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The Secondary Effects of Pencycuron on the Formation of Giant Protoplasts and the Lipid Peroxidation of *Rhizoctonia solani* AG4

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The secondary effects of pencycuron on cell membrane of *Rhizoctonia solani* AG4 were investigated by the observation of giant protoplast formation and lipid peroxidaiton. Compared to protoplasts initially produced from the strains, protoplasts of *R. solani* R-C (sensitive strain) and Rh-131 (non-sensitive strain) increased in their size by 2.0-3.5 times 12 h after incubation in potato-dextrose broth containing novozyme (7 mg/ml) and β-glucuronidase (60 μl/ml) with 0.6 M mannitol (pH 5.2). The increase of protoplast size in R-C was slightly inhibited from 13.8 μm without pencycuron to 10.3 μm with 1.0 μg/ml of pencycuron. However, the size of giant protoplast of Rh-131 was not affected by the pencycuron treatment. Both strains R-C and Rh-131 did not exhibit the lipid peroxidation 12 h after the application of 1.0 μg/ml pencycuron. The remarkable peroxidation of membrane lipid was observed only in R-C 24 h after pencycuron application, but not in Rh-131. Although the inhibition of giant protoplast formation and the membrane lipid peroxidation were observed only in the sensitive strain R-C by pencycuron, it is difficult to conclude that these are the primary mechanism of pencycuron. The mild activity of pencycuron on the inhibition of giant protoplast formation and late membrane lipid peroxidation in the fungicide-sensitive strain did not coincide with the dramatic activity of pencycuron in *R. solani*. Therefore, our results suggest that inhibition of giant protoplast formation and membrane lipid peroxidation is the secondary effect of pencycuron.

Keywords : giant protoplasts, lipid peroxidation, pencycuron, secondary effect.

Rhizoctonia solani Kühn (teleomorph; *Thanatephorus cucumeris* (Frank) Donk) is a destructive and widespread plant pathogen with a very wide host range including rice, turfgrass, vegetables, and many other field crops (Cu et al., 1996; Burpee and Martin, 1992; Keinath, 1995; Doupnik, 1993). Until now *R. solani* is regarded as an unspecialized fungus. Currently, more than 10 anastomosis groups (AGs) were reported from the isolates of *R. solani*. Some isolates of *R. solani,* even in the same AG, vary considerably in pathogenicity and culture characteristics. Occasionally, each AG showed the different sensitivity to a certain fungicide (Kataria et al., 1991).

Pencycuron, which was used for the control of the *Rhizoctonia* diseases, showed diverse activities against each AG of *R. solani*. Pencycuron exerts the specific activity *in vitro* to inhibit mycelial growth at different concentrations depending on the AGs of *R. solani*. The pencycuron sensitivity was observed in AG1, 2, and 3, but AG5 was shown to be far less sensitive (Kim et al., 1996). It appeared that there are both pencycuron-sensitive and non-sensitive strains in AG4. The AG-specific activity of pencycuron in *R. solani* suggested that the pencycuron target site might be specific. Yamada et al. (1988) reported that pencycuron inhibited neither the biosynthesis of sterol and chitin nor the activity of trehalase. The fungicide did not affect significantly the other macromolecule biosynthesis such as nucleic acid and proteins. Leroux et al. (1990) reported that *Botrytis cinerea* and *Pseudocercosporella herpotricoides* isolates resistant to benzimidazole fungicides, an inhibitor of microtubule assembly, were sensitive to pencycuron. However, inhibition of tubulin assembly was not observed by *in vitro* assay with tubulin extracted from pencycuron-sensitive isolate of *R. solani* (Roh et al., 1999). Ueyama et al. (1990) found changes in the mycelial morphology and in the cytoskeletal microtubules of the hyphal tip of the pencycuron-sensitive isolate by immunofluorescence-staining method and microscopical observation. It was implicated that the target site of pencycuron would be in plasma membrane of sensitive *R. solani,* even though the fungicide directly inhibited the tubulin assembly. Kim et al. (1996) reported the effects of pencycuron on the osmotic stability of protoplasts and the fluidity of lipid membrane of *R. solani*. When pencycuron was treated during the short period of time, dramatic changes in membrane fluidity were observed (Kim and Yamaguchi, 1996). The change of membrane fluidity should in turn influence many cellular physiological functions.

