Effects of Ozone on the Sporulation, Germination, and Pathogenicity of Botrytis cinerea

Charles R. Krause and T. Craig Weidensaul

Research Plant Pathologist, Agricultural Research Service, U. S. Department of Agriculture, Nursery Corps Research Laboratory, Delaware, OH 43015; and Director of the Laboratory for Environmental Studies and Associate Professor of Plant Pathology, The Ohio State University, Columbus, and Ohio Agricultural Research and Development Center, Wooster, OH 44691.

Portion of a dissertation submitted by the senior author to the Dept. of Plant Pathology, The Ohio State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Accepted for publication 6 July 1977.

ABSTRACT

KRAUSE, C. R. and T. C. WEIDENSAUL. 1978. Effects of ozone on the sporulation, germination, and pathogenicity of Botrytis cinerea. Phytopathology 68: 195-198.

Studies were made to determine if *Botrytis cinerea* conidia remain viable when grown in vivo and in vitro in the presence of ambient ozone levels and whether ozonized conidia retain pathogenicity. Ozone at 599 $\mu g/m^3$ (0.30 ppm) for two 6-hr periods significantly inhibited *B. cinerea* sporulation and germination in vivo and in vitro. Conidia ozonized in vitro at 299 $\mu g/m^3$ (0.15 ppm) for two 6-hr periods caused less infection than non-ozonized conidia based on total lesion area. No Botrytis lesions were induced on detached leaves inoculated with ozonized conidia. Results indicated that ozonization of conidia produced in vivo and in vitro decreased germination of conidia, germ tube length, pathogenicity, and/or virulence.

Additional key word: Pelargonium hortorum.

Air pollutants directly affect plant pathogenic fungi (9, 10, 13, 19). Couey and Uota (2) reported that sulfur dioxide inhibits spore germination of *Botrytis cinerea* Pers. Couey (1) also reported similar studies on germination of *Alternaria* spp. conidia. Saunders (18) found that sulfur dioxide inhibited germination of conidia of *Diplocarpon rosae.*

Ozone inhibited mycelial growth of fungus colonies and decreased sporulation (5, 8, 11). Hibben (6) found that 199 to 499 $\mu g/m^3$ (0.10 to 0.25 ppm) ozone reduced *Botrytis allii* conidial germination.

Hibben and Stotzky (7) have shown that differential spore sensitivity to ozone depended on the fungal species, spore morphology, moisture, substrate, and ozone dosage. They found that after exposing agar cultures of various fungi to 1,997 $\mu g/m^3$ (1 ppm) for 6 hr, thick-walled multi-cellular spores of *Alternaria* and *Stemphylium* spp. germinated at the same rate as did spores of nontreated controls. However, at the same ozone dose the germination rate of thin-walled, hyaline spores of *Rhizopus stolonifer*, *Trichoderma viride*, *Aspergillus niger*, *Penicillium egyptiacum*, and *Botrytis* allii decreased. Rich and Tomlinson (16) concluded that ozone may injure conidiophores while stimulating the in situ germination of attached *Alternaria solani* conidia either by the destruction of a germination inhibitor or by altering the permeability of the cell wall to gases.

00032-949X/78/000030$03.00/0

Despite numerous studies relating to effects of ozone on fungi, only Krause and Weidensaul (10) have reported on the effect of this pollutant on conidia produced in vivo and in vitro.

The objective of this study was to determine if *B. cinerea* conidia remain viable and pathogenic when produced in the presence of high ambient ozone levels. *Botrytis cinerea* was chosen for this study since it readily infects geranium leaves and grows and sporulates well on artificial media.

MATERIALS AND METHODS

Rooted cuttings of geranium, *Pelargonium* × *hortorum* Bailey 'Sincerity' were established in 14 cm (diam) plastic pots in a soil-peat-perlite (2:1:1, v/v) mixture. Plants were grown for 2 mo in charcoal-filtered air under a 16-hr day at 19,370 lux. Cuttings were maintained at 27 C during the day and 16 C at night. Plants were fertilized every 2 wk with 500 ml of commercial fertilizer (20-20-20) and watered every 2 days.

Botrytis cinerea was cultured in the dark 10 days on V-8 juice agar at 68 C. Suspensions of conidia were prepared by flooding culture plates with 15 ml of sterile, deionized-distilled water containing two drops of Tween-20. Contents of several plates were collected in a sterile 500-ml Erlenmeyer flask. The resulting suspension was filtered through four layers of sterile gauze. The suspension of conidia was collected by filtering through a Millipore filter (0.2 μm) and washed with sterile, deionized-distilled water. The conidia were resuspended

in sterile, deionized-distilled water sufficient to produce a suspension containing 7,000 to 8,000 spores/ml. Only fresh suspensions of conidia were used for inoculations to avoid storage effects on virulence of conidia and pathogenicity. Plants to be inoculated were placed in a mist chamber adjusted to apply mist for 2 sec at 10-min intervals. Each plant was sprayed with approximately 10 ml of conidia suspension and then incubated for 72 hr in darkness at 22 C.

