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Effect of Prochloraz on Electrolytic Leakage and Spore Germination of *Puccinia recondita* Causing Wheat Leaf Rust

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The effects of prochloraz on membrane permeability and germination of uredospores of *Puccinia recondita* were investigated to determine its potential mode of action on wheat leaf rust control activity. Disease control activity of ergosterol biosynthesis inhibitors (EBIs) and their activities on uredospore membrane permeability and germination were examined with wheat leaf rust pathogen, both *in vitro* and *in vivo*. While wheat leaf rust was not controlled by prochloraz, electrolytic leakage and spore germination of *P. recondita* uredospore was the highest with the use of prochloraz among the eight fungicides tested. Prochloraz stimulated uredospore of *P. recondita* to germinate at a higher ratio. Although certain EBIs, such as hexaconazole, showed excellent control activity, their effects on uredospore membrane permeability and germination was much inferior to prochloraz. Therefore, results of this study suggest that effects of EBIs on membrane permeability and germination of uredospore are not always correlated with their disease control activity.

Keywords : Disease control activity, membrane permeability, prochloraz, *Puccinia recondite*, uredospore germination.

Fungicides inhibiting fungal ergosterol biosynthesis belong to an important group of antifungal agents used both in plant protection and in the medical field. Their biological activities have been described against a broad range of plant pathogens in a very low concentration, and are caused by the inhibition of ergosterol biosynthesis pathway, which has been reported in many plant pathogenic filamentous fungi (Hong et al., 1989; Kato et al., 1975; Kerkenaar et al., 1984; Sherald and Sisler, 1975; Siegel and Ragsdale, 1978).

The ergosterol biosynthesis inhibiting fungicides (EBIs) are very heterogeneous with respect to their chemical structure. Nevertheless, most of these can be grouped into three major classes such as: azoles; pyridines and pyrimidines with hydroxy functions; and morpholines. The primary mode of action of EBIs in azoles and in pyridines and pyrimidines is the inhibition of C14 demethylation in sterol biosynthesis (Siegel, 1981). Alternately, the morpholines were presented to interfere with the Δ^{14} reductase and/or $\Delta^{8} \rightarrow \Delta^{7}$ isomerase (Baloch et al., 1984). After treatment of EBIs, changes of fine structure of germ tubes according to blocking ergosterol biosynthesis and accumulating abnormal sterols have been frequently observed. Richmond (1984) reported the alteration of fine structure in germinating conidia of *Botrytis cinerea* by treatment of triadimefon, resulting in the production of stubby and swollen germ tube and striking changes in the process of septum formation. The ultrastructural changes of cell membrane in germ tubes of *Puccinia graminis* f. sp. *tritici* were published by Dahmen et al. (1988). The abnormal cell membrane caused by treatment of EBIs induced changes of membrane permeability and abnormal distribution of chitin in plasma membrane (Sancholle et al., 1984; Steel et al., 1989).

Prochloraz, known as a fungicide inhibiting ergosterol biosynthesis, is included in the imidazole group. Prochloraz gave excellent control to plant disease caused by *Septoria* spp., *Rhynchosporium secalis*, eyespot by *Pseudocercosporella herpotrichoides*, and net blotch by *Pyrenophora teres*, while it showed poor control activity against several rust diseases caused by *Puccinia* spp. (Copping et al., 1984).

In this study, the activity of prochloraz against *Puccinia recondita*, assessed in a greenhouse test with seedlings of wheat plant, was confirmed to be poor and less than the other EBI fungicides tested. In spite of the low fungicidal activity of prochloraz in the greenhouse, a dramatic change of electrolytic leakage in uredospore of *P. recondita* was attributed to the prochloraz treatment. Although prochloraz has been commercialized for the protection of plant diseases, it is not clear whether the effect on cell membranes is related with the biological activity.

