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Inhibitory Effects of Atmospheric Ozone on *Magnaporthe grisea* Conidia

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Direct effects of atmospheric ozone on conidia of the rice blast pathogen, *Magnaporthe grisea*, were investigated to evaluate ozone-induced effects on infection potential of the rice blast fungus. Acute ozone exposure (200 nl l^{-1}, 8 h day^{-1}, 3 days) during sporulation significantly affected conidial morphology, appressorium formation, and disease development on rice leaves. Ozone caused reduction in conidial size and change in conidial shape. Relative cytoplasmic volume of lipids and vacuoles were increased in ozone-exposed conidia. Inhibition of appressorium formation and simultaneous increase in endogenous level of polyamines were found in ozone-exposed conidia. The inverse relationship between appressorium formation and level of polyamines implies that ozone-mediated increase in intracellular level of polyamines may inhibit appressorium formation in rice blast fungus. Furthermore, rice plants inoculated with ozone-fumigated conidia exhibited less severe disease development than those with unfumigated conidia. This result suggests that the anti-conidial consequence of acute ozone will eventually weaken the rice blasts potential for multiple infection cycle. This further suggests that consequently, rice blast can be transformed from an explosive disease to one that has limited epidemiological potential in the field.

Keywords **:** appressorium, polyamines, rice blast disease, vacuoles.

Ozone is considered as the most economically important air pollutant causing growth reduction, foliage injury, and yield loss in several vegetations (Heagle, 1989; Miller, 1987). In addition to direct effects on plants, ozone may also influence plant response to other stresses such as pathogens. Early reviews (Dowding, 1988; Heagle, 1973), as well as more recent comprehensive review (Manning and Von Tiedemann, 1995), have listed many empirical observations where plant disease was either enhanced or decreased by ozone. By suppressing the pathogen, plant exposed to ozone becomes more resistant (Laurence, 1981; Rusch and Laurence, 1993). Suppression of plant pathogens by ozone was reported on *Botrytis cinerea* (Heagle, 1982) and *Marssonina tremulae* (Beare et al., 1999).

Currently, ozone concentrations are rapidly increasing in Korea, with the highest ozone episodes recorded during summer when rice blast disease occurs severely (Yun et al., 1999). Previous study showed that acute ozone exposure of 200 nl l^{-1} for 8 h per day for 5 days severely damaged actively growing aerial mycelia of *Magnaporthe grisea*, the causal agent of rice blast (Hur et al., 2000). This implies that ozone-induced suppression of the fungus can influence rice blast severity under acute ozone exposure, which can simultaneously affect rice plants and rice blast pathogens in the field. Conidia produced on the infected areas can be spread to other plants and can cause more infection. However, no attempt has been made to evaluate the direct effect of acute ozone on *M. grisea* conidia and the subsequent change in the infection of the fungal pathogen on rice plants. Thus, in this study, ozone-induced effects on conidial morphology, appressorium formation related with intracellular levels of polyamine, and infection of *M. grisea* were investigated.

Materials and Methods

Fungal isolate and cultural conditions. Race KJ 201 of *M. grisea* was used throughout the experiments and was routinely maintained on potato dextrose agar (PDA) (Difco, Detroit, USA). The cultures were kept in a 25°C-incubator under white light (16 h photoperiod). For the preparation of conidia suspensions, the cultures were grown on oatmeal agar (50 g of oatmeal per liter). After a 7-day incubation, aerial mycelia of the plates were removed with a sterilized razor blade and then the scraped culture plates were exposed to ozone for 3 days under fluorescence light to promote conidiation (24 h photoperiod). When the ozone exposure was terminated, conidia were harvested by flooding the plates with 10 ml distilled water.

Ozone exposure. The fumigation system and ozone exposure have been described previously by Hur et al. (2000). Ozone was generated by passing cylinder-stored oxygen through a 220-V, single phase O_3 generator and delivered to fumigation chambers

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via a set of fine needle valves. Acute ozone of 200 nl l^{-1} was fumigated for 8 h day^{-1} (from 08:00 to 16:00) for 3 days. Ozone concentrations in the chambers were monitored continuously during the exposure with an ultraviolet (UV) photometric ozone analyzer (Model 400, API, San Diego, USA) installed with internal zero/span calibrator.

