

## Ozone-Enhanced Leaching of Onion Leaves in Relation to Lesion Production by *Botrytis cinerea*

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### ABSTRACT

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Analysis of dew from leaves of onion plants exposed or not exposed to 0.18 ppm (353  $\mu\text{g}/\text{m}^3$ ) ozone ( $\text{O}_3$ ) for 4 hr indicated a substantial increase in leachate from  $\text{O}_3$ -injured cells. The electrical conductivity and carbohydrate concentration were greater in dew from exposed plants than in dew from unexposed plants. Conidia of *Botrytis cinerea* suspended in dew from the

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oldest nonsenescent leaf (leaf 1) of  $\text{O}_3$ -exposed plants induced significantly more lesions per square centimeter of leaf surface when used to inoculate leaf 1 of unexposed plants than did conidia suspended in dew from leaf 1 of unexposed plants.

Ozone ( $\text{O}_3$ ) can predispose potato (*Solanum tuberosum* L.), geranium (*Pelargonium hortorum* Bailey), broad bean (*Vicia faba* L.), and onion (*Allium cepa* L.) to increased foliar infection by *Botrytis cinerea* Pers. (4-6,9). Manning et al (5,6) observed that such predisposition of potato and geranium was associated with areas of  $\text{O}_3$ -induced foliar necrosis, and they hypothesized that these necrotic areas could serve as infection courts for the fungus. However, predisposition of broad bean foliage to *B. cinerea* as reported by Magdycz (4) was not associated with  $\text{O}_3$ -induced necrosis of the foliage. Rist and Lorbeer (9) have described a similar lack of association between  $\text{O}_3$ -induced necrotic areas on onion leaves and predisposition to *B. cinerea*. In cases where necrosis obviously was not required for predisposition, an alternative to the necrotic infection court mechanism of predisposition can be proposed. It is known that  $\text{O}_3$  can injure the plasmalemma of leaf cells, causing these membranes to leak (3), and that exogenously supplied nutrients can stimulate lesion production by *B. cinerea* on onion leaves (1). Therefore, it was postulated that lesion induction by *B. cinerea* on leaves of onion plants exposed to  $\text{O}_3$  was stimulated by nutrients that leaked out of  $\text{O}_3$ -injured leaf cells (9).

The purpose of the current study was to test this nutrient leakage hypothesis. It was assumed nutrients that leak through  $\text{O}_3$ -injured cell membranes would leach and accumulate in dew on the leaf surfaces. If this assumption is valid, the concentration of substances in dew from exposed plants would be higher than that in dew from unexposed plants. Therefore, dew collected from leaves of plants exposed to  $\text{O}_3$  was compared to dew collected from leaves of unexposed plants to determine if the dew from the exposed plants exhibited a higher electrical conductivity, contained a higher carbohydrate concentration, and stimulated greater lesion production by *B. cinerea* than dew from unexposed plants.

### MATERIALS AND METHODS

**Cultural conditions.** Plants of the  $\text{O}_3$ -sensitive onion cultivar Autumn Spice (9) were grown from seed in 10.12-cm-diameter

(4-in.) plastic pots filled with greenhouse soil and maintained in a growth chamber (16-hr photoperiod of 25,000 lux, from 0600 to 2200 hours, and a 24/18 C day/night regime). Plants were between 8 and 10 wk old when used. Four days prior to the initiation of an experiment, the plants were shaded 60% with shade cloth, which enhanced their sensitivity to  $\text{O}_3$  (*unpublished*).

**Ozone exposures.** All  $\text{O}_3$  exposures were conducted in a chamber that consisted of two compartments separated by a removable partition (9).  $\text{O}_3$  was introduced into one compartment while the other was kept free of  $\text{O}_3$ . Approximately 16 hr before initiation of an exposure period, the plants were placed in each compartment. The partition between the two compartments was removed to ensure that both sets of plants received uniform conditions of temperature and relative humidity prior to exposure, and was replaced immediately before the  $\text{O}_3$  exposure. Exposures generally were conducted from 1000 to 1400 hours. The chamber was maintained at 24/18 C (day/night) and 70% relative humidity (RH). The photoperiod (~16,000 lux) was initiated 4-5 hr prior to the exposure period and terminated at 2200 hours. Concentrations of  $\text{O}_3$  were monitored continuously with a Mast  $\text{O}_3$  meter routinely calibrated as described previously (9) to prevent the tendency for meter drift to become a factor in the experiments. The concentration was maintained for 4 hr at 0.18 ppm (353  $\mu\text{g}/\text{m}^3$ )  $\pm$  0.015 ppm (29.4  $\mu\text{g}/\text{m}^3$ ) for all experiments.

