Correlation of Lipid Peroxidation in *Botrytis cinerea* Caused by Dicarboximide Fungicides with Their Fungicidal Activity

Hee Jae Lee,* Gyung Ja Choi, and Kwang Yun Cho

Korea Research Institute of Chemical Technology, Jang-dong 100, Yusung-ku, P.O. Box 107, Taejon 305-606, Korea

Dicarboximide fungicides iprodione, vinclozolin, and procymidone were examined for their capacity to inhibit mycelial growth, to cause cellular leakage, and to cause lipid peroxidation on *Botrytis cinerea* isolate BC2. All three fungicides effectively inhibited the mycelial growth of the fungi. The IC_{50} values were found to be about 2 μM for all three fungicides, indicating that the fungicidal activity of the individual fungicides was almost the same. The fungicides caused significant cellular leakage and lipid peroxidation on the fungi in a concentration-dependent manner. Fungicidal activity of the three individual fungicides on inhibiting mycelial growth of the fungi correlated positively well with both cellular leakage and lipid peroxidation that were caused by the respective fungicides. Positive correlations were also found between the degree of cellular leakage and lipid peroxidation following treatment with the fungicides. Our results support the view that dicarboximide fungicides exert their fungicidal activity mainly through membrane lipid peroxidation and subsequent cellular leakage from the treated fungi.

Keywords: *Botrytis cinerea; cellular leakage; dicarboximide fungicides (iprodione, vinclozolin, procymidone); lipid peroxidation*

INTRODUCTION

Dicarboximide fungicides are widely used to control a number of fungi, especially *Botrytis cinerea,* pathogenic to vegetables, soft fruits, vines, and flower crops (Leroux and Fritz, 1984; Pommer and Lorenz, 1995). Although their common structure is an *N*-3,5-dichlorophenyl cyclic imide, they can be divided into three groups according to their cyclic amide component: hydantoins, oxazolidinediones, and succinimides (Leroux and Fritz, 1984; Edlich and Lyr, 1992; Pommer and Lorenz, 1995). The dicarboximide fungicides exert their fungicidal effects by causing rapid collapse or burst of treated fungal hyphae (Edlich and Lyr, 1992, 1995). They are known to have more inhibitory effect on mycelial growth than on spore germination (Hisada and Kawase, 1977; Pappas and Fisher, 1979; Choi et al., 1996). In addition, they rapidly disintegrate the mitochondrial inner membranes of treated fungi (Edlich and Lyr, 1995).

Membrane lipid peroxidation triggered by active oxygen species has been suggested to be a major cause for the fungitoxic effect of the dicarboximide fungicides (Edlich et al., 1988; Edlich and Lyr, 1992, 1995; Steel and Nair, 1993). We have also demonstrated that the dicarboximide fungicide vinclozolin causes significant lipid peroxidation in a dicarboximide-susceptible isolate of *B. cinerea* (Choi et al., 1996). The fungicide caused lipid peroxidation on the fungi in a concentration-dependent manner, but little or no lipid peroxidation occurred in a dicarboximide-resistant isolate of the fungus treated with the fungicide. Furthermore, α-tocopherol, a naturally occurring antioxidant in biological systems, prevented lipid peroxidation in the susceptible isolate. However, the precise mechanism of action of the dicarboximide fungicides is still ambiguous. The source(s) of active oxygen species which initiate the lipid peroxidation remains to be determined unequivocally.

No comprehensive study has been published to demonstrate the capacity of dicarboximide fungicides to cause lipid peroxidation in *B. cinerea*. Furthermore, little information is available to show a relationship between lipid peroxidation and fungal death following the treatment of the dicarboximide fungicides. In this study, we report that the dicarboximide fungicides iprodione, vinclozolin, and procymidone all cause significant lipid peroxidation and subsequent cellular leakage from a dicarboximide-susceptible isolate of *B. cinerea* and that their ability to cause lipid peroxidation correlates well with their fungicidal activity.