Exposure chambers at the Laboratory for Environmental Studies, Ohio Agricultural Research and Development Center, Wooster, were used in the study. Chambers measured 101 × 101 × 76 cm and were under slight negative pressure and negative air flow at about three air changes per min. Standard environmental parameters were 320 lux, 22 C, and 80% relative humidity. A Model 1100 McMillan chemiluminescent ozone meter was used to monitor ozone concentrations constantly. Each chamber was equipped with a sprinkling system that sprayed water into the chambers for 6 sec each hr. Incubation "tents" made of aluminum foil were provided in each chamber to prevent water spray from contaminating *B. cinerea* culture plates.

Ten inoculated plants were placed in exposure chambers along with ten 5-day-old plate cultures of *B. cinerea* growing on a standard V-8 juice agar. The chambers were sealed and the ozone concentrations were adjusted to 0.00 $\mu g/m^3$ (charcoal-filtered air), 299 $\mu g/m^3$ (0.15 ppm) and 599 $\mu g/m^3$ (0.30 ppm). The exposures consisted of two 6-hr periods on consecutive days. The treatments in the experiment were: (i) inoculated plants exposed to filtered air, (ii) culture plates exposed to filtered air, (iii) inoculated plants treated with ozone at 299 $\mu g/m^3$ (0.15 ppm), (iv) culture plates treated with ozone at 299 $\mu g/m^3$ (0.15 ppm), (v) inoculated plants treated with ozone at 599 $\mu g/m^3$ (0.30 ppm), and (vi) culture plates treated with ozone at 599 $\mu g/m^3$ (0.30 ppm).

At the end of the ozone exposure, the presence of spores on buds, leaves, or stems was observed for each inoculated plant treatment. Conidia from each culture plate and each plant were harvested immediately after fumigation with a sterile, deionized-distilled water wash. The spore suspensions from each treatment were pooled. Conidia from each treatment were filtered, washed, and resuspended as previously described.

The percent germination and germ tube length of half the conidial suspension were determined by the hanging-drop method (15) 0, 24, and 48 hr after fumigation. A second germination test involved water agar (17). Five ml of sterile 3% water agar and 2 ml of conidial suspension were pipetted aseptically into a sterile 100 × 15-mm plastic culture plate and mixed together. There were 10 replicates of each treatment. Spores were incubated in the dark at 20 C. Germination percentage and germ tube length were recorded when the agar solidified and then after 24 and 48 hr.

The remaining half of the washed conidial suspension was tested for its ability to induce local lesions. Three geranium leaves were placed in a 25 × 150 mm plastic petri plate on moist filter paper. Ten drops of the conidial suspension were placed randomly on each leaf. After replacement of the lids, plates were incubated in darkness at 20 C for 4 days.

Infection was evaluated by measuring *B. cinerea* lesion areas and then calculating percentage of the leaf area affected. Total leaf area was determined using a Model AAM-5 Hayashi Denko electronic area meter. *Botrytis* cinerea lesions were then excised from the leaf and the amount of noninfected tissue remaining was measured. Random isolations from lesions confirmed the presence of *B. cinerea*.

A Student's *t*-test was performed on percentage germination and germ tube length treatment means to detect significant differences between germination tests, sources of conidia and incubation times. A one-way analysis of variance was performed on the data to assess pathogenicity of treated conidia and a Duncan's multiple range test was used to separate treatment means (3).

RESULTS

Although the results were not quantified, *Botrytis cinerea* sporulated more profusely on plants exposed to ozone-free air than on those exposed to ozone. Sporulation occurred on buds, leaves, and stems on plants fumigated with 299 $\mu g/m^3$ (0.15 ppm) ozone for two 6-hr periods on consecutive days. However, on plants exposed to 599 $\mu g/m^3$ (0.30 ppm) ozone, sporulation occurred only on infected stems. Infection, although not quantified, appeared to increase on all inoculated plants at each ozone dose during the fumigation period. Typical *B. cinerea* symptoms of brown, water-soaked lesions and gray conidiophores were observed. Microscopic identification and random isolations from suspected *B. cinerea* infections confirmed the presence of the pathogen.

As the ozone level increased, germination and germ tube length decreased on conidia grown in the presence of ozone at 299 and 599 $\mu g/m^3$ (Table 1). A paired *t*-test showed no significant differences in treatment means comparing the hanging drop and water agar tests in any of the treatments. No significant differences in germination or germ tube length were found between in vitro or in vivo sources of conidia fumigated at the same ozone concentrations. Germination of conidia was significantly greater in both samples grown in filtered air. Conidia grown on culture plates failed to germinate when exposed to ozone at 599 $\mu g/m^3$ (0.30 ppm) for two 6-hr periods.