The peroxidation of lipid membrane has been believed to

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be the primary mechanism of fungicidal activity of certain fungicides, such as aromatic hydrocarbon group and dicarboximide fungicides, in *Botrytis cinerea* as well as in *Mucor mucedo* (Lyr, 1988; Choi et al., 1996). It is not tested yet if pencycuron activity on *R. solani* is related with lipid peroxidation of fungal lipid bilayer membrane. An incubation system with cellulase and driselase for giant protoplast formation from fungal mycelium was also developed to study a mode of action for a fungicide (Teraoka et al., 1992). This was to test that if a fungicide inhibits the synthesis of cell membrane, it inhibits the formation of giant protoplasts. An experiment with protoplasts produced from *Pyricularia oryzae* P-2 showed that the giant protoplast formation system is suitable to investigate the fungicidal effect on plasma membrane of various fungi. In this study, we employed the system of fungal giant protoplast formation to investigate the effect of pencycuron on plasma membrane of both pencycuron-sensitive and pencycuron-non-sensitive strains of *R. solani*. In addition, we performed the lipid peroxidation assay of *R. solani* cytoplasmic membrane to test the effect of pencycuron on lipid bilayer membrane of *R. solani*.

Materials and Methods

Culture method. Strains of *R. solani* AG4 were supplied from Natioanl Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries in Japan. All strains were maintained at 4°C for 5 days after incubation on potato-dextrose agar (PDA) at 25°C. Both pencycuron-sensitive strain R-C and pencycuron-non-sensitive strain Rh-131 of *R. solani*, were used 3 days after incubation on PDA at 25°C.

Preparation of test solution. Pencycuron was supplied from Nihon Bayer Agrochem. The fungicide was routinely dissolved in dimethyl sulfoxide (DMSO) to make the stock solution. To investigate the effect of pencycuron on the formation of giant protoplasts and lipid peroxidation, the pencycuron solution was treated in protoplast suspensions and mycelia of *R. solani* to the indicated concentrations. The final concentration of DMSO was adjusted to 1%.

Formation of giant protoplasts. Fungal protoplasts were prepared by a modified method of Hashiba and Yamada (1982). For the generation of protoplasts, 3 mycelial discs (diameter; 5 mm) of *R. solani* strains were inoculated in 50 ml of potato-dextrose broth (PDB), and shaked for 3 days at 25°C with 150 rpm. The fungal broth culture was homogenated with polytron (Ultra-Turrax Type TP18) 3 times each for 1 min to prepare mycelial suspension of each strain. Two mililiters of each homogenated mycelial suspension was transferred into 20 ml of fresh PDB in petri dishes. They were incubated without shaking at 25°C for 2 days for *R. solani* strain R-C, and for 1 day for strain Rh-131, respectively. Grown mycelia were harvested and washed 3 times with 0.6 M mannitol by centrifugation at $3,000 \times g$ for 10 min. Mycelia of *R. solani* R-C and Rh-131 were resuspended in enzyme solution containing novozyme (7 mg/ml) and β-glucuronidase (60 μl/ml) with 0.6 M mannitol (pH 5.2). The enzyme solutions were placed on a shaking incubator with 60 rpm at 34°C for 3 h. The culture was filtered through a 20-μm nylon mesh to remove mycelial debris and the filterate was centrifuged with 0.6 M mannitol twice at $1,000 \times g$ for 5 min to remove enzymes. The pellets containing protoplasts of *R. solani* R-C and Rh-131 were resuspended in PDB containing 0.6 M mannitol with the enzymes (novozyme, 7 mg/ml; β-glucuronidase, 60 μl/ml) to induce giant protoplasts. Pencycuron was treated in the protoplast suspension at indicated concentrations.

Measurment of lipid peroxidation. Lipid peroxidation was estimated by using a slight modification of the thiobarbituric acid (TBA) method as previously described (Buege and Aust, 1978). Three mycelial discs (diameter; 5 mm) of *R. solani* R-C and Rh-131 were inoculated in petri dishes containing 10 ml of PDB and incubated without shaking at 25°C for 3 days. Cultures were homogenated with polytron (Ultra-Turrax Type TP18) 3 times each for 1 min to prepare mycelial suspension. Pencycuron was added at indicated concentrations in 50 ml of PDB where 2 ml homogenate of *R. solani* was inoculated. Following 2 day incubation, mycelia were harvested and washed with 0.1 M phosphate buffer (pH 8.0) by centrifugation twice at $3,000 \times g$ for 10 min. The harvested mycelia were pulverized with a mortar and pestle using liquid nitrogen and resuspended in 0.1 M phosphate buffer (pH 8.0). The suspension was centrifuged at $8,000 \times g$ for 20 min to remove mycelial debris. One mililiter of supernatant was combined with 2 ml of stock reagent (15% trichloroacetic acid (w/v), 0.375% tribarbituric acid (w/v), 0.25 N hydrochloric acid) and mixed thoroughly. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at $1,000 \times g$ for 10 min. The absorbance of the supernatant was determined at 535 nm to estimate the amount of malondialdehyde, a product of lipid peroxidation. The malondialdehyde concentration of the samples could be calculated by using an extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$.