MATERIALS AND METHODS

Chemicals. Technical grade dicarboximide fungicides iprodione [3-(3,5-dichlorophenyl)-*N*-isopropyl-2,4-dioxoimidazolidine-1-carboxamide (**1**)], vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione (**2**)], and procymidone [*N*-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide (**3**)] depicted in Figure 1 were generous gifts from Korea Samkong Co., Ltd. (Seoul, Korea). Potato dextrose broth (PDB) was obtained from Difco Laboratories (Detroit, MI). All biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Organic solvents were obtained from Oriental Chemical Co. (Seoul, Korea).

Fungal Cultures. *B. cinerea* isolate BC2 which was collected from tomato fields in Korea was used in the experiments. The isolate has previously been found to be susceptible to the dicarboximide fungicides (Kim et al., 1993; Choi et al., 1996). Spores from the isolate were inoculated on PDB media at a concentration of 1.3×10^6 spores/mL and then incubated

* Author to whom correspondence should be addressed. Present address: Institute of Biotechnology, Chonnam National University, Kwangju 500-757, Korea (phone, 82-62-520-6434; fax, 82-62-524-1785).

S0021-8561(97)00501-3 CCC: $15.00
Published on Web 01/15/1998

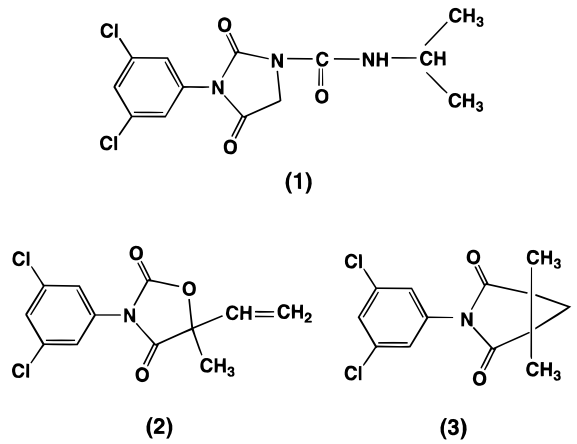

Figure 1. Chemical structures of dicarboximide fungicides iprodione (**1**), vinclozolin (**2**), and procymidone (**3**).

under white light conditions (35 μmol/m^2/s) with agitation (200 oscillations/min) at 25 °C for 2 days. The fungal cultures in the logarithmic phase of growth were harvested by centrifugation at 5000*g* for 10 min and washed twice in 3-(*N*-morpholino)propanesulfonic acid (MOPS; 1 mM, pH 7.0) using the same volume as the fungi were originally cultured in. The resuspended mycelia in MOPS buffer (1 mM, pH 7.0), with a volume of 20 mL, were dispensed into each fresh conical flask.

Fungicide Treatments. The fungal cultures were treated with iprodione, vinclozolin, or procymidone at various concentrations ranging from 0 to 33 μM and then incubated with shaking at 25 °C for 48 h. The fungicides were dissolved in acetone and added to the treatment culture. A control contained the same amount of acetone without the fungicides. The final concentration of acetone in the culture was less than 1 vol %. No detrimental effects of acetone alone on the fungi were detected (data not shown). Three replicates were used for each treatment.

Mycelial Growth Inhibition. Fungicide inhibition of mycelial growth, measured as a dry weight relative to the control, was determined at 48 h after incubation in PDB media with different concentrations of the fungicides. The mycelia in control cultures have a dry weight of 120 mg. The fungal cultures were treated with the fungicides at a concentration range from 0 to 33 μM at 6 h after incubation of the spores. Almost all spores were found to have germinated by the incubation stage. The treated mycelia were incubated under the same conditions used in the fungal cultures.

Cellular Leakage. The fungi washed with MOPS buffer (1 mM, pH 7.0) were treated with the individual fungicides at the same concentration range used under the above incubation conditions. Cellular leakage from the fungal tissues was measured by the detection of electrolyte leakage into the incubation medium with a conductivity meter (Denki Kagaku Keiki Co., Ltd., Japan) following the method of Lee et al. (1995). The measurements were made after incubating the fungal mycelia with the fungicides for 48 h, since no further cellular leakage has previously been observed beyond 48 h with vinclozolin (Choi et al., 1996). Because of differences in background conductivity of the different treatment solutions, results are expressed as changes in conductivity from the initial measurement.

