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Effects of Atmospheric Ozone on the Rice Blast Pathogen *Pyricularia grisea*

Jae-Seoun Hur*, Ki Woo Kim[1], Pan Gi Kim[1], Sung-Chul Yun[2] and Eun Woo Park[2]

**Department of Environmental Education, Sunchon National University, Sunchon 540-742, Korea*

[1]National Instrumentation Center for Environmental Management, Seoul National University, Suwon 441-744. Korea

[2]School of Applied Biology and Chemistry, Seoul National University, Suwon 441-744. Korea

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The direct effects of acute O_3 on the growth, sporulation and infection of *Pyricularia grisea*, rice blast pathogen, were investigated to understand the interactions between ozone and the pathogen. Acute exposure of 200 nl l^{-1} ozone for 8 h significantly reduced conidia germination on water agar. Ozone exposure of 200 nl l^{-1} for 8 h per day for 5 days had no effect on increase in colony diameter, but severely damaged actively growing aerial mycelia. However, the damage to mycalia was recovered during the following 16 h exposure of unpolluted air. Conidial production was also stimulated by the acute ozone exposure for 5 days. The conidia exposed to the acute ozone for 5 days normally germinates but slightly reduce appressoria formation on rice leaf. However, the conidia produced by artificial stimulation under the same ozone concentration for 10 days showed significant reduction in appressoria formation on a hydrophobic film. This study suggests that the acute ozone could inhibit appressoria formation as well as vegetative growth of the pathogen, resulting in decrease in rice blast development in the field during summer when high ozone episodes could occur occasionally.

Keywords **:** acute ozone, conidia, germination, appressorium formation, vegetative growth.

Ozone is considered the most economically important air pollutant causing growth reduction, foliage injury and yield loss in several vegetation (Heagle, 1989; Miller, 1987). In addition to direct effects of ozone on plant, it may also influence plant response to other stresses such as pathogens. Ozone exposure renders plant more vulnerable to infection by providing infection courts (Manning et al., 1970). On the other hand, the plant exposed to ozone can be more resistant to a pathogen by decreasing the suitability of the plant as a host or suppressing the pathogen (Laurence, 1981; Rusch and Laurence, 1993). Suppression of plant pathogens by ozone was reported on *Botrytis cinerea* and *Marssonina tremulae* (Beare et al., 1999; Heagle, 1982). For example, conidia germination of *M. tremulae* was significantly deceased after acute exposure to 200 nl l^{-1} for 8 h per day over 15 days. Therefore, direct effects of ozone on plant pathogens need to be investigated to understand the impact of ozone on plants which are under influence of the pathogen at the same time.

Pyricularia grisea (T. T. Herbert) Barr is the causal agent of rice blast, one of the destructive pathogens of rice throughout the rice-growing areas of the world. Ozone concentrations are rapidly increasing in Korea during the last few years and the highest ozone episodes were often recorded during summer when rice blast disease occurs severely (Yun et al., 1999). Relative high concentrations of ozone during summer were known to cause invisible injury such as yield reduction as well as visible damage to rice palnts (Sohn and Lee, 1997; Wahid et al., 1995). Given the potential for concurrent impact of rice blast and ozone on rice plants during summer, investigation on the interaction between ozone and *P. grisea* is needed. Thus, we examined the direct effects of ozone on the growth, sporulation and infection of *P. grisea*.

Materials and Methods

Ozone exposure. The fumigation system consisted of two 0.016 m^3 clear-cast closed glass chambers housed in two growth chambers. Air was pushed into the system by a small diaphragm pump (Daegun electric Co., Seoul, Korea) and passed through activated charcoal filters and the following impinger filled with sterilized distilled water. Activated charcoal filters maintained the ozone concentration in the chambers at less than 10 nl l^{-1}. The impinger maintained the relative humidity over 80% enough to prevent water loss from culture plates placed in the glass chambers. Air entered the bottom of each chamber and exited through the chamber top. Airflow was balanced to give approximately 3 air changes min^{-1} in each chamber. Ozone was generated by passing cylinder-stored oxygen through a 220-V, single phase O_3 generator and delivered to the chamber via a set of fine needle values. Acute ozone exposure at the level of 200 nl l^{-1} was given over 5 days for 8 hr (08:00-16:00) each day.

