium azide (6.5 μg/ml) completely inhibited the oxygen consumption of mycelia after 1 hr of incubation (Table 1).

Effect on leakage of cellular substances. When mycelia were incubated with 20 μg/ml of ferimzone in deionized water, a rapid increase of conductivity in incubation medium was observed after about 4 hr of incubation, and the conductivity reached about fourfold of the untreated control after 30 hr of incubation (Fig. 7). In the same experiment, the pH of the incubation medium was decreased by the ferimzone treatment apparently in correspondence with increase in conductivity and reached around 4 after 22 hr of incubation while pH in the untreated control remained constant at around 7 (Fig. 7). In contrast to the effects on the change in conductivity and pH, ferimzone did not stimulate the leakage of ninhydrin positive substances from cells (data not shown).

DISCUSSION

Many systemic fungicides including benzimidazoles (1,14,19), dicarboximides (2), and ergosterol biosynthesis inhibitors (EBIs) (7) are known to induce morphological alterations in fungi directly or indirectly depending on their mode of action. EBIs also induce alterations in cell wall architecture of hyphae of *Penicillium italicum* and *Ustilago maydis* (7) and *P. oryzae* (T. Okuno, *unpublished data*) as revealed in stronger response to the stilbene type fluorescent brighteners. In the presence of ferimzone, spores of *P. oryzae* germinated normally and produced hyphal cells containing nucleus and septa. The application of fluorescent probes did not reveal any affection of ferimzone in response to them. Instead, ferimzone caused the granulation and localization of cytoplasm. In this regard, mode of action of ferimzone is not like many other systemic fungicides.