Lipid Peroxidation. The degree of lipid peroxidation was estimated by the rate of malondialdehyde (MDA) production using a slight modification of the thiobarbituric acid (TBA) method as previously described (Buege and Aust, 1978; Slater, 1984). The fungi were treated with the fungicides and incubated in the same manner as used for the cellular leakage measurement. The MDA concentrations were determined from the incubation media as well as from the fungal mycelia.

The treated fungi were collected by centrifugation at 5000*g* for 10 min. The fungal mycelia were pulverized in a mortar and pestle using liquid nitrogen and then resuspended with 5 mL of a solution of 0.5% TBA in 20% trichloroacetic acid (TCA).

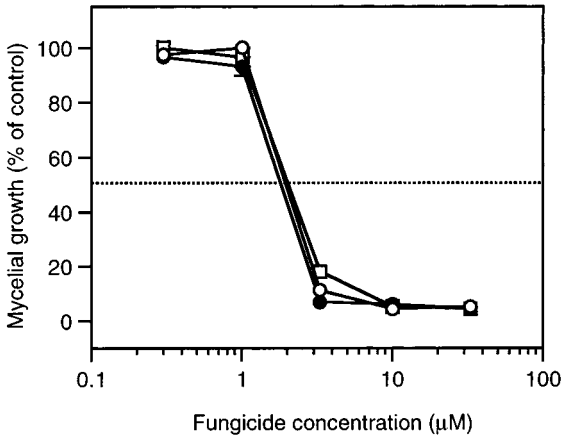

Figure 2. Effects of (○) iprodione, (●) vinclozolin, and (□) procymidone on mycelial growth of *B. cinerea* isolate BC2. Fungicide inhibition of mycelial growth, measured as a dry weight relative to the control, was determined 48 h after incubation of the fungi with the individual fungicides. Error bars are ±1 SE of the means. In some cases the error bar is obscured by the symbol.

The suspension was centrifuged at 20000*g* for 15 min, and supernatants were collected. The supernatants were heated in a boiling water bath for 25 min and allowed to cool in an ice bath. Following the centrifugation at 20000*g* for 15 min, the resulting supernatants were used for determination of MDA. The aliquots of the liquid incubation media were also subjected to the same procedure used for the fungal mycelia, after 2 mL of a solution of 0.5% TBA in 20% TCA was added to 5 mL of the aliquot as before (Choi et al., 1996). Absorbance at 532 nm for each sample was recorded and corrected for nonspecific turbidity at 600 nm using a Beckman DU-65 spectrophotometer. MDA concentration was calculated using a molar extinction coefficient of 156 mM^{-1} cm^{-1} (Buege and Aust, 1978). The MDA concentrations as a function of protein concentration from both fractions of the fungi and the liquid medium were pooled and then regarded as the total MDA produced by the fungi (Choi et al., 1996). Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

RESULTS

Fungicidal Activity. Iprodione, vinclozolin, and procymidone all significantly inhibited mycelial growth of *B. cinerea* isolate BC2 (Figure 2). Dose−response curves for the inhibition of mycelial growth by the individual fungicides were almost the same (Figure 2). The mycelial growth was completely inhibited by concentrations higher than 3 μM. The IC_{50} values of all three fungicides were approximately 2 μM.

Cellular Leakage. All fungicides caused electrolyte leakage from the fungal mycelia depending on the fungicide concentrations (Figure 3). The capacity of the individual fungicides to cause cellular leakage was not significantly different, although vinclozolin appeared to be slightly less effective than the other two fungicides. The cellular leakage increased with increasing concentrations of all the fungicides up to 10 μM, but further cellular leakage at higher concentrations occurred only with procymidone.