This study identified the biological activity of prochloraz against wheat leaf rust caused by *P. recondite* by investigating the protective activities on wheat seedlings grown in the greenhouse and the changes of membrane permeability

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of uredospores *in vitro*. In addition, the morphological changes of uredospores were investigated with a light microscope after prochloraz application into spore suspensions.

Materials and Methods

Fungicides and test solution. Eight fungicides inhibiting ergosterol biosynthesis (Table 1) and benomyl were used in this test. To prepare test solutions at the indicated concentrations, each fungicide was dissolved in dimethyl sulfoxide (DMSO), followed by diluting with the solution containing 250 μg/ml of Tween 20. All fungicides used in this study were technical. The final concentration of DMSO was below 1%.

Maintenance of rust fungi and inoculum preparation. Uredospores of *P. recondita* were suspended in 250 μg/ml of Tween 20 solution to make 0.1 g of uredospores per 100 ml of final solution. To maintain rust fungi known as obligate fungal pathogen, the prepared uredospore suspension of *P. recondita* was inoculated to the first leaves of wheat by spraying. After keeping the wheat plants in saturated humidity chamber at 20°C for 24 hours, the plants were moved to a growth chamber (20°C, >65% RH) to induce the rust disease. For evaluating the biological activity of EBIs against wheat leaf rust and their effects on the membrane permeability of *P. recondita,* uredospores were harvested from lesions developed on the first leaves of wheat, and used as an inoculum in this study.

Determination of fungicidal activity. Fungicidal activities of each fungicide were detected on the first stage seedlings of wheat in a greenhouse. Ten (10) ml of each fungicide solution prepared as above was sprayed onto four pots, in each of which five wheat seedlings were planted. The treated plants were kept in a greenhouse for 1 day, following inoculation of rust fungi by spraying 2.5 ml of uredospore suspension per test pot. Untreated control was sprayed only with the Tween 20 solution containing 1% of DMSO. Treated plants were continuously placed in saturated humidity chamber for 24 hours and moved into the growth chamber to induce leaf rust. All tests were replicated three times. The disease control activity of fungicides against wheat leaf rust was evaluated with control value (CV) calculated by the formula CV (%) = [(A–B)/A] × 100, where A and B represent the disease severity on the treated and untreated plants, respectively.

Determination of membrane permeability. Membrane permeability was determined by measuring the electrolytic leakage in spore suspension of *P. recondita* applied with EBIs. The spore suspension was adjusted to 1 mg uredospore/ml with 250 μg/ml of Tween 20 solution. Each fungicide dissolved in DMSO was applied to 10 ml of uredospore suspension in 100-ml Erlenmeyer flask at the indicated concentrations. The final concentration of DMSO was adjusted below 1%. Uredospore suspensions treated with or without EBIs were incubated on rotary shaker (125 rpm) at 20°C. The electrolytic conductivity of uredospore suspension was measured at 1 hour interval by transferring 4.5 ml of aliquots to the well of conductivity meter (Denki Kagaku Keiki Co., LTD, Model AOC-10) equipped with two platinum electrodes.

Observation of spore germination. Uredospore suspension of *P. recondita* adjusted to 1 mg of uredospore/ml of Tween 20 solution was dropped onto a well of a slide glass. To observe the uredospore germination, slide glasses were placed in a plastic box spread with three soaked pieces of paper towel. After incubation at 20°C for 3 hours, the percentage of uredospore germir ation was determined from microscopic examination of at least 100 spores per replicate, with three replicates per treatment.