Teflon tubing was used for all the air sampling. Ozone concentra-

*Corresponding author.
Phone) +82-661-750-3383, Fax) +82-661-750-3308
E-mail) jshur1@sunchon.ac.kr

tions 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. The analyzer was calibrated before exposure.

Fungal isolate and cultural conditions. Race KI 197 of *P. grisea* was used throughout the experiments and was routinely maintained on potato dextrose agar (PDA) (Difco, Detroit, USA) The cultures were kept in a 20°C incubator under white light (16 hr photoperiod). For preparation of conidia suspensions, the cultures were grown on oatmeal agar (50 g of oatmeal per liter) at 20°C under fluorescence light to promote conidiation. Conidia were collected from 10-day-old cultures by flooding the plates with sterilized distilled water and the conidia concentration of the resultant suspension was adjusted to approximately 10^4-10^5 conidia ml^{-1}.

Ozone effects on conidial germination on water agar. The conidia suspension of *P. grisea* was sprayed on water agar plates (20 g l^{-1}) containing streptomycin sulphate (100 mg l^{-1}). Conidia were exposed to 200 nl l^{-1} ozone for 8 hr. Five plates were removed at 16 h after termination of the exposure in each chamber and flooded with lactophenol blue staining solution (Beare et al., 1999). Five 10-mm diameter plugs were taken from each plate using a cork borer and examined under a compound microscope at ×400 magnification. Conidial germination was counted by observing 500 conidia for each plug.

Ozone effects on fungal growth. Mycelial discs (5 mm in diameter) of *P. grisea* were removed from the margin of actively growing mycelial mat on PDA and transferred to fresh PDA (25 g l^{-1}) plates. When the diameter of mycelial mat reached approximately 1.5 cm in a 20°C incubator, the plates were moved to the ozone chamber and exposed to 200 nl l^{-1} ozone for 8 hr per day over 5 days. After termination of the exposure, the diameter of the mycelial mat, including that of the original inoculum plug, was measured and conidia were harvested by flooding the plates with 5 ml distilled water to examine ozone effect on conidia production. The conidia suspension was also used to investigate the germination and appressorium formation of the conidia pre-exposed to ozone on rice leaf discs. Small pieces of the mycelial mat were taken to investigate ozone damage to fungal hyphae under a scanning electron microscope (SEM).

Specimen preparation for scanning electron microscopy. Agar plugs were removed with a razor blade from the PDA culture plates after the ozone exposure. The specimens were mounted to metal stubs using carbon tapes and plunged into liquid nitrogen for cryo-fixation for 10 sec. The specimens were then placed in a cryo-chamber of a cryo-transfer system (CT1500, Oxford Instruments, UK) maintained at -170°C, and transferred to a cold specimen stage of a scanning electron microscope (JSM-5410LV, JEOL, Japan) Sublimation was performed by heating and maintaining the cold specimen stage at -70°C for 3 min, and the process was monitored at 5 kV. The specimens were then sputter-coated with gold and observed with the scanning electron microscope at 20 kV.

Ozone effects on conidial germination on rice leaf discs. The rice plants of cultivar Dongin, which was previously found to be moderately sensitive to ozone (Sohn and Lee, 1997), were grown in a 27°C growth chamber and exposed to a photosynthetic photon flux density of 1500 μmol $m^{-2}s^{-1}$ (14 hr photoperiod) supplied by metal halide lamps with a 5 cm water layer to reduce heat. Ten-mm diameter leaf discs of the 6th leaf were obtained from approximately 40-day old rice plants. The conidia suspension containing approximately 5×10^4 conidia ml^{-1} was prepared as previously mentioned. Tween 20 was added to the conidia suspension at the rates of 0.01% (v/v). The conidia suspension was sprayed onto the surface of leaf discs using a hand-hold sprayer. Five discs were placed on water agar (20 g l^{-1}) and incubated at 25°C for 24 hr. The discs were removed and placed into a clearing solution of a 3:1 mixture of ethanol and glacial acetic acid for 24 hr. All discs were washed in distilled water and stained with lactophenol blue for 10 min before being examined under a compound microscope (Beare et al., 1999). The total number of conidia and germinated conidia per leaf disc was counted along with the number of appressorium-forming conidia. Twenty-five leaf discs were used for the counting in ozone or filtered-air treatment.