Lipid Peroxidation. All fungicides caused significant lipid peroxidation, as measured by MDA production, on the fungi in a concentration-dependent manner (Figure 4). With 33 μM fungicides, for example, levels of MDA were approximately 3-fold higher than in control. There was a general pseudolinear increase with a logarithmic increment of the fungicide concentration.

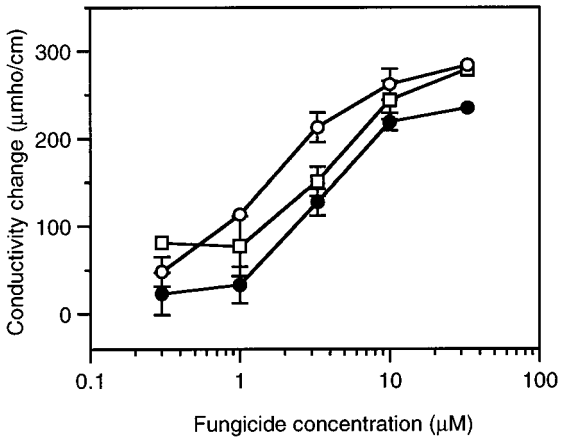

Figure 3. Effects of (○) iprodione, (●) vinclozolin, and (□) procymidone on cellular leakage from *B. cinerea* isolate BC2. Cellular leakage from fungal tissues was determined 48 h after incubation with the individual fungicides. Values are the difference between treated and control tissues. Error bars are ±1 SE of the means. In some cases the error bar is obscured by the symbol.

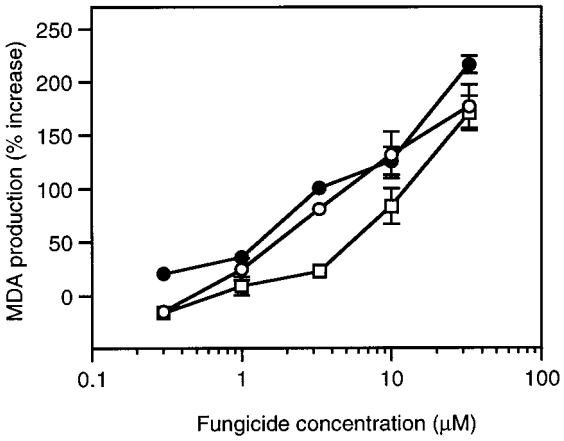

Figure 4. Effects of (○) iprodione, (●) vinclozolin, and (□) procymidone on MDA production from *B. cinerea* isolate BC2. The levels of MDA were determined 48 h after incubation of the fungi with the fungicides. Error bars are ±1 SE of the means. In some cases the error bar is obscured by the symbol.

Correlations of Cellular Leakage and Lipid Peroxidation with Fungicidal Activity. Mycelial growth inhibition correlated positively with the degree of cellular leakage observed for the respective fungicides (Figure 5). Similar relationships were found between fungicidal activity of iprodione and vinclozolin and MDA production caused by the respective fungicides (Figure 6A,B). In procymidone-treated fungi, however, the correlation was poorer (Figure 6C). Cellular leakage also increased with the level of MDA produced by the treatment of the fungicides (Figure 7).

DISCUSSION

Dicarboximide fungicides iprodione, vinclozolin, and procymidone effectively inhibited mycelial growth of *B. cinerea* isolate BC2, with similar IC_{50} values for all three fungicides. Since the dicarboximide fungicides are known to have a less inhibitory effect on spore germination than on mycelial growth of many fungi including *B. cinerea* (Hisada and Kawase, 1977; Pappas and Fisher, 1979; Choi et al., 1996), the inhibition of spore germination by the three fungicides was not examined in this study.