Table 1. Activities of several fungicides inhibiting sterol biosynthesis against wheat leaf rust caused by *Puccinia recondita*

Fungicides[a]	Concentration (μg/ml)	Control value[b] (%)	EC_{50}[c] (μg/ml)	Molecular Weight
Hexaconazole	12.5	92.2 ± 7.7	1.4	314.2
	25.0	100		
	50.0	100		
	100.0	100		
Triadimefon	12.5	11.1 ± 19.2	35.7	293.8
	25.0	44.4 ± 19.2		
	50.0	61.1 ± 19.2		
	100.0	82.2 ± 15.0		
Flusilazole	12.5	66.7	3.5	315.4
	25.0	83.3		
	50.0	96.7		
	100.0	98.2 ± 1.5		
Fenarimol	12.5	50.0	6.9	331.2
	25.0	83.3		
	50.0	97.7 ± 1.7		
	100.0	99.8 ± 0.2		
Nuarimol	12.5	0	34.0	314.7
	25.0	27.8 ± 25.5		
	50.0	83.3		
	100.0	96.7		
Prochloraz	12.5	0	88.0	376.7
	25.0	0		
	50.0	27.8 ± 9.6		
	100.0	61.1 ± 19.2		
Triflumizole	12.5	11.1 ± 19.2	45.3	345.7
	25.0	22.2 ± 19.2		
	50.0	55.6 ± 9.6		
	100.0	77.8 ± 9.6		
Triforine	12.5	0	52.0	435.0
	25.0	16.7		
	50.0	50.0 ± 16.7		
	100.0	75.6 ± 8.4		

[a] Fungicides were applied by spraying 1 day before inoculation.
[b] Following investigation of disease severity (%) on the first leaf of wheat treated or non-treated, control value (CV) was calculated by the formula CV (%) = [(A–B)/A] × 100, where A and B represent the disease severity on the untreated and treated plants, respectively. Mean of control value (CV) from three replicates ± standard deviation.
[c] Effective concentration to inhibit 50% of disease severity compared with untreated control.

Results

Activity of fungicides against wheat leaf rust in the greenhouse test. Disease control activities of fungicide against wheat leaf rust were evaluated in the greenhouse (Table 1). Hexaconazole showed the most excellent activity among eight fungicides tested, which showed EC_{50} value of 1.4 μg/ml. Even at 25 μg/ml of hexaconazole, wheat leaf rust caused by *P. recondita* was perfectly controlled by protective spraying. However, severe phytotoxic symptom appeared on the wheat, which include dwarfing of plants and scorch of the first leaf treated with fungicide at a high concentration such as 100 μg/ml. From prochloraz treatment, poor control activity was observed even at 100 μg/ml while the same phytotoxic symptom that appeared from hexaconazole treatment was observed. Both flusilazole and fenarimol showed similar degree activity, of which EC_{50} values were 3.5 and 6.9 μg/ml, respectively.

Effect of fungicides on membrane permeability. Electrolytic leakage was monitored by measuring the conductivity change in uredospore solution of *P. recondita* treated with or without each fungicide for 6 hours (Fig. 1). Conductivity change of uredospore solution of *P. recondita* was the highest with prochloraz treatment, while nuarimol did not cause any effect on conductivity change of uredospore solution. Treatment of 30 and 300 μM prochloraz on *P. recondita* uredospore increased electrolytic leakage over time until 6 hours. While 300 μM of prochloraz increased electrolyte rapidly within an hour to reach almost maximum conductivity change, 30 μM of prochloraz gradually increased conductivity change, and the conductivity change by 30 μM prochloraz treatment became almost the same as that by 300 μM prochloraz treatment after 5 hours. The other three fungicides used in this study, triadimefon, flusilazole and hexaconazole, showed medium level of conductivity change over time at the 300 μM level of each fungicide. However, treatment of 30 μM of the three fungicides did not cause any significant electrolytic leakage on the uredospore solution of *P. recondita*.

To investigate the relationship between disease control activity and membrane permeability, prochloraz, which showed the highest electrolytic leakage among tested EBIs in spite of very poor disease control activity against wheat leaf rust, was compared with hexaconazole showing the best disease control activity. As depicted in Fig. 2, continuous increase of electrolytic leakage to above 20 μS/cm was observed at 100 and 300 μM of prochloraz treatment. Even at 33 μM, electrolyte of uredospores leaked slowly until 3 hours of incubation, and subsequently electrolytic leakage was increased. The conductivity in 300 μM of hexaconazole treatment rapidly increased until 3 hours, and reached slowly to 13.7 μS/cm 6 hours after treatment. With 100 μM, hexaconazole showed very slow increase of electrolytic leakage of only 2.8 μS/cm. However, treatment of hexaconazole below 33 μM did not cause any significant leakage of electrolyte.