Ozone effects on appressoria formation. The culture plates prepared as above were exposed to 200 nl l^{-1} ozone for 8 hr per day for one week under dark condition. After 7-day exposure, aerial mycelia of the culture plates were removed with a sterilized razor blade and then the scraped culture plates were further exposed to the ozone for 3 days under fluorescence light to promote conidiation (24 hr photoperiod). Conidia production was stimulated during the last 3-day exposure of acute ozone. When ozone exposure was terminated, conidia were harvested by flooding the plates with 10 ml distilled water. Several droplets (100 μl) of the conidia suspension (5×10^4 conidia ml^{-1}) were placed on a hydrophobic and transparent over-head-project (OHP) film, sealed in a moistened box, and incubated at 25°C for 24 hr Preliminary investigation showed that the film was suitable for the observation and formation of conidial appressoria. At every 6-hour interval during the incubation, the percentages of germinated and germinating conidia induced to form appressoira were determined from direct microscopic examination of 1800 conidia on three films in ozone or filtered-air treatment.

Results

Vegetative growth of the fungus was severely altered by acute ozone exposure of 200 nl l^{-1}. The fungal cultures grown under filtered-air formed white-colored mycelial mat with dense aerial mycelia (Fig. 1A and C). When the fungal cultures were exposed to ozone, dark gray-colored mycelial mat was formed with loose aerial mycelia (Fig. 1B and D). Compared with intact hyphae of *P. grisea* exposed to filtered-air (Fig. 1E), severely damaged hyphae were found in the cultures exposed to ozone (Fig. 1F). Ozone caused the deformation of fungal hyphae such as swelling, shrinking, distortion and disruption. Ozone damage to vegetative growth of the mycelia was obvious during the 8 h-exposure of ozone. Alternately arranged bands of gray-colored and loose mycelia and white-colored and dense mycelia were