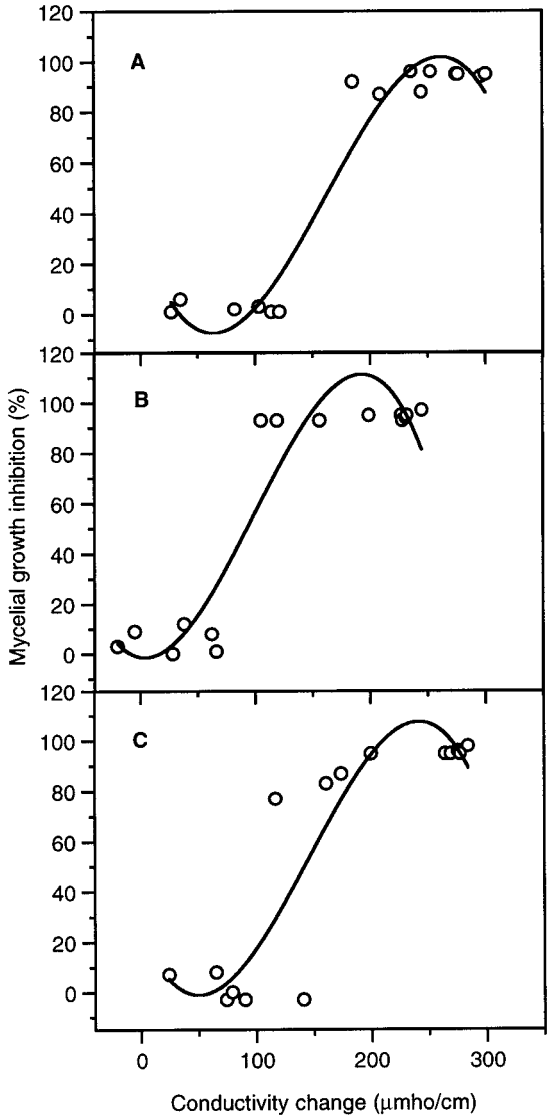

Figure 5. Relationships between effects of (A) iprodione, (B) vinclozolin, and (C) procymidone on cellular leakage from *B. cinerea* isolate BC2 and their inhibitory effects on mycelial growth. Lines are trinomial regressions with *r* of 0.98, 0.94, and 0.90 for A, B, and C, respectively.

The involvement of lipid peroxidation in the mechanism of dicarboximide fungicides has been hypothesized (Lyr and Edlich, 1986; Edlich et al., 1988; Lyr, 1988; Edlich and Lyr, 1992, 1995; Steel and Nair, 1993; Choi et al., 1996), although other effects of the fungicides, such as inhibition of cell division (Georgopoulos et al., 1979), DNA synthesis (Hisada and Kawase, 1977; Pappas and Fisher, 1979), and fatty acid synthesis (Pappas and Fisher, 1979), have also been proposed as possible mechanisms of action. The increases of MDA observed with increasing fungicide concentrations in the present work confirm the previous findings demonstrating that the dicarboximide fungicides cause membrane lipid peroxidation in *Mucor mucedo* (Lyr and Edlich, 1986; Lyr, 1988), *Ustilago maydis* (Lyr, 1988), and *B. cinerea* (Choi et al., 1996). The cellular leakage caused by the fungicides appeared to result from the lipid peroxidation process, as suggested by Choi et al. (1996). It is reinforced by the fact that relationships between cellular leakage and MDA production from the fungal mycelia were sigmoidal (Figure 7), implying that lipid peroxidation precedes cellular leakage. Furthermore,