Effect of EBIs on spore germination. Since some EBIs

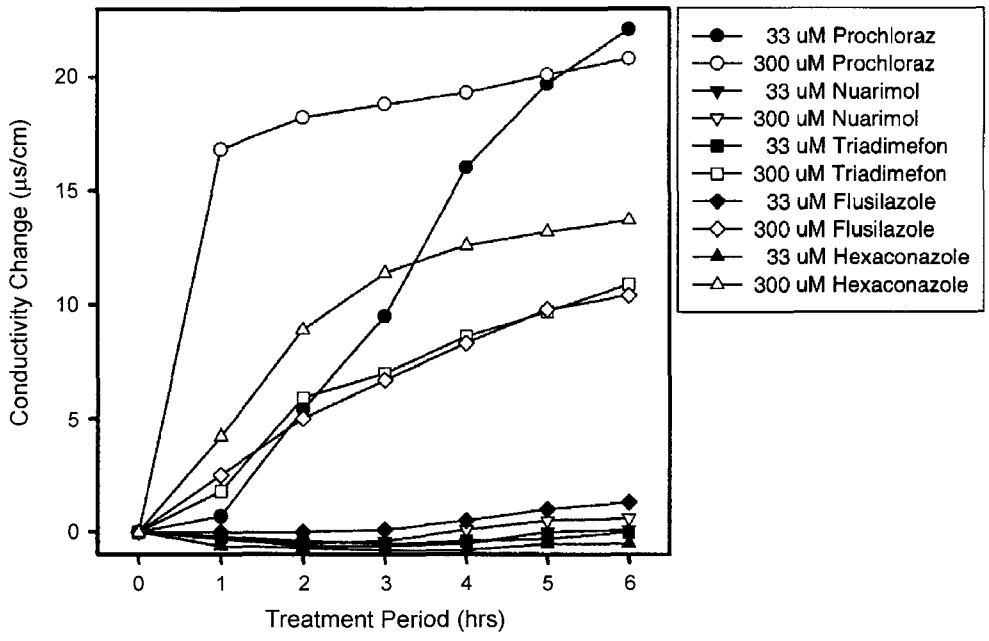

Fig. 1. Effect of several fungicides inhibiting ergosterol biosynthesis on membrane permeability of germinating uredospores of *Puccinia recondita*.