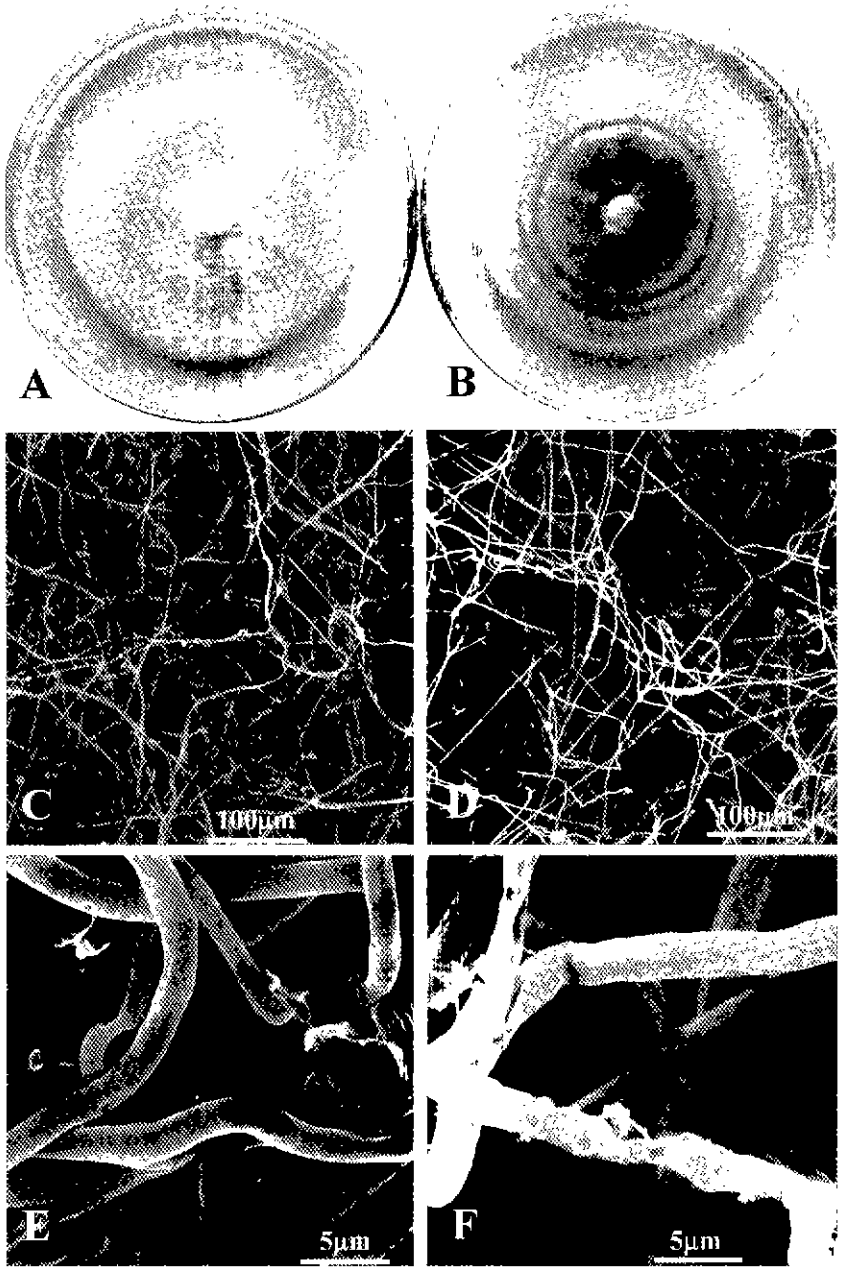

Fig. 1. Ozone-induced damage to aerial mycelia of *Pyricularia grisea*. The fungal cultures were exposed to filtered air (A, C, E) or 200 nl l^{-1} O_3 for 8 hr per day (B, D, F) for 5 days. (A): evenly growing fungal colony with white-colored acrial mycelia, (B): alternately arranged bands of gray-colored aerial mycelia under acute ozone, (C): densely developed aerial mycelia, (D): loosely developed aerial mycelia under ozone exposure, (E): intact fungal hyphae, and (F): severe deformation of fungal hyphae such as swelling, shrinking, distortion and disruption under acute ozone.

produced on PDA during the 5-day exposure period (Fig. 1B).

Ozone exposure to germinating conidia of *P. gisea* caused significant reduction in the rate of conidial germination on water agar (Table 1). Production of more than two germ-tubes appeared to be inhibited under ozone but the effect was not significant.

There was no difference in the diameter of mycelial mat of the fungus between the cultures under ozone exposure and filtered-air (Table 2). However, production of conidia was significantly stimulated by acute ozone (Table 2). Ozone exposure enhanced conidia production as much as 1.8 times compared with filtered-air. Unlike the reduction of conidia germination on water agar under ozone exposure (Table 1), conidia harvested from the cultures previously exposed to acute ozone exhibited no significant difference

Table 1. Effects of acute ozone exposure on conidia germination of *Pyricularia grisea* on water agar

	Germinated conidia	Conidia with more than 2 germ tubes
Control	91.5* ± 2.76	26.2 ± 1.50
Ozone	83.6 ± 6.59	25.8 ± 1.79

Spore suspension was sprayed on distilled water agar (20 g l^{-1}) and exposed to 200 nl l^{-1} ozone for 8 h. Spore germination was observed at 16 hr after termination of the exposure. The data represent the means and standard deviations of 25 replicates. The asterisk indicates significant difference between control and ozone exposure at $P < 0.001$

Table 2. *Pyricularia grisea* fungal growth and conidia production on agar plates during acute ozone exposure, and spore germination and appressorium formation on rice leaf discs after ozone exposure

	on potato dextrose agar (during ozone exposure)[a]	
	Mycelial diameter (mm)	Number of conidia ($\times 10^4$ conidia ml^{-1})
Control	63.2 ± 0.7	4.71 ± 1.11
Ozone	63.6 ± 0.4	8.61* ± 2.07
	on rice leaf discs (after ozone exposure)[b]	
	Germinated conidia (%)	Appressorium-forming conidia (%)
Control	73.78 ± 10.76	36 93 ± 9.87
Ozone	76.32 ± 9.28	27.21 ± 10.46

[a] *P. grisea* was exposed to 200 nl l^{-1} ozone (8 h day^{-1}) for 5 days. Conidia were collected by flooding the plates with 5 ml sterilized distilled water.