Results

Inhibition on the formation of giant protoplasts. The giant protoplasts of *R. solani* R-C and Rh-131 were observed under a light microscope 12 h after incubation. The size of protoplasts increased up to 2.0 to 3.5 times compared to that of initial protoplasts, and the giant protoplasts of Rh-131 were bigger than those of R-C. As shown in Table 1, the capability of the formation of giant protoplasts in R-C and Rh-131 was not affected by applying pencycuron. Protoplasts applied with 1.0 μg/ml pencycuron turned giant protoplasts with the size of 10.3 μm and 36.1 μm for R-C and Rh-131, respectively. However, the size of giant protoplasts of R-C was negatively correlated with applied pencycuron concentrations. In contrast, the size of giant protoplast of Rh-131 increased with pencycuron treatment. This result indicated that pencycuron slightly inhibited the process of giant protoplast formation in the pencycuron-sensitive strain

Table 1. Effect of pencycuron on the formation of giant protoplasts from protoplasts of *Rhizoctonia solani* R-C sensitive to pencycuron and Rh-131 non-sensitive to pencycuron

Pencycuron (μg/ml)	Size of protoplasts[a] (μm)			
	R-C		Rh-131	
	0 h	12 h	0 h	12 h
0	6.7 ± 1.8[b]	13.8 ± 3.0	10.3 ± 3.3	28.6 ± 7.6
0.2		12.3 ± 3.3		33.4 ± 9.3
1.0		10.3 ± 3.6		36.1 ± 7.5

[a] Protoplast size was measured after 12 h incubation in potato dextrose broth containing the enzyme solution containing 0.6 M mannitol with celluase and β-glucuronidase.
[b] Values are the means and standard deviations of three experiments, each with three replicates.

R-C, while the fungicide has a positive effect on the giant protoplast formation in the non-sensitive strain Rh-131.

Effect of pencycuron on the lipid peroxidation. The concentration of MDA produced by the fungi was determined as an estimate of lipid peroxidation following treatment of pencycuron. The amount of MDA produced from both strains R-C and Rh-131 was not different in 12 h between the two strains irrespective of pencycuron treatment. A remarkable difference of lipid peroxidation was observed from R-C after 24 h treatment of 1 μg/ml pencycuron (Fig. 1). However, the fungicide did not influence the lipid peroxidation of Rh-131. The generation of MDA from R-C was correlated with the concentration of pencycuron after 48 h incubation of fungal mycelia with the fungicide (Fig. 2). The maximum lipid peroxidation by the fungicide in R-C was shown at 1 μg/ml of pencycuron treatment. These results indicate that pencycuron cause the peroxidation of lipid membrane selectively in the strain R-C sensitive to pencycuron.

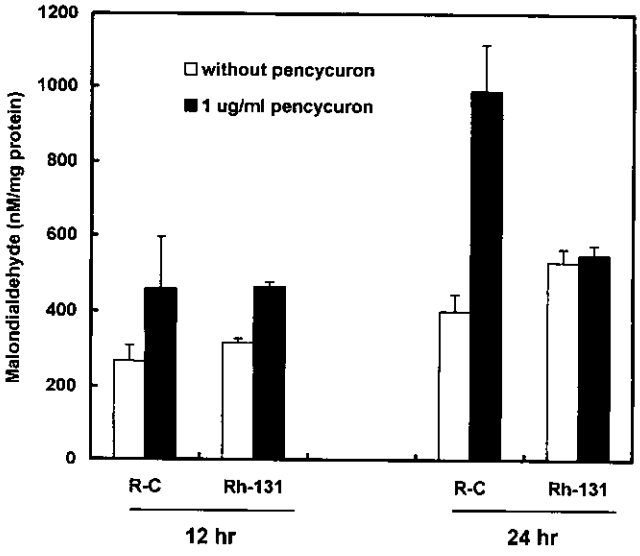

Fig. 1. Lipid peroxidation in *Rhizoctonia solani* R-C (sensitive strain) and Rh-131 (non-sensitive strain) grown in potato dextrose broth with 1.0 μg/ml pencycuron or without pencycuron. The measurement of lipid peroxidation was conducted with the estimation of malondialdehyde in mycelia after incubation for 12 h and 24 h.