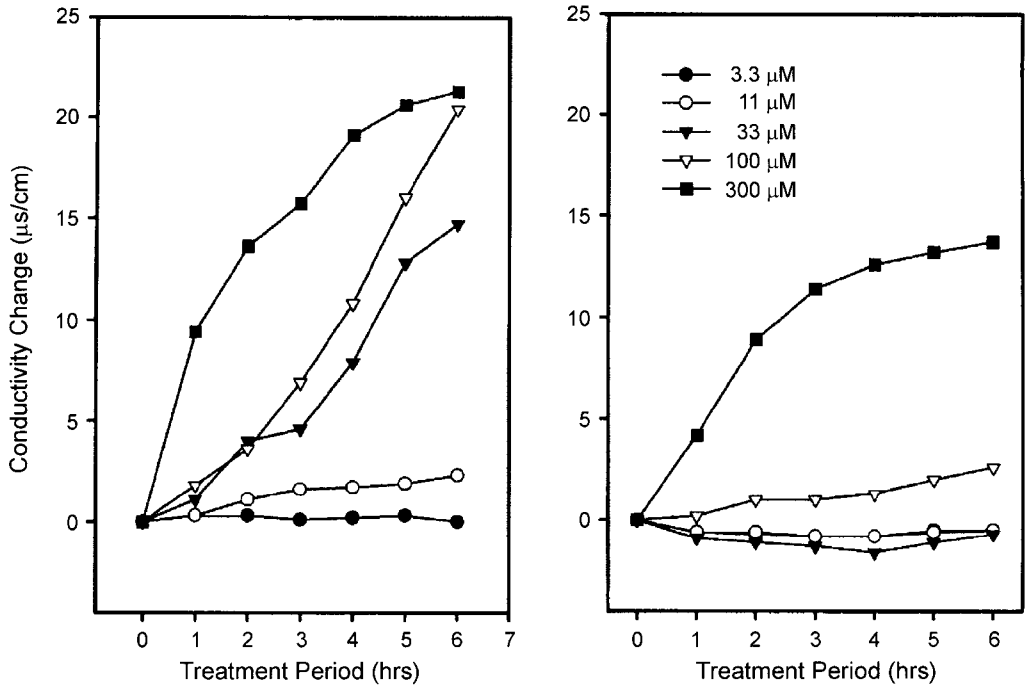

Fig. 2. Effect of prochloraz (left) and hexaconazole (right) on electrolytic leakage from plasma membranes of *Puccinia recondita* by the lapse of incubation time.

Table 2. Effect of fungicides on the germination of uredospore of *Puccinia recondita*

Fungicides	Concentration (μM)	Germination Ratio (%)
Prochloraz	200	0
	100	75.1 ± 5.6
	50	35.4 ± 7.2
Hexaconazole	200	0.3 ± 0.1
	100	1.1 ± 0.5
	50	2.8 ± 1.0
Benomyl	200	7.5 ± 3.2
	100	0.9 ± 0.8
	50	1.9 ± 1.1
Untreated Control		3.2 ± 0.7

showed various effects on membrane permeability of uredospore of *P. recondita,* effects of EBIs on uredospore germination was investigated. Uredospore germination was very low in distilled water containing 250 μg/ml of Tween 20 without fungicide after incubation for 3 hours (Table 2). Uredospore germination ratio decreased gradually by increasing the concentration of hexaconazole treated on uredospore. However, prochloraz dramatically raised the germination ratio. Nevertheless, as shown in Table 2, germination ratio of *P. recondita* increased up to 35.4 and 75.1% at 50 and 100 μg/ml of prochloraz, respectively, and the length of germ tube in 50 μg/ml of prochloraz treatment was inhibited by above 50% compared with that of non-treated control (Fig. 3).

Discussion

EBIs used in this report could be classified by the activity against wheat leaf rust. While prochloraz and triflumizole, and triforine, which belong to the imidazole and piperazine group, respectively, showed weak activity against *Puccinia recondita*, the others exhibited excellent control activity (Coping et al., 1984). It has been reported that the chemical structure of EBIs against wheat leaf rust was related with their biological activity, because it affected the binding affinity of fungicide on target enzyme, uptake and transport in plant, and on metabolism in solution.

At high concentrations, EBIs somewhat caused phytotoxicity on crops. Except for triforine, the application of EBIs tested in this study resulted in the phytotoxicity of wheat, of which the symptom was dwarfing. Especially, leaf spraying of prochloraz caused severe dwarfing symptom in wheat, although soil-drenching treatment of prochloraz did not cause any. It was assumed that the degree of prochloraz phytotoxicity by application method was dependent on this fungicides penetration nature into plants and the sensitivity of gibberellin biosynthesis.