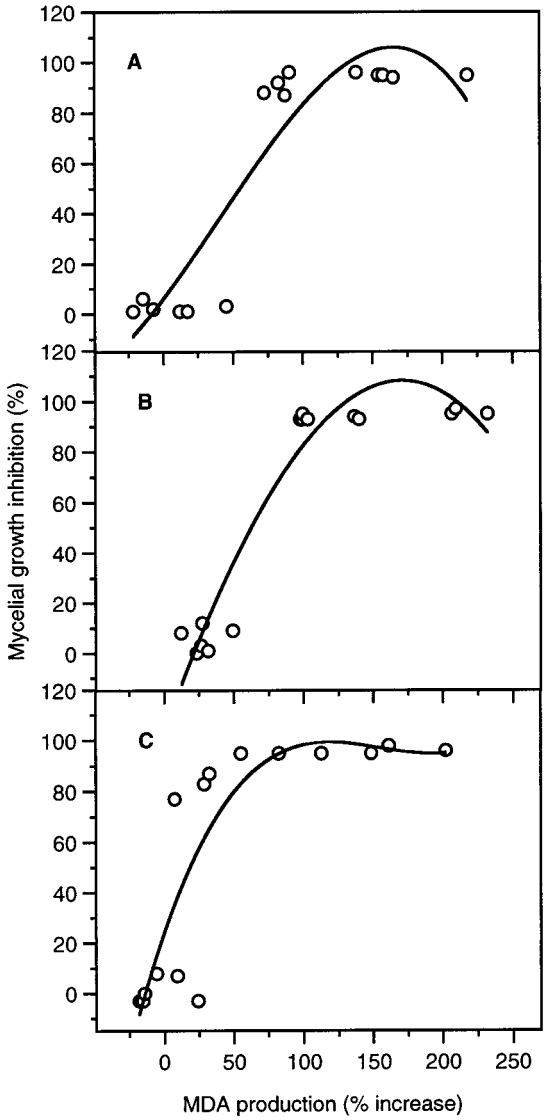

Figure 6. Relationships between effects of (A) iprodione, (B) vinclozolin, and (C) procymidone on MDA production in *B. cinerea* isolate BC2 and their inhibitory effects on mycelial growth. Lines are trinomial regressions with *r* of 0.93, 0.96, and 0.87 for A, B, and C, respectively.

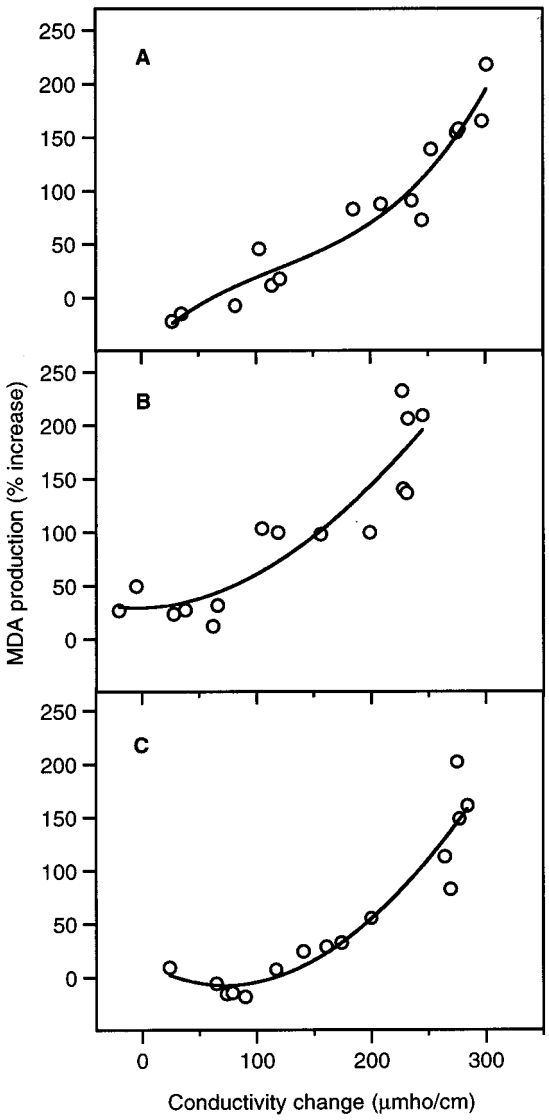

Figure 7. Relationships between effects of (A) iprodione, (B) vinclozolin, and (C) procymidone on cellular leakage from *B. cinerea* isolate BC2 and MDA production. Lines are trinomial regressions with *r* of 0.97, 0.91, and 0.95 for A, B, and C, respectively.

both the cellular leakage and MDA production correlated with the fungicidal activity of the three fungicides, respectively (Figures 5, 6). However, the correlations between fungicidal activity and lipid peroxidation were poor when the level of lipid peroxidation was high (Figure 6), suggesting that lipid peroxidation at a certain level is sufficient to cause fungal death.