Incubation time affected the germination and germ tube length of control conidia. After 24 hr, the mean germination was 88%, but increased to 96.6% after 48 hr. Germ tube length of control conidia greatly increased between the 24-and 48-hr observations, but germ tubes of ozonated conidia did not.

Ozonization of conidia reduced their ability to produce lesions on detached leaves (Table 2). Conidia from infected plants caused larger lesions than did conidia originating from culture plates, whether treated with ozone at 299 $\mu g/m^3$ (0.15 ppm) or filtered air. However, no Botrytis lesions were produced by inoculation with conidia grown with ozone at 599 $\mu g/m^3$ (0.30 ppm) from either source of conidia.

DISCUSSION

Germination of conidia germ tube length, and

TABLE 1. Effects of ozone on development of *Botrytis cinerea* conidia

Ozone concentration ($\mu g/m^3$)	Source of conidia	Germination method	Germination after incubation for:		Germ tube length after incubation for:	
			24 hr (%)	48 hr (%)	24 hr (μm)	48 hr (μm)
0.0	Infected plant	HD[x]	88.0 c[z]	96.6 c	36.0 c	105.0 c
		WA[y]	87.0 c	98.4 c	34.0 c	117.0 c
0.0	Culture plate	HD	96.5 c	99.1 c	38.0 c	126.0 c
		WA	97.1 c	100.0 c	41.0 c	129.0 c
299	Infected plant	HD	47.1 d	56.0 d	10.0 d	15.0 d
		WA	48.2 d	58.5 d	7.0 d	11.0 d
299	Culture plate	HD	51.6 d	60.1 d	5.0 d	9.0 d
		WA	54.8 d	58.5 d	4.0 d	7.0 d
599	Infected plant	HD	2.0 e	2.1 e	1.2 d	1.9 d
		WA	1.9 e	2.2 e	2.0 d	2.5 d
599	Culture plate	HD	0.0 e	0.0 e	0.0 d	0.0 d
		WA	0.0 e	0.0 e	0.0 d	0.0 d

[x]Hanging-drop test (based on 10 replications).
[y]Water agar test (based on 10 replications).
[z]Values followed by the same letter within a given column are not different, $P = 0.05$, by Duncan's multiple range test.

TABLE 2. Effect of ozone on size of *Botrytis cinerea* lesions on inoculated detached leaves

Ozone concentration ($\mu g/m^3$)	Source of conidia	Lesion area (% of total leaf area)
0.0	Infected plant	55.6 A[z]
299	Infected plant	23.9 B
599	Infected plant	0.0 C
0.0	Culture plate	43.2 D
299	Culture plate	9.9 E
599	Culture plate	0.0 C

[z]Means followed by the same letter are not significantly different from one another, $P = 0.05$ by Duncan's multiple range test.

pathogenicity of *B. cinerea* conidia produced in vivo and in vitro were decreased by exposure to ozone. In addition, sporulation occurred only on diseased stems after fumigation with ozone at 599 $\mu g/m^3$ (0.30 ppm) for two 6-hr periods. At this level, ozone decreased conidial viability of a pathogen growning saprophytically (in vitro) or parasitically (in vivo). Parasitism therefore can be limited through the direct effect of acute ozone doses on the parasite.

Magdycz (12) demonstrated that ambient level of ozone stimulated germination of *B. cinerea* spores when the gas was bubbled through an aqueous spore suspension. Heagle (4) pointed out, however, that "Poor aeration may be one reason for decreased effect on spores in a suspension. Much of the ozone probably breaks down in solution and never directly contacts the spores." In this study aerial cultures growing on agar plates and infected plants were exposed to ozone and the developing conidia were harvested.

The mode of action of ozone on fungi is not certain. Since ozone attacks cellular membranes of higher plants, perhaps fungal membranes could be similarly affected. If that is true, exposed conidia membranes could experience decreased differential permeability. Perhaps ozone increases conidiophore respiration, resulting in prematurely formed and nonviable conidia.

Ozone exposure of conidia to 599 $\mu g/m^3$ (0.30 ppm) for two 6-hr periods totally inhibited their ability to infect detached leaves. Since the infection process involving *B. cinerea* is thought to be primarily enzymatic (14), ozone at certain doses may inhibit, directly or indirectly, enzyme activity of the fungus thus resulting in less maceration of cells and possibly decreased infection.

Since this study involved only ozone, the additive, antagonistic and (possibly) synergistic effects with other air pollutants are unknown. If ozone actually decreases pathogenicity, virulence, or sporulation of the gray mold fungus, at levels that are not injurious to plants, the presence of some ozone may be beneficial. Ozone may be adversely affecting the progress of certain plant diseases. During thermal inversions when ambient ozone levels often rise, sporulation of viable conidia could be decreased sufficiently to affect the epidemiology of the disease.

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