Morphology and appressorium formation of the exposed conidia. Upon termination of ozone exposure, conidia were harvested by flooding the plates with 10 ml distilled water. One or two droplets of the conidia suspension were placed on clean glass slides to measure their length and width under a microscope. One hundred conidia per treatment were examined to determine their size. Several droplets (200 µl) of the conidia suspension (approximately 5×10^4 conidia ml^{-1}), prepared as above, were placed on a hydrophobic and transparency film (CG3300, 3 M, Italy), sealed in a moistened box, and incubated at 25°C for 18 h. The percentages of germinated and germinating conidia induced to form appressoria were determined by the direct microscopic examination of 1,000 conidia on three films in ozone or filtered-air treatment.

Ultrastructural examination. Ozone-exposed conidial suspensions prepared as above were centrifuged and the remaining pellets were processed for microscopy. Those exposed to filtered air were also processed as control. Specimens were fixed with modified Karnovskys fixative consisting of 2% (V/V) glutaraldehyde and 2% (V/V) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) overnight, and washed with the same buffer three times for 10 minutes per step. These were then postfixed with 1% (W/V) osmium tetroxide in the same buffer at 4°C for 2 h and washed twice with distilled water. The postfixed specimens were *en bloc* stained with 0.5% uranyl acetate at 4°C overnight. They were dehydrated in a graded ethanol series (30, 50, 70, 80, 95 and 100%) and subjected three times in 100% ethanol for 10 minutes per step. The specimens were further treated with propylene oxide as a transitional fluid twice for 30 minutes, and embedded in Spurrs medium. Transverse sections were made with a diamond knife using an ultramicrotome (MT-X, RMC, Tucson, USA). Ultrathin sections (approximately 70 nm thick) were mounted on copper grids and double-stained with 2% (W/V) uranyl acetate and with Reynolds lead citrate for 7 minutes. Sections were examined with transmission electron microscope (JEM-1010, JEOL, Tokyo, Japan) operated at an accelerating voltage of 80 kV.

Extraction and analysis of polyamines. Ozone-exposed conidial suspensions prepared as above were centrifuged and the remaining pellets were harvested, washed with distilled water, and dried on filter paper for 1 h. Air-dried conidia (2-3 mg) were ground in ice-cold 5% perchloric acid. The homogenates were placed in an ice bath for 1 h and centrifuged at $15,000 \times g$ for 30 minutes. Supernatant fraction was used for the analysis. Free and conjugated polyamines were extracted, dansylated, solvent-purified, separated by TLC, and quantified using a spectrophotofluorimeter (model RF-1501, Shimadzu, Japan) as described previously (Tiburico et al., 1985).

Inoculation protocol. The rice plants were grown in a 27°C growth chamber and exposed to a photosynthetic photon flux density of 1500 µmol $m^{-2}s^{-1}$ (14 h photoperiod) supplied by metal halide lamps. Conidia suspension containing approximately 10^4 conidia ml^{-1} was prepared as previously mentioned. Tween 20 was added to the conidia suspension at the rate of 0.01% (v/v). Forty-day-old rice cultivar of Hwachung was inoculated with the conidia suspension. Sixteen rice plants were used for each treatment. The conidia suspension was directly sprayed onto the leaves of the target plants using a handheld sprayer. The plants inoculated with ozone or filtered-air exposed conidia were sealed with plastic bags to maintain 100% relative humidity at 25°C for 18 h before being returned to the growth chamber. After the plastic bags have been removed, the plants were incubated in the chamber at approximately 70% relative humidity. To quantify leaf injury, the percentage of leaf number and leaf area with necrotic lesions of rice blast after 7 days incubation was estimated. The number of lesions per plant was also counted. The percentages of diseased leaf area were measured with the use of transparent films printed with quadrates (5×5 or 2×2 mm). Each leaf was assessed separately, and the experiment was replicated three times.

For statistical analysis of data, the standard procedures of ANOVA were used (Minitab 10.2 Statistical Software).

Results

Morphological changes in ozone-exposed conidia. Table 1 shows the mean size of conidia formed under ozone exposure. Acute ozone exposure caused significant decrease in conidial length and increase in conidial width. As a result, smaller and rounder conidia were produced under ozone exposure compared with those produced under filtered-air exposure. Ultrastructural changes were also found in exposed conidia as shown in Fig. 1. The relative cytoplasmic volume of lipids and vacuoles was increased in fumigated conidia. Furthermore, electron-opaque accumulations in the vacuoles were observed in the exposed conidia, more likely related to ozone exposure.