[b] The conidia were harvested immediately after termination of the ozone exposure and sprayed on 10-mm diameter rice leaf discs. The inoculated discs were incubated at 25 °C for 24 hr.

The data represent the means and standard deviations of 25 replicates. The asterisk indicates significant difference between control and ozone exposure at $P < 0.001$.

from the cultures under filtered-air in conidia germination and appressoria formation on rice leaf discs (Table 2). However, significant reduction of spore germination and appressoria formation on hydrophobic OHP films was found in the conidia produced by artificial stimulation under acute ozone exposure (Table 3). More than 90% of conidia were germinated at 6 hr incubation and formed appressoria at 24 hr incubation on the films. Interestingly, significant difference in appressoria formation between the condia produced under ozone and filtered-air was consistently observed throughout the incubation period but reduction of appressoria formation was more pronounced at early incubation time than at late incubation time. At 6 hr incubation, almost 40% of reduction in appressoria formation was found in the condia produced under acute ozone, compared with those under filtered-air. The germination of conidia exposed to ozone was also significantly different from those

Table 3. Effect of acute ozone exposure during condinia production of *Pyricularia grisea* on spore germination and appressoria formation on hydrophobic OHP films

Incubation time (Hour)	Germinated conidia (%)		Appressorium-forming conidia (%)	
	Ozone	Control	Ozone	Control
6	93.0 ± 1.76	95.1*** ± 1.68	21.8 ± 7.76	37.9*** ± 6.86
12	96.3 ± 1.87	97.3 ± 0.78	79.3 ± 2.09	84.3*** ± 2.11
18	97.1 ± 1 .31	98.6* ± 1.38	83.1 ± 1.50	86.0*** ± 2.09
24	99.2 ± 0.83	99.9** ± 0.30	90.7 ± 2.96	93.3** ± 0.77

After 7-day exposure of *P. grisea* cultures to 200 nl l^{-1} ozone (8 hr day^{-1}) under dark condition, aerial mycelia were removed and then the scraped culture plates were further exposed to the ozone for 3 days under fluorescence light to promote conidiation. Conidia were collected by flooding the plates with 10 ml sterilized distilled water. Droplets of the conidia suspension were placed on hydrophobic OHP films, sealed in moistened box and incubated at 25°C for 24 hr. Spore germination and appressoria formation were observed at every 6 hr during the incubation.

The data represent the means and standard deviations of 18 replicates. The asterisks indicate significant difference between control and ozone exposure at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

exposed to filtered-air, but the difference was less than 2.2 % throughout the incubation period. It was obvious that conidia exposed to ozone could normally germinate but less successfully form appressoria than those exposed to filtered-air.

Discussion

It was clearly demonstrated in this study that growth of *P. grisea in vitro* was severely inhibited by 200 nl l^{-1} ozone exposure (8 h day^{-1}, for 5 days). The findings were different from the previous report showing most fungi were tolerant to ozone (Manning and Tiedemann, 1995). Relatively high ozone dose (ozone concentration and exposure duration) was employed in this study. Nevertheless, the responses of *Marssonina tremulae*, casual agents of leafspot disease of poplar, to acute ozone exposure at the level of 200 nl l^{-1} (8 hr day^{-1}, for 7 days) were comparable to the responses of *P.* grisea to the same ozone exposure. Mycelial growth of *M. tremula* was unaffected, whereas severely damaged aerial mycelia of *P. grisea* were clearly observed under SEM. Unlike *P. grisea*, high tolerance of *M. tremula* against the acute ozone was postulated by the fact that *M. tremula* hyphae are bounded by a bi-layered electron dense wall which may act as an initial barrier to the pollutant (Beare et al., 1999).