Contradictory reports showing no lipid peroxidation in the dicarboximide fungicide-treated *U. maydis* microsomal membranes (Orth et al., 1992) and *B. cinerea* mycelia (Steel and Nair, 1993) have also been published. Even in these cases, interestingly, the protective effects of α-tocopherol from the fungicides have usually been observed. Thus, two different interpretations might be possible on this contradiction. One is that α-tocopherol protects the dicarboximide fungicide-treated fungi by its hydrophobic nature rather than its antioxidant activity. The other is that the measurement of lipid peroxidation using MDA as an indicator varies with the system employed.

Orth et al. (1993) reported that α-tocopherol could form hydrophobic micelles with the aromatic hydrocarbon fungicide tolclofos-methyl, lowering the actual concentration of the fungicide within the cell and thereby protecting the fungicide-treated *U. maydis*. Supposing that α-tocopherol is not hydrophobic, however, it may not reach membranes of cells and organelles. Thus, the hydrophobic nature of the α-tocopherol may be necessary for its action as an antioxidant in situ. Burton et al. (1983) speculated that the long-chain phytyl group on α-tocopherol allows the compound to partition into hydrophobic membranes of cells and organelles, where it presumably exerts its antioxidant activity in the prevention of lipid peroxidation. In addition, we are not aware of any reports providing the evidence that α-tocopherol protects the fungi from the dicarboximide fungicides by forming hydrophobic micelles with the fungicides.

On the other hand, the second interpretation seems more plausible, since reported levels of MDA production by the dicarboximide fungicides have been quite low and variable (Lyr, 1988; Edlich and Lyr, 1992, 1995; Orth et al., 1992; Steel and Nair, 1993). For example, the MDA levels in *M. mucedo* have been reported to range

from 152% to 336% of control with vincolzolin treatment at the same concentration of 5 mg/L (Lyr, 1988; Edlich and Lyr, 1992) and thus have not correlated with the growth inhibition. However, we have recently observed a sufficient level of MDA production in vinclozolin-treated *B. cinerea* with a more refined MDA measuring system than the others employed (Choi et al., 1996). Samples for the MDA measurements should be taken from both the fungicide-treated mycelia and the aliquots in which the fungal mycelia were incubated, since the MDA molecules are readily leached out from the fungal mycelia into the incubation media when the fungal plasma membranes are damaged (Choi et al., 1996). Strong positive correlations were found between lipid peroxidation caused by the dicarboximide fungicides and their fungicidal activity on inhibiting mycelial growth of *B. cinerea* as shown in Figure 6. This result along with our previous findings (Choi et al., 1996) imply that lipid peroxidation is obviously related to fungal death.

In summary, our results show that dicarboximide fungicides iprodione, vinclozolin, and procymidone are all effective to inhibit mycelial growth, to cause cellular leakage, and to cause lipid peroxidation on *B. cinerea* isolate BC2. They also demonstrate that the fungicidal activity of the three fungicides correlates well with their capacity to cause cellular leakage and lipid peroxidation. Thus, they support the hypothesis that the mechanism of action of dicarboximide fungicides is associated with membrane lipid peroxidation.

LITERATURE CITED

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248−254.

Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. *Methods Enzymol*. **1978**, *52*, 302−310.

Burton, G. W.; Joyce, A.; Ingold, K. U. Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes? *Arch. Biochem. Biophys*. **1983**, *221*, 281−290.

Choi, G. J.; Lee, H. J.; Cho, K. Y. Lipid peroxidation and membrane disruption by vinclozolin in dicarboximide-susceptible and -resistant isolates of *Botrytis cinerea*. *Pestic. Biochem. Physiol*. **1996**, *55*, 29−39.

Edlich, W.; Lyr, H. Target sites of fungicides with primary effects on lipid peroxidation. In *Target Sites of Fungicide Action*; Köller, W., Ed.; CRC Press Inc.: Boca Raton, FL, 1992; pp 53−68.