Inhibition of appressorium formation in ozone-exposed conidia. There was significant difference in the appressorium formation of germinated conidia between ozone and filtered-air exposure. The appressoria formed in unfumigated conidia were well melanized and distinguishable from the germtube (Fig. 2). More than 90% of unfumigated

Table 1. Effect of acute ozone exposure (200 nl l^{-1}, 8 h day^{-1}, 3 days) during conidia production on conidial length and width of *Magnaporthe grisea*[a]

	Conidia treated with		% of ozone to filtered-air
	Ozone	Filtered-air	
Length (µm)	22.22 ± 3.14*[b]	28.71 ± 4.08	77.4
Width (µm)	9.05 ± 1.22*	8.34 ± 1.08	108.5

[a] The data represent the means and standard deviations of 100 conidia.
[b] The asterisks indicate significant difference between ozone and filtered air at $P < 0.001$.

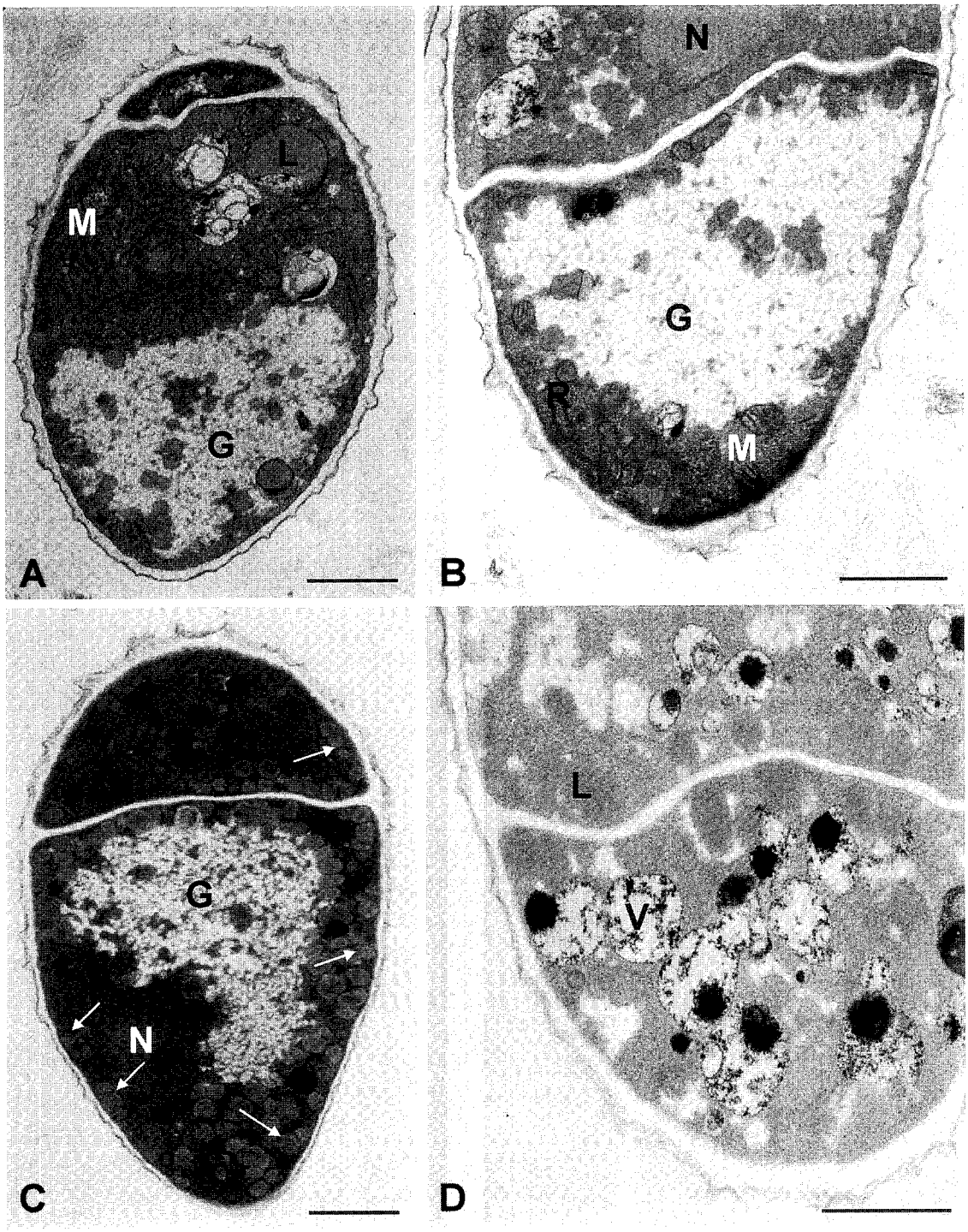

Fig. 1. Conidia of *Magnaporthe grisea* exposed to acute ozone (200 nl l^{-1}, 8 h day^{-1}, 3 days) during condia formation (bar = 1 μm). A-B: filtered-air exposure, C-D: ozone exposure. G = glycogen; L = lipid body; M = mitochondria; N = nucleus; R = rough endoplasmic reticulum. Cytoplasmic lipid droplets (arrows) are relatively abundant in ozone-exposed conidia.