**Dew production and collection.** Approximately 3 hr after an exposure period, the exposed and unexposed plants were placed in a dew chamber (11). Dew that formed on the leaf surfaces during the subsequent 14 hr was collected by removing individual leaves, inserting each leaf tip first into a 25  $\times$  225-mm test tube, and manually rolling the leaf base between the thumb and forefinger. Each leaf and the inner wall of the test tube containing the leaf were rinsed by pipetting 2 ml of reagent grade water (Milli-Q water purification system, Millipore Corp., Bedford, MA) into the test tube and shaking the test tube briefly. The test tubes then were allowed to stand for 10 min while the dew and rinse water drained to the bottom of the tube. In this way, dew samples were obtained that consisted of 2 ml of water and ~0.05 ml of dew from a single leaf. Samples from exposed plants were compared with samples from unexposed plants by measuring their electrical conductivity and carbohydrate concentration and testing the effect of the dew on lesion production by *B. cinerea*. Each procedure was conducted twice.

**Electrical conductivity of dew samples.** The electrical conductivity of each dew sample was determined with a YSI model

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31 conductivity bridge (Yellow Springs Instrument Co., Inc., Yellow Springs, OH). The small volume of the individual samples prevented complete immersion of the conductivity cell. Therefore, the vent slots of the cell were sealed with Parafilm, the cell was inverted and clamped to a ring stand, and the individual samples were pipetted into the cell.

**Carbohydrate concentration of dew samples.** The total carbohydrate concentration of the dew samples was determined by using anthrone reagent as described by Morris (7) but modified slightly by reducing the volume of reagent mixed with each sample from 8 to 4 ml. With this modification, individual dew samples (each slightly >2 ml) could be assayed separately. A 2-ml aliquot of each dew sample was pipetted into a 15 × 125-mm test tube containing 4 ml of anthrone reagent. Each test tube was shaken for ~5 sec. The tubes then were maintained at room temperature for 15 min to allow for complete development of the blue reaction product. The absorption of light at 620 nm by each sample was determined with a Perkin-Elmer Hitachi 200 spectrophotometer (Coleman Instruments Division, Dale Brook, IL). Absorption was directly proportional to the concentration of carbohydrate in the samples. The concentrations of carbohydrate were expressed in glucose equivalents per milliliter as determined by using a standard absorption curve prepared with solutions of glucose at 0, 10, 20, 30, 40, and 50 µg/ml. Since the mean volume of the dew collected from a single leaf was only 0.05 ml and the dew was dispersed in 2.0 ml of water, the carbohydrate concentrations obtained using the standard curve were multiplied by a factor of 40 to correct for the 40-fold dilution of the dew in each sample. This resulted in an estimate of the actual concentration of carbohydrate in the dew prior to removal from the leaf surface. The mean volume (0.05 ml) of dew collected from a single leaf was estimated by consolidating the portion of each dew sample remaining in the 25 × 225-mm test tube after the 2-ml aliquot was removed for use in the anthrone reaction and by dividing the volume of the resulting consolidated sample by the total number of samples.