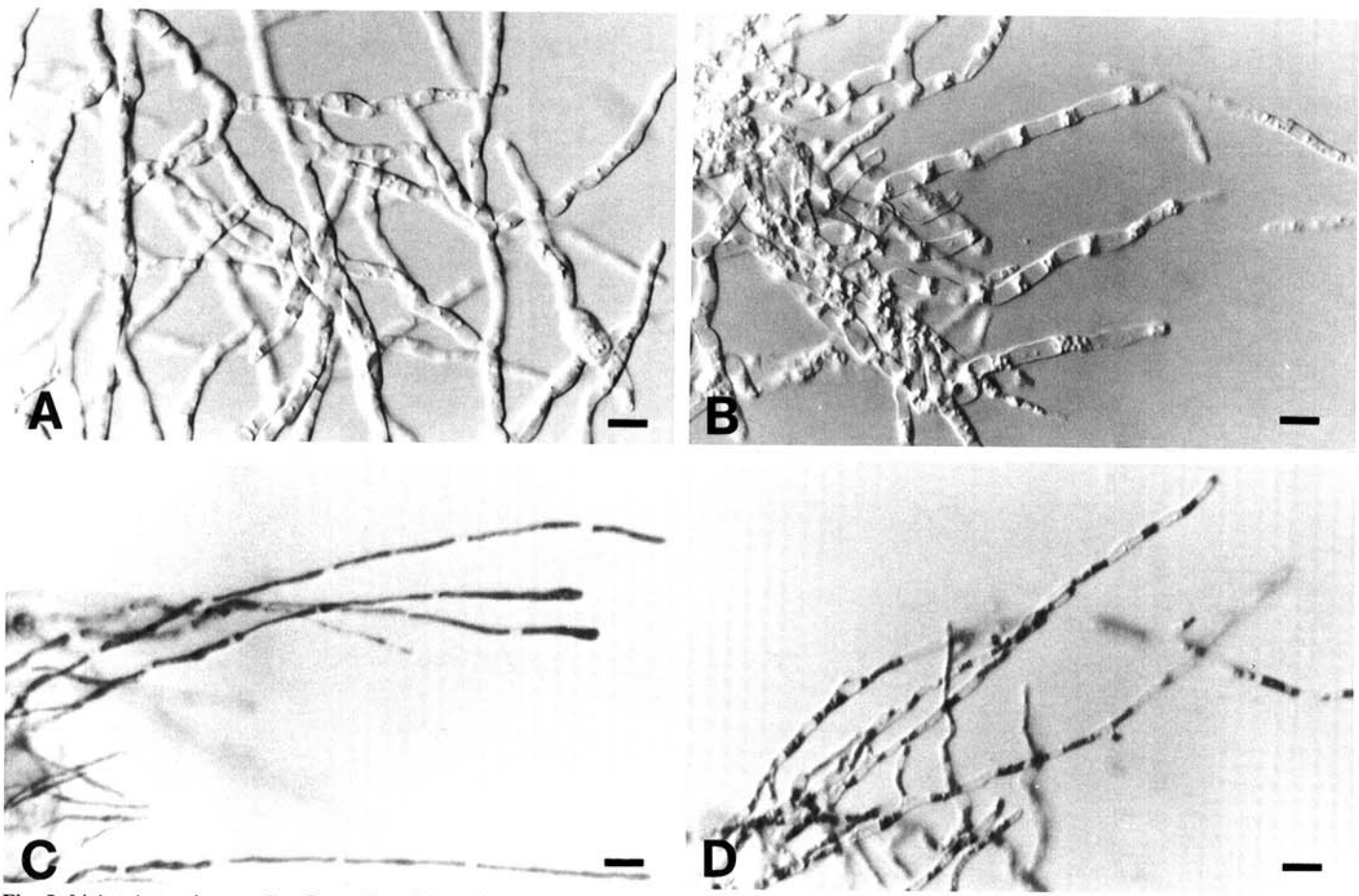

Fig. 5. Light photomicrographs of mycelia of *Pyricularia oryzae*. Mycelia were incubated in the absence and the presence of 20 μg/ml of ferimzone at 28 C for 48 hr. **A and C,** control; **B and D,** treated with ferimzone. Specimens were stained with cotton blue (C and D). Bar represents 10 μm.

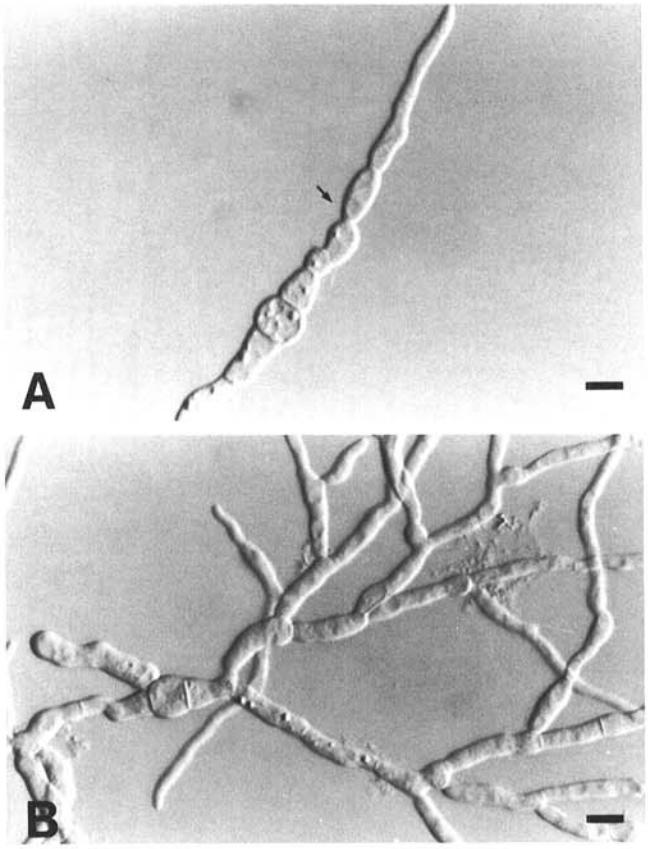

Fig. 6. Restoration of the morphological influence of ferimzone in *Pyricularia oryzae*. **A,** Spores and **B,** mycelia incubated with 20 μg/ml of ferimzone at 30 C for 48 hr were washed, incubated in the toxicant-free medium for additional 24 hr. Note the regrowth of hyphae (arrow in A) and decrease of the granulation and localization of cytoplasm (B) compared with the morphology shown in Figure 4B and Figure 5B. Bar represents 10 μm.

TABLE 1. Effect of ferimzone on the respiratory activity of mycelia of *Pyricuralia oryzae*[y]

Time after treatment (hr)	% of the untreated control in O_2 consumption rate[z]		
	Chemicals		
	Ferimozone	Oligomycin	Sodium azide
1	103.1 b	0 a	0 a
2	98.3 b	0 a	0 a
3	100.7 b	···	···
4	98.0 b	···	···
5	102.2 b	···	···

[y] Mycelia were incubated in 0.15 M phosphate buffer, pH 6.4, for 4 hr and then incubated in the buffer containing 0.1 M glucose in the absence and the presence of 25 μg/ml of ferimzone, 10 μg/ml of oligomycin, and 6.5 μg/ml of sodium azide at 30 C.