conidia formed appressoria within 18 h of incubation (Table 2). However, ozone exposure noticeably inhibited appressorium formation (Fig. 2). The inhibition in appressorium formation of the fumigated conidia was consistently observed throughout the incubation period and was more pronounced at earlier incubation time (Table 2). At 6 h of incubation, almost 90% of inhibition in appressoria formation was found in ozone-exposed conidia, compared

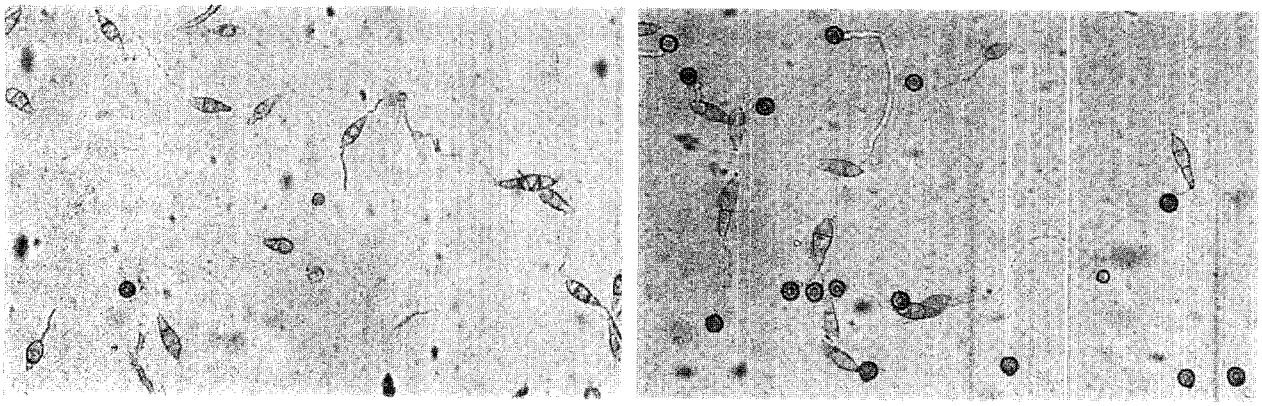

Fig. 2. Inhibition of appressorium formation in ozone-exposed conidia (left). The appressoria formed in unfumigated conidia (right) were well melanized and distinguisible from germtube.

Table 2. Effect of acute ozone exposure (200 nl l^{-1}, 8 h day^{-1}, 3 days) during conidia production of *Magnaporthe grisea* on appressoria formation on a hydrophobic and transparency film[a]

Incubation time (h)	Appressorium-forming conidia (%)		% of ozone to filtered-air
	Ozone	Filtered-air	
6	5.6 ± 2.6*[b]	51.1 ± 6.7	11.0
12	13.7 ± 6.2*	67.2 ± 9.5	20.4
18	46.8 ± 9.9*	90.2 ± 4.3	51.9

[a]The data represent the means and standard deviations of 1,000 conidia.
[b]The asterisks indicate significant difference between ozone and filtered air at $P < 0.001$.

with filtered-air-exposed conidia.

Increase in polyamines level in ozone-exposed conidia. The main effect of ozone on the endogenous level of conidial polyamines is presented in Table 3. Four polyamines of putrescine, cadaverine, spermindine, and spermine were present at a detectable level in fumigated conidia. In unfumigated conidia, however, only spermidine and spermine were found at a detectable level. Endogenous levels of spermidine and spermine were significantly increased under ozone exposure.

Table 3. Effect of acute ozone exposure (200 nl l^{-1}, 8 h day^{-1}, 3 days) during conidia production of *Magnaporthe grisea* on endogenous level of conidial polyamines[a]

Treatment	Polyamines (μM gfw^{-1})			
	Putrescine	Cadaverine	Spermidine	Spermine
Ozone	2.95 ± 0.71	4.32 ± 0.52	7.02 ± 0.53*[b]	0.82 ± 0.08*
Filtered-air	N.D[c]	N.D	0.30 ± 0.07	0.08 ± 0.02

[a]The data represent the means and standard deviations of 10 replicates.
[b]The asterisks indicate significant difference between ozone and filtered air at $P < 0.001$.
[c]D: not detectable.