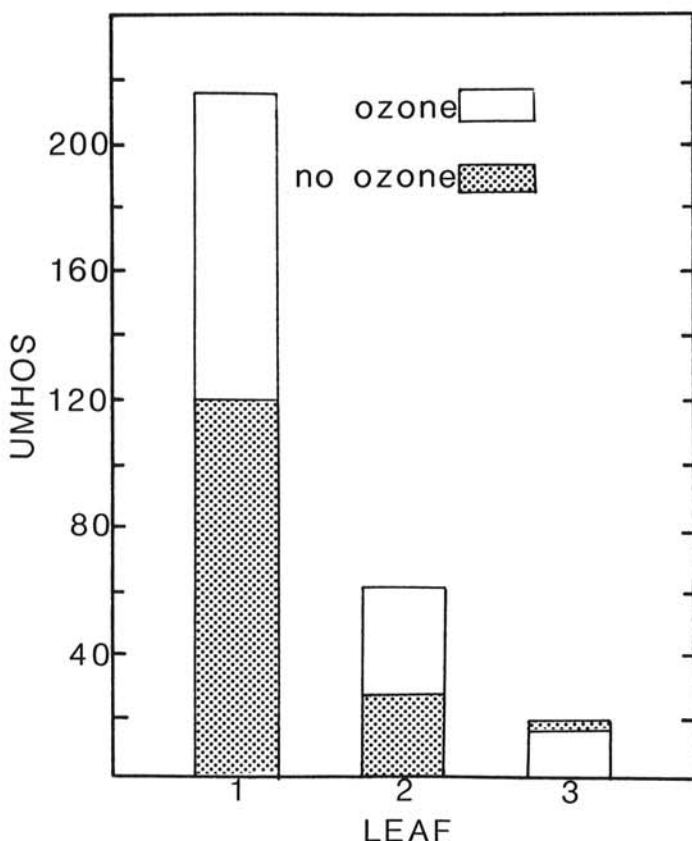


Fig. 1. Electrical conductivity of dew samples collected from the first, second, and third oldest nonsenescent leaves (leaves 1, 2, and 3, respectively) of onion plants exposed or not exposed to 0.18 ppm ozone for 4 hr. Statistical parameters are presented in the text.

**Inoculum preparation and inoculation.** One-day-old conidia of *B. cinerea* used to inoculate onion plants were produced by the following technique. Single conidia were transferred to plastic petri dishes (9-cm diameter) containing 15 ml of potato-dextrose agar (PDA, Difco Laboratories, Detroit, MI). The dishes were sealed with Parafilm and placed in an incubator at 21 C under fluorescent lights (Sylvania F20T12/CW; Sylvania Lighting Center, Danvers, MA) with a 14-hr photoperiod for 3–14 days. One day before inoculum was required, the conidia in a sporulating culture were removed with a sterile vacuum apparatus consisting of a 300-ml sidearm flask connected to a vacuum pump and were discarded. The culture dishes were resealed and returned to the incubator for 18–30 hr. The conidia produced during this period were collected with the vacuum apparatus and suspended in dew samples from leaves of O<sub>3</sub>-exposed or unexposed plants.

The isolate of *B. cinerea* utilized in this study was obtained from blighted onion florets collected in Orange County, NY. Virulence was maintained by frequent transfer of single conidia from sporulating onion leaves to fresh media.

In all inoculations, individual leaves of intact plants were sprayed with conidial suspension until their surfaces were covered with a fine mist. This was accomplished by using a Preval aerosol atomizer (Precision Valve Corp., Yonkers, NY).

**Effect of dew on lesion production.** Unexposed plants were inoculated with conidia suspended in dew collected from either exposed or unexposed plants. Four suspensions of 1-day-old conidia were prepared in the following manner. Two suspensions (50 ml each) were obtained by combining the individual dew samples collected from the oldest nonsenescent leaf (leaf 1) of 25 plants exposed to O<sub>3</sub> and by then combining the samples collected from the next oldest leaf (leaf 2) of the same plants. These consolidated samples were placed separately in the 300-ml sidearm flask attached to the vacuum pump and conidia from sporulating cultures of *B. cinerea* were drawn into the flask until the concentration approximated 3 × 10<sup>4</sup> conidia per milliliter. This process was repeated using samples from leaves 1 and 2 of 25 plants not exposed to O<sub>3</sub>. The suspensions prepared with dew from leaf 1 of exposed or unexposed plants were used to inoculate leaf 1 of other unexposed plants. The suspensions prepared with dew collected from leaf 2 of exposed or unexposed plants were used to inoculate leaf 2 of other unexposed plants. Plants from all inoculations were incubated simultaneously for 72 hr under a 16-hr photoperiod (17,000 lux) at 24 C/60% RH day, 18 C/100% RH night. The numbers of lesions that developed per square centimeter of leaf surface area were determined.

**Statistical procedures.** Analyses of variance and statistical contrasts between pertinent means were performed with an S.A.S. general linear model program (S.A.S. Institute, Cary, NC).

## RESULTS

**Symptomology.** O<sub>3</sub>-induced injury to onion leaves was ultimately expressed as necrotic stippling similar to that described previously (9). Lesions induced by *B. cinerea* were also similar to those previously described, and did not arise directly from O<sub>3</sub>-induced necrotic specks (9).

**Electrical conductivity of dew samples.** A set of 15 plants was exposed to 0.18 ppm O<sub>3</sub> for 4 hr and then placed in the dew chamber with a set of 15 unexposed control plants. Dew was collected from the first, second, and third oldest nonsenescent leaves (leaves 1, 2, and 3, respectively) of each plant 14–16 hr after initiation of the dew period. This experiment was conducted twice with similar results which were combined (Fig. 1). The mean conductivity of samples from leaves 1 and 2 of plants exposed to O<sub>3</sub> was significantly greater (*P* = 0.01) than that of the samples from corresponding leaves of unexposed plants (Fig. 1). No significant difference in conductivity was detected between samples from leaf 3 of exposed plants and samples from leaf 3 of unexposed plants.