Edlich, W.; Lyr, H. Mechanism of action of dicarboximide fungicides. In *Modern Selective Fungicides: Properties, Applications, and Mechanisms of Action*; Lyr, H., Ed.; Gustav Fisher Verlag: Jena, 1995; pp 119−131.

Edlich, W.; Lorenz, G.; Lyr, H.; Pommer, E.-H. Studies on the biochemical basis of resistance against dicarboximide fungicides. *Proc. Br. Crop Prot. Conf. Pests Diseases* **1988**, *1*, 391−396.

Georgopoulos, S. G.; Sarris, M.; Ziogas, B. N. Mitotic instability in *Aspergillus nidulans* caused by the fungicides iprodione, procymidone, and vinclozolin. *Pestic. Sci*. **1979**, *10*, 389−392.

Hisada, Y.; Kawase, Y. Morphological studies on antifungal action of *N*-(3′,5′-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide on *Botrytis cinerea*. *Ann. Phytopathol. Soc. Jpn*. **1977**, *43*, 151−158.

Kim, B. S.; Choi, G. J.; Cho, K. Y. Responses to several fungicides of *Botrytis cinerea* isolates resistant to benzimidazole and dicarboximide fungicides. *Korean J. Plant Pathol*. **1993**, *9*, 98−103.

Lee, H. J.; Duke, M. V.; Birk, J. H.; Yamamoto, M.; Duke, S. O. Biochemical and physiological effects of benzheterocycles and related compounds. *J. Agric. Food Chem*. **1995**, *43*, 2722−2727.

Leroux, P.; Fritz, R. Antifungal activity of dicarboximides and aromatic hydrocarbons and resistance to these fungicides. In *Mode of Action of Antifungal Agents*; Trinci, A. P. J., Ryley, J. F., Eds.; Cambridge University Press: Cambridge, 1984; pp 207−237.

Lyr, H. Lipid peroxidation: A side effect of sterol demethylation inhibitor fungicides in *Mucor mucedo* (L.) Fres and *Ustilago maydis*. *Pestic. Biochem. Physiol*. **1988**, *32*, 197−204.

Lyr, H.; Edlich, W. Lipid peroxidation, a consequence of the mechanism of action of aromatic hydrocarbon and dicarboximide fungicides and a side effect in DMI fungicides. *Proc. Br. Crop Prot. Conf. Pests Diseases* **1986**, *1*, 879−885.

Orth, A. B.; Sfarra, A.; Pell, E. J.; Tien, M. An investigation into the role of lipid peroxidation in the mode of action of aromatic hydrocarbon and dicarboximide fungicides. *Pestic. Biochem. Physiol*. **1992**, *44*, 91−100.

Orth, A. B.; Sfarra, A.; Pell, E. J.; Tien, M. Assessing the involvement of free radicals in fungicide toxicity using α-tocopherol analogues. *Pestic. Biochem. Physiol*. **1993**, *47*, 134−141.

Pappas, A. C.; Fisher, D. J. A comparison of the mechanisms of action of vinclozolin, procymidone, iprodione, and prochloraz against *Botrytis cinerea*. *Pestic. Sci*. **1979**, *10*, 239−246.

Pommer, E.-H.; Lorenz, G. Dicarboximide fungicides. In *Modern Selective Fungicides: Properties, Applications, and Mechanisms of Action*; Lyr, H., Ed.; Gustav Fisher Verlag: Jena, 1995; pp 99−118.

Slater, T. F. Overview of methods used for detecting lipid peroxidation. *Methods Enzymol*. **1984**, *105*, 283−293.

Steel, C. C.; Nair, N. G. The physiological basis of resistance to the dicarboximide fungicide iprodione in *Botrytis cinerea*. *Pestic. Biochem. Physiol*. **1993**, *47*, 60−68.

Received for review June 16, 1997. Revised manuscript received October 28, 1997. Accepted November 20, 1997.

JF970501C