[z] Means of three separate experiments with the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

The antifungal activity of ferimzone against *P. oryzae* was exerted prominently in inhibiting mycelial growth, including appressorial germination. Because appressorial germination of *P. oryzae* is very sensitive to various conditional factors including chemicals (13) and nutrients such as sugars (T. Okuno, *unpublished data*), the antifungal character of ferimzone should be considered prominently through the inhibition of mycelial growth. The sensitivity of mycelia to ferimzone was not influenced greatly by the age of mycelia, unlike IBP (4) and isoprothiolane (11). These results are consistent with the prominent curative control activity of ferimzone against rice blast disease in plants.

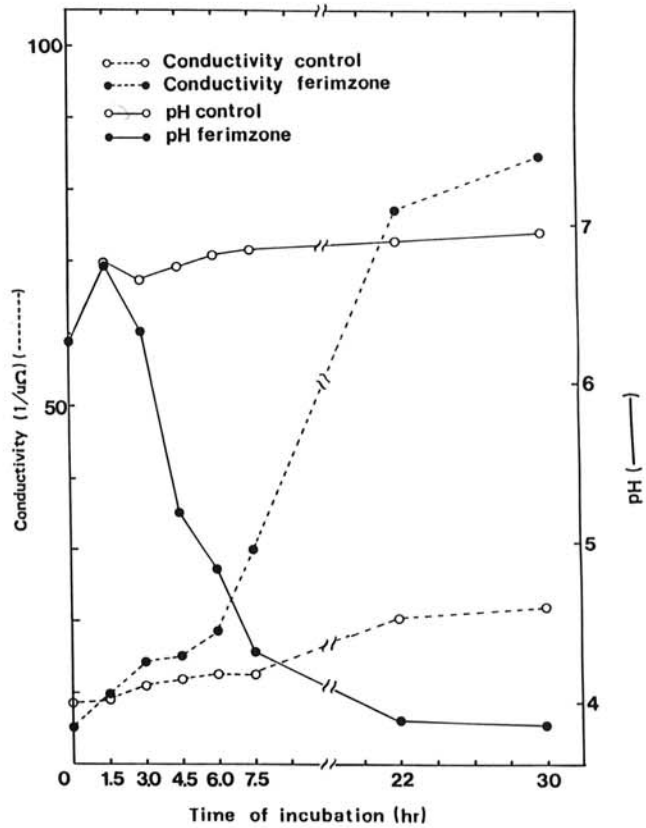

Fig. 7. Effects of ferimzone on the conductivity and pH of incubation medium. Mycelia were incubated in deionized water in the absence and the presence of 20 μg/ml of ferimzone at 30 C for indicated times. The clarified supernatant obtained by centrifugation and filtration of mycelial suspension were used for the measurement of conductivity and pH. Each point is an average of two replicates.

The binding of ferimzone to cellular target components appears to be rather loose because rinsing of mycelia treated with ferimzone for several days restored growth. In this regard ferimzone behaves much like metalaxyl (6).

The failure of ferimzone to influence the respiratory activity of *P. oryzae* suggests that ferimzone does not primarily affect energy metabolism. This would also not be inconsistent with the fungistatic nature of the toxicant.

Ferimzone enhanced the leakage of electrolytes, which decreased pH without affecting the leakage of amino acids from mycelia (Fig. 7). Polyene macrolide antibiotics such as nystatin, tetrin A, and filipin are known to disrupt membrane integrity by forming complex with sterol molecules in the membrane (8,16) and to cause the leakage of cellular substances. The mechanism of action of ferimzone does not seem similar to that of the antibiotics because although nystatin causes the leakage of electrolytes from mycelia of *P. oryzae* it did not decrease pH of medium. Also, the antifungal activity of ferimzone was not influenced by the addition of sterols (T. Okuno, *unpublished data*). Ferimzone may act like ionophore specific to acidic electrolytes.

In consideration of the fact that ferimzone does not inhibit the synthesis of cellular constituents such as DNA, RNA, protein, cell walls, and any lipid components (12), the effects on membrane function are likely responsible for the antifungal action of ferimzone.

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