**Carbohydrate concentration of dew.** A set of 15 plants was exposed to 0.18 ppm O<sub>3</sub> for 4 hr and then placed in the dew chamber with a similar set of unexposed plants. Dew was collected from leaves 1 and 2 of each plant ~18 hr after placement in the dew

TABLE 1. Average numbers of lesions per square centimeter of onion leaf surface inoculated with conidia of *Botrytis cinerea* suspended in dew from foliage of onion plants not exposed to ozone or exposed to 0.18 ppm ozone for 4 hr<sup>a,b,c</sup>

Leaf <sup>d</sup>	Dew from exposed plants	Dew from nonexposed plants	Significance level (P)
1	1.50	0.36	0.0290
2	0.23	0.15	0.5456

<sup>a</sup>Statistical contrasts were made using the "estimate" option on an S.A.S. general linear model program (S.A.S. Institute, Cary, NC 27511). Contrasts were conducted between the means from the second and third columns for each row of the table.

<sup>b</sup>The data are the mean numbers of lesions observed for 30 replicates (one plant per replicate).

<sup>c</sup>Conidia were suspended in dew after it was collected from onion leaves.

<sup>d</sup>Leaf 1 and 2 represent the first and second oldest nonsenescent leaf on the onion plants, respectively.

chamber. The total carbohydrate content of each sample then was determined. This experiment was conducted twice with similar results which were combined (Fig. 2). The carbohydrate content of dew from leaves 1 and 2 of exposed plants was significantly higher than that from the corresponding leaves of unexposed plants.

**Effect of dew on lesion production.** Significantly more lesions ( $P = 0.05$ ) developed per square centimeter of leaf surface on leaf 1 of plants inoculated with conidia suspended in dew from leaf 1 of  $O_3$ -exposed plants than on leaf 1 of plants inoculated with conidia suspended in dew from leaf 1 of unexposed plants (Table 1). No significant differences occurred between the numbers of lesions that developed on leaf 2 of plants inoculated with conidia suspended in dew from leaf 2 of  $O_3$ -exposed plants and the numbers of lesions that developed on leaf 2 of plants inoculated with conidia in dew from leaf 2 of unexposed plants. This experiment was conducted twice with similar results which were combined (Table 1).

## DISCUSSION

The nutrient leakage hypothesis has been proposed to explain  $O_3$ -induced predisposition of plant foliage to greater infection by *B. cinerea* (4,9,12). Until the present report, however, data that directly supported or challenged this hypothesis were lacking. Three tenets of this hypothesis as it relates to the predisposition of onion foliage to *B. cinerea* are: that  $O_3$  can cause onion leaf cells to leak, some of the leaking materials are nutrients, and these nutrients can stimulate infection by the pathogen. Data from the current study supported these three tenets.

Leakage of cellular solutions out of  $O_3$ -injured cells and into the intercellular spaces of plant leaf tissues results in the development of dark green or "water-soaked" areas in those tissues (3). Results of experiments (2,8) with bean (*Phaseolus vulgaris* L.), cotton (*Gossypium hirsutum* L. 'Acala SJ-1'), lemon (*Citrus limon* Burm.), and tobacco (*Nicotiana tabacum* L.) that involved the use of radioactively labeled substances show that such leakage results from alteration of the normal semipermeability of the plasmalemma (2,8). Leakage of electrolytes from plant cells injured by toxins also has been demonstrated (10). In the latter case, the electrical conductivity of suspensions of toxin-treated protoplasts was greater than that of a similar suspension of nontreated protoplasts (10). In the current study, ozone-induced leakage of electrolytes from onion leaves into dew formed on the leaves was demonstrated by the greater electrical conductivity of dew from leaves 1 and 2 of plants exposed to  $O_3$  in contrast to that of dew from the corresponding leaves of unexposed plants (Fig. 1). No difference in conductivity existed between dew from leaf 3 and exposed versus unexposed plants. This pattern of electrolyte leakage from leaves 1 and 2 but not leaf 3 of plants exposed to 0.18 ppm  $O_3$  for 4 hr parallels the pattern where leaves 1 and 2, but not leaf 3 were predisposed to *B. cinerea* by similar doses of  $O_3$  (9).