Prochloraz has been presented to inhibit sterol biosynthesis by binding to cytochrome-P450-dependent sterol 14a-demethylase, subsequently blocking ergosterol of fungi

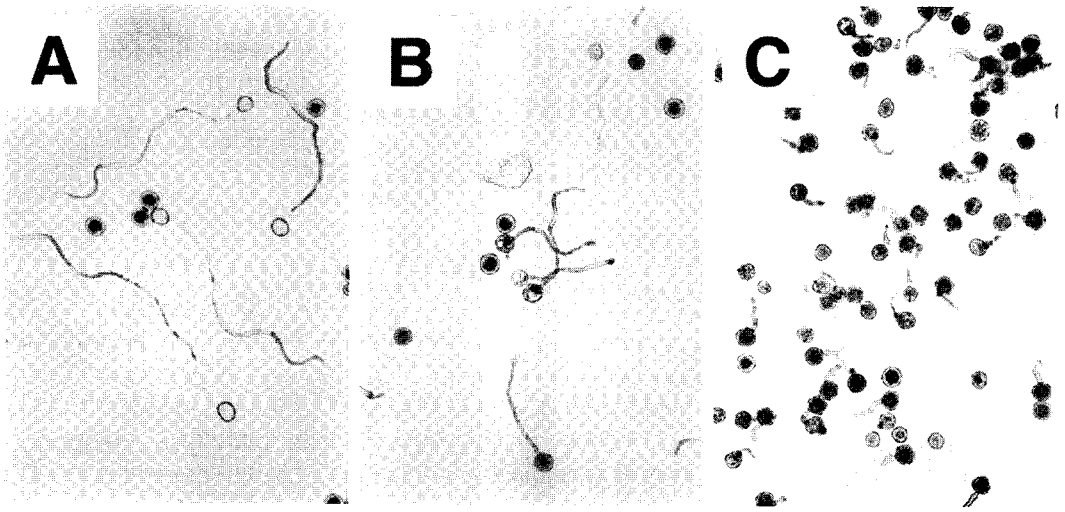

Fig. 3. Germination of uredospores of *Puccinia recondita* in the solution containing 250 μg/ml of Tween 20 with or without prochloraz inhibiting ergosterol biosynthesis. (**A**) Untreated control; (**B**) 50 μg/ml of prochloraz treatment; (**C**) 100 μg/ml of prochloraz treatment.

and accumulating abnormal sterols, such as lanosterol and 24-methylene dihydrolanosterol (Coping, 1984; Yanosaka et al., 1991; Stehmann et al., 1994; Kapteyn et al., 1994). It is assumed that the depletion of functional sterol, ergosterol, and accumulation of abnormal sterols lead to a disruption of membrane functions and to growth inhibition. Secondary effect of EBIs is the alteration of membrane properties such as permeability, and the irregular and excessive chitin deposition at the hyphal tips and at the septae of various target fungi (Sancholle et al., 1984; Dahmen et al., 1988). Treatment with 33 μM of prochloraz for 2 hours significantly caused electrolytic leakage from germinating uredospore of *P. recondita*, although at that concentration, it could not control leaf rust by protective application on wheat in the greenhouse. It was shown that control activity of prochloraz on wheat leaf rust was not correlated with its effect on electrolytic leakage.

As shown in Table 2, prochloraz increased the uredospore germination ratio. Generally uredospores, which were maintained at below -25°C under drying condition, must take a heat shock at 45-50°C for 2.5-15 minutes to break the dormancy of spores of *Puccinia recondita* (Choi et al., 1993). Rust spores, stimulated with a heat shock, could germinate with a higher ratio rather than non-treated ones. Like heat-treatment, prochloraz also accelerated the uredospore germination of *P. recondita* remarkably, but its mechanism has not been elucidated yet.

Considering the results of this study, the effect of prochloraz on the cell membrane might have been followed by electrolytic leakage and stimulation of germination in uredospores of *P. recondita*. In spite of low control activity, prochloraz caused considerable increase of the electrolytic leakage of uredospores, because the number of germinating uredospore increased for the stimulation of its germination ratio by the fungicide treatment. However, the direct effect of prochloraz on the rust pathogen *in vitro*, such as germination and electrolytic leakage of uredospore, was not correlated with the disease control activity on wheat leaf rust in the greenhouse test.

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