Interestingly, ozone exposure had no effect on the increase in diameter of *P. grisea* colony on PDA. The lack of ozone effect on the increase in colony diameter may be due to the immersed nature of the mycelia within the agar medium. The avoidance of mycelia from the pollutant by

directing fungal growth within the agar medium could explain no difference in colony diameter between the fungal cultures under filtered-air and ozone throughout the ozone exposure. However, aerial mycelia which were in direct contact with ozone were found to be severely damaged. The avoidance may have also happened to many fungi growing on the agar medium and could partly explain the reported tolerance of many fungi such as *Alternaria oleraceae* whose colony growth was only inhibited by a high ozone dose (600 nl l^{-1}, for 4 days) (Treshow et al., 1969). Therefore, it must be carefully considered that the estimation of ozone damage to fungi *in vitro* can be variable depending on characteristics of fungal growth and culture media, and the high tolerance of many fungi growing on agar media during ozone exposure would be overestimated.

Alternated band arrangement of aerial mycelium with different colors and density resulted from the interruption of ozone exposure for 16 hr followed by 8 hr exposure during the 5 days of ozone exposure. Formation of the bands indicates that fungal growth of *P. grisea* was inhibited under presence of ozone for the 8 h and recovered under the following unpolluted air for 16 h. The result suggests that *P. grisea* could be damaged during high ozone episodes in summer but the adverse effect of ozone could be masked by recovering mycelial growth of the fungus under relatively lower ozone levels of ambient air in the field.

Stimulation of conidia germination under low ozone doses has been shown in the cases of other fungi and is due to increases in permeability of spore wall to water through ozone-induced oxidation of cell wall components (Beare et al., 1999; Hibben and Stotzky, 1969). However, conidia germination of *P. grisea in vitro* was suppressed by acute ozone. Conidia of *P. grisea* are small (15.8-27.0 μm long, 6.8-10.2 μm wide) and hyaline (Ou, 1985). Such spores tend to be more sensitive to pollutant effects than large and pigmented spores (Manning and Tiedemann, 1995). It is well known that spore germination is inhibited to some extent by substances contained within the spores themselves (Agrios, 1988). Therefore, it may be suggested that ozone-induced degradation of cell wall of conidia could accelerate release of the inhibitors contained within conidia.

Conidia production of the fungus *in vitro* was stimulated by acute ozone. Nutrients in the growth medium and light conditions affect sporulation of *P. grisea in vitro* (Leung and Shi, 1994). Currently, the mechanisms for the ozone-induced stimulation of conidia production is not determined. Presumably, reproductive growth of the fungus may have been stimulated to compensate for severe retardation of vegetative growth caused by acute ozone.

Conidia produced under acute ozone seemed to be unaffected in conidia germination but significantly inhibited in appressoirum formation. Appressorium, the specialized infection structure of many plant pathogenic fungi, has been known to be induced by environmental stimuli including thigmotropic and chemical signals (Podila et al., 1993). Recently, polyamines were suggested to inhibit the appressorium formation of *P. grisea* by regulating the level of intracellular cyclic AMP (Choi et al., 1998). Polyamines also play important roles in preventing cells from oxidative stress by eliminating active oxidants induced by ozone (Bors et al., 1989; Smith, 1985). Increased concentration of intracellular polyamines has been well established in the mechanisms regulating oxidative damages in ozone-exposed cells (Hur and Wellburn, 1994; Rowland-Bamford et al., 1989; Wellburn and Wellburn, 1996). Therefore, it might be possible that the intracellular level of polyamines was elevated in the conidia under acute ozone and the increased level of polyamines could attribute to suppression of appressorium formation.

It can be concluded in this study that *P. grisea* was sensitive to acute ozone which has occasionally recorded during high ozone episodes in summer. Consequently, the acute ozone would severely inhibit appressoria formation as well as vegetative growth of the pathogen, resulting in decrease in rice blast development in the field during summer.

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