The increase of electrolytes in dew from leaves of exposed plants supports the assumption that  $O_3$  can cause onion leaf cells to leak but does not mandate that such leakage involves substances

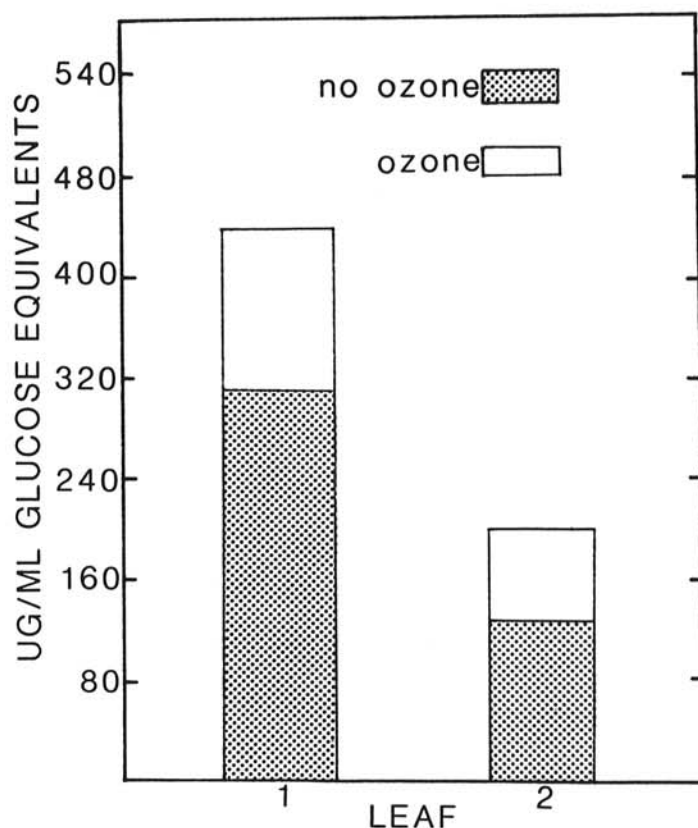


Fig. 2. Carbohydrate content of dew collected from the first and second oldest nonsenescent leaves of onion plants exposed or not exposed to 0.18 ppm ozone for 4 hr. Statistical parameters are presented in the text.

capable of stimulating lesion production by *B. cinerea*. However, the presence of a stimulating factor in dew from leaves of plants exposed to  $O_3$  was confirmed by the increase in lesion production by conidia suspended in dew from leaf 1 of exposed plants compared to lesion production by conidia suspended in dew from leaf 1 of unexposed plants (Table 1). A discrepancy exists, however, in that only dew from leaf 1 and not dew from leaf 2 of exposed plants stimulated lesion production, whereas leaves 1 and 2 of exposed plants were both predisposed to *B. cinerea* (9). The stimulating factor in dew from leaf 2 of exposed plants may have been diluted to a level below its effective concentration by the 2-ml water rinses used during collection of the individual dew samples. Although dew from leaf 1 was collected in the same fashion, it may not have been diluted below its effective concentration. However, it is possible that a different response could have occurred if undiluted dew had been used.

The stimulating factor in dew present on leaves of exposed plants was not identified in the current study, but possibly it was a nutrient that leaked out of  $O_3$ -injured leaf cells and which subsequently was leached to the leaf surface by dew. Alternatively,  $O_3$ -injured cells may release substances which themselves are injurious, and injury resulting from such substances could conceivably enhance infection by *B. cinerea*. However, the carbohydrate content of dew from leaves 1 and 2 of plants exposed to  $O_3$  was significantly greater than that of dew from the corresponding leaves of plants not exposed to  $O_3$ . Therefore, carbohydrate leaking from  $O_3$ -injured cells is a reasonable candidate for the stimulant. The nature of the carbohydrate present is of importance in this suspect/pathogen interaction. Yoder and Whalen (13) reported that a minimum of 100  $\mu\text{g/ml}$  glucose was required to stimulate germination of conidia of *B. cinerea* whereas Clark and Lorbeer (1) reported maximum germination in the presence of 30  $\mu\text{g}$  of sucrose per milliliter. The actual sugars present in dew were not determined in the present study. An increase in amino acid content of dew, which would have increased the electrical conductivity reading while

carbohydrates would not, also may have stimulated lesion production on plants exposed to O<sub>3</sub> either in conjunction with carbohydrate or alone.

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