Table 4. Effect of acute ozone exposure (200 nl l^{-1}, 8 h day^{-1}, 3 days) during conidia production of *Magnaporthe grisea* on disease incidence and severity on rice leaves[a]

	Inoculation of conidia treated with		% of ozone to filtered-air
	Ozone	Filtered-air	
Diseased leaves (%)[c]	41.00 ± 5.06*[b]	74.98 ± 6.96	54.7
Number of lesion[d]	47.88 ± 5.00*	90.25 ± 17.6	53.0
Diseased area (%)[e]	1.20 ± 0.22*	5.59 ± 1.05	21.5

[a]The data represent the means and standard deviations of 16 replicates.
[b]The asterisks indicate significant difference between ozone and filtered air at $P < 0.001$.
[c]Diseased leaves (%) = (number of diseased leaves/number of total leaves per plant) × 100.
[d]Total number of lesions per plant.
[e]Diseased area (%) = (total area of lesions/total leaf area per plant) × 100.

Inhibition of conidial infection in ozone exposed conidia. The inhibitory effect of ozone on conidial infection in rice leaves is shown in Table 4. The rice leaves inoculated with fumigated conidia exhibited less severe damage than those with unfumigated conidia. Typical symptom of necrotic lesions was found within 60 h on the leaves inoculated with unfumigated conidia. However, the necrotic spots were observed more than 72 h after inoculation of fumigated conidia. The percentage of diseased leaves was as much as 54% in the rice plants inoculated with fumigated conidia, lower compared with those with unfumigated conidia at 7 days after inoculation. The percentage of diseased area and total number of necrotic lesions per plant were also much lower in fumigated conidia inoculation than in unfumigated conidia inoculation.

Discussion

The disease cycle of rice blast involves three distinct phases: infection, colonization, and sporulation. Conidia produced on a lesion, which developed on susceptible host after successful infection, provide the inoculum for the next infection cycle. The polycyclic nature of the disease makes each phase a key determinant of the severity of blast epidemics. Therefore, ozone-induced interference during distinct phases can reduce the epidemiological potential for the pathogenic fungus. This is the first report which emphasizes that acute ozone exposure (200 nl l^{-1}, 8 h day^{-1}, 3 days) to *M. grisea* conidia during sporulation can alter conidial potential for rice blast epidemics by affecting conidia formation, ultrastructural morphology, biochemical properties regulating appressorium formation, and infection and subsequent disease development processes.

Acute ozone exposure during sporulation produced abnormally shaped conidia. Fumigated conidia were much shorter and wider than unfumigated conidia. Compared with unfumigated conidia showing the normal shape of pyriform to obclavate, fumigated conidia were shaped like turbinate. Hence, fumigated conidia became smaller and rounder than unfumigated conidia after acute ozone exposure. The requirement of additional energy used for compensatory and repair processes of ozone damage to newly forming conidia is possibly responsible for the small conidia formation in ozone treatment. Hamer and Givan (1990) previously reported that the abnormal spore shape caused a reduction in pathogenicity on rice. Therefore, it is possible that ozone-induced changes in conidial size and shape can affect conidial potential for rice blast disease.

Ozone-induced changes in the ultrastructure of *M. grisea* conidia were evident in the relative cytoplasmic volume of lipids and vacuoles. These observations were also found in lichen-forming endosymbiotic algae of *Flavoparmelia caperata* and *Usnea hirta* (Tahanen et al., 1997). When the lichen were exposed to 30-300 nl l^{-1} ozone for 3-5 days, cytoplamsic lipid droplets and vacuoles of endosymbiotic algae were significantly increased at higher ozone concentrations. Therefore, an increase in the cytoplasmic volume of lipid and vacuoles may be a good indication of ozone-induced ultrastructural changes.