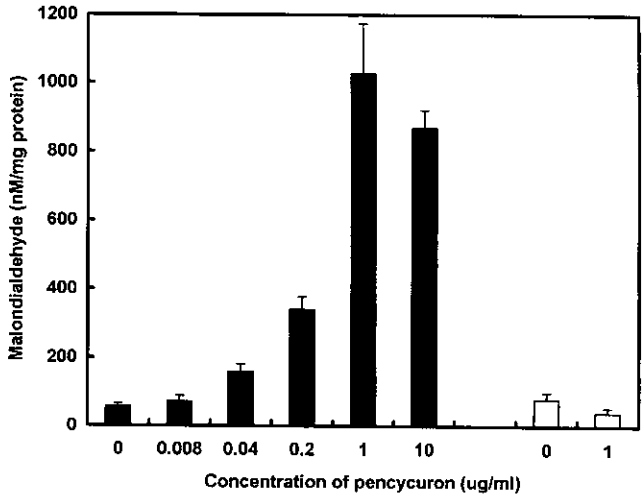

Fig. 2. Effect of pencycuron on the lipid peroxidation of *Rhizoctonia solani* R-C (sensitive strain; ■) and Rh-131 (non-sensitive strain; □). The amount of malondialdehyde was measured 48 h after incubation in potato-dextrose broth amended with pencycuron at indicated concentrations.

Discussion

The action mechanism of pencycuron has been remained obscure despite of a few documentations. It has been known that some strains of *Botrytis cinerea* and *Pseudocercosporella herpotrichoides* resistant to carbendazim exhibited a higher sensitivity to pencycuron (Leroux & Gredt, 1990). Ueyama et al. (1990) observed under a fluorescent microscope that pencycuron caused deterioration of microtubule arrays, a cytoskeleton in hyphal tips of *R. solani*. However, inhibitory effect of pencycuron on the assembly of tubulin to microtubules was not confirmed in *in vitro* assay system.

We have previously observed a different inhibitory activity by pencycuron on the mycelial growth of *R. solani* strains which belong to AG 4. According to the specific activity of pencycuron to *R. solani* even in a same anastomosis group, it was suggested that the target site of pencycuron would be very unique. In spite of the specific activity of pencycuron against each anastomosis group of *R. solani*, there has been no report such as the appearance or the artificial selection of resistant isolates. By using two *R. solani* AG4 strains, R-C (sensitive to pencycuron) and Rh-131 (non-sensitive), our previous studies demonstrated that pencycuron influenced the fluidity of lipid membranes, subse-

quently being likely to alter the function of plasma membrane (Kim et al., 1996; Kim and Yamaguchi, 1996).

In this study, we investigated the additional effect of pencycuron on plasma membrane, specifically the effect on the formation of giant protoplasts and the lipid peroxidation. Our results indicated that pencycuron affected giant protoplast formation only in pencycuron-sensitive strain R-C but not in pencycuron-nonsensitive strain Rh-131. According to Teraoka et al. (1992), the formation of giant protoplasts may require a newly synthesized cell membrane at least. The mechanism of cell membrane synthesis is thought to be the same as that of hyphal growth. Electron microscopic studies of the apices of actively growing hyphae have led to the vesicular hypothesis of hyphal tip growth. Vesicles, which contain materials utilized in hyphal formation as well as possibly enzymes involved in cell wall synthesis or softening of pre-existing wall material, migrate to hyphal apex and fuse with plasma membrane. Ultimately plasma membranes increase at the fusion site equal to the membrane surface of the incorporated vesicles. The inhibition of the formation of giant protoplasts in R-C caused by pencycuron was thought partly to result from the inhibition of the fusion between vesicles and plasma membrane. However, even with 1.0 μg/ml pencycuron, the size of protoplasts of isolate R-C was larger than that produced initially from *R. solani* R-C. It is indicated that the inhibition of the formation of giant protoplasts is not the primary mode of action of pencycuron as a fungicide, because mycelial growth of R-C is completely inhibited with 1.0 μg/ml pencycuron.

In *Botrytis cinerea*, lipid peroxidation preceded cellular leakage following treatment of vinclozolin and eventually led to the destruction of lipid membrane after prolonged incubation (Choi et al., 1996). Our sturdy showed that the lipid peroxidation caused by pencycuron clearly happened 24 h after treatment in *R. solani* R-C. After 48 h it became remarkable and dependent upon applied concentrations. Despite the definite change of lipid peroxidation, it was difficult to conclude that lipid peroxidation is the primary mechanism of pencycuron, because the remarkable alteration of the fluidity of lipid membrane as a fungicide happened considerably before the lipid peroxidation. As depicted in Figure 1, lipid peroxidation was slight even in the pencycuron-sensitive strain 12 h after treatment. Generally, the peroxidation of lipid membranes happens after active oxygen species are sufficiently generated and/or natural protective mechanisms, such as detoxifying enzymes and endogenous antioxidants, are overloaded. It is not clear how active oxygen species are produced in strain R-C and whether there is a relationship between the binding site of pencycuron and the production of active oxygen species. However, a possibility that alteration of membrane fluidity by the fungicide causes subsequently lipid peroxidation is not excluded, because the lipid peroxidation is a late process occurring by pencycuron treatment.

Our results clearly suggest that pencycuron has the secondary effects on fungal cell membrane biosynthesis and on lipid bilayer viability in *R. solani*, although they are not the primary fungicidal mechanism.

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