Interestingly, ozone-exposed conidia significantly reduced appressorium formation on hydrophobic surface. An appressorium is a specialized infection structure, which adheres tightly to and then penetrates its host. The differentiation and maturation of appressoria are critical steps for successful infection. Appressorium formation in plant-pathogenic fungi is induced by environmental stimuli including thigmotropic and chemical signals (Hoch et al., 1987; Podila et al., 1993). Recent studies on intracellular signaling systems involved in appressorium differentiation in *M. grisea* suggested that polyamines were related to inhibition of appressorium formation by reducing intracellular cyclic AMP levels in this fungus (Choi et al., 1998). They found that the addition of 1-5 mM of polyamines (putrescine, spermidine and spermine) to conidia suspension significantly impaired appressorium formation in a dose-dependent manner, while exogenous addition of cyclic AMP restored appressorium formation inhibited by polyamines. The inverse relationship between appressorium formation and endogenous level of polyamines was also confirmed in this study. Ozone fumigation during sporulation significantly increased the total amount of polyamines in conidia. Compared with unfumigated conidia, fumigated conidia accelerated biosynthesis and the conversion of polyamines. Spermidine, known to be the major component of *M. grisea* conidial polyamines (Choi et al., 1998), showed the most significant difference between fumigated and unfumigated conidia. In particular, endogenous level of spermine, the most potent inhibitor of appressoria formation in *M. grisea*, increased as much as eight times in fumigated conidia.

Polyamines (putrescine, spermidine, and spermine) are ubiquitous organic cations of low molecular weight that bind to polyanionic macromolecules like DNA, RNA, and phospholipids in cells (Igarashi et al., 1982). In fungi, polyamines have been reported to be important in cell differentiation during spore germination, dimorphic transition, and conidiation (Khurana et al., 1996; Orlowski, 1995; Reyna-Lopez and Ruiz-Herrera, 1993). Polyamines also play important roles in preventing cells from oxidative stress by eliminating active oxidants induced by ozone (Bors et al., 1989; Smith, 1985). It was well known in many higher plants that ozone exposure induced endogenous polyamines biosynthesis and/or conversion of polyamines pool as a scavenging response to active oxidants formed by ozone (Wellburn and Wellburn, 1996). The scavenging response may also be true in ozone-injured conidia. Therefore, it can be presumed that ozone exposure during sporulation can increase intracellular level of polyamines by triggering endogenous polyamine biosynthesis and/or conversion. Consequently, the ozone-induced increase in intracellular level of polyamines can inhibit appressorium formation by regulating intracellular cyclic AMP levels, an important regulator of appressorium formation (Lee and Dean, 1993; Mitchell and Dean, 1995).

The relationship between ozone-induced inhibition of appressorium formation and decreased infection was confirmed in an inoculation test. The inoculation of *M. grisea* conidial suspension (race KJ 201) on the leaves of compatible rice cultivar Hwachung exhibited remarkable differences in disease incidence and development between fumigated and unfumigated conidia. Distinguishable necrotic

spots were detected at least 12 h earlier in rice plants inoculated with unfumigated conidia than those with fumigated conidia. Evaluation of disease severity at 7 days after inoculation made it possible to speculate that successful infection and disease development of the fungus was inhibited by acute ozone. The percent of lesion area developed on the leaves treated with unfumigated conidia was almost five times higher than those with fumigated conidia. It was previously reported that conida production from different types of lesions was variable, and that small lesions produced fewer spores and took longer to produce than large lesions (Ou, 1985). The results strongly suggest that ozone-induced inhibition of appressoria formation can adversely affect conidial infection and subsequent disease development process of rice blast pathogen.

In this study, rice plants were moistened for 18 h after inoculation, in which more than 90% of unfumigated conidia germinated and formed appressoria, while less than 50% of fumigated conidia formed appressoria. Considering that the period of leaf wetness to ensure germination and appressorium formation for successful infection of *M. grisea* conidia is only less than 12 h in the field, limited number of ozone-damaged conidia can germinate and form appressoria within the leaf wetness period. If this suppression of appressorium formation and consequent infection could be induced by acute ozone exposure in the field, the direct effect of ozone on the rice blast pathogen and disease cycle can considerably affect disease severity and disease development in the field. Therefore, evaluation of rice blast damage to rice plants in the field should consider the potential of direct ozone injury to the pathogen, as well previous exposure of the rice fields to acute ozone episodes where exposure is highly expected.

It was clearly demonstrated that *M. grisea* conidia were severely affected by acute ozone exposure (200 nl l^{-1}, 8 h day^{-1}, 3 days) during conidia formation. This study suggests that, if ozone exposure occurs during sporulation, acute ozone can inactivate *M. grisea* conidia and consequently, can reduce infection potential for rice blast fungus. Therefore, it can be speculated that the anti-conidial consequence of acute ozone will eventually weaken the rice blasts potential for multiple infection cycle. Furthermore, rice blast can be transformed from an explosive disease to one that has limited epidemiological potential in the field.

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