

## The Role of Ethylene in Anthracnose of Cucumber, *Cucumis sativus*, Caused by *Colletotrichum lagenarium*

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### ABSTRACT

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Cucumber (*Cucumis sativus*) seedlings pretreated with ethylene gas had 21% more anthracnose lesions than did controls, following inoculation with *Colletotrichum lagenarium*. Ethylene fumigation 24 hr after inoculation increased lesion size by 36%. Silver thiosulfate (0.1 mM), an inhibitor of ethylene action, decreased lesion number and lesion size. Silver thiosulfate did not inhibit germination, growth, or the sporulation capacity of *C. lagenarium*. However, scanning electron microscopy showed an inhibition of *C. lagenarium* conidial germination when silver thiosulfate (1 mM) was applied to the leaf surface. Increased ethylene production

was observed after 3 days, followed by increased peroxidase activity at 6 days. Isoelectric focusing gels indicated that pI 4 and 6.5 isozymes were enhanced by the invading pathogen. Since ethylene failed to induce disease resistance and silver thiosulfate failed to induce susceptibility, it was concluded that ethylene-induced proteins, such as peroxidase, do not play a role in limiting disease development in the cucumber-*C. lagenarium* system. However, ethylene action appears necessary for lesion development and senescence.

*Additional keywords:* silver thiosulfate, peroxidase isozymes, chlorophyll degradation.

A number of reviews on the role of ethylene in disease resistance in plants have appeared (4,7). The subject is difficult to interpret, because ethylene may have a promotive, inhibitory, or no effect on disease development. Some examples of these observations follow. Promotion of disease development: lemons-*Diplodia natalensis* Pole-Evans (6); strawberry-*Botrytis cinerea* Pers. ex Fr. (14); Barley-*Helminthosporium sativum* Pammel, C. M. King & Bakke (11); and various flowering ornamentals-unspecified fungi (29). Inhibition of disease development: mung bean-*Rhizoctonia solani* Kühn (5); tangerines-*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (9); tomato-*Verticillium albo-atrum* Reinke & Berthier (23); and tobacco-tobacco mosaic virus (27). No effect on disease development: tomato-*Fusarium oxysporum* Schlecht. (16); potato-*Phytophthora infestans* (Mont.) de Bary (19); pepper-*Erwinia carotovora* (21); and celery-*Botrytis cinerea* (24). In a comprehensive study showing both increased and decreased disease development after ethephon treatment of various plants, Dehne and Spengler (12) reported that ethephon increased the resistance of cucumber to *Erysiphe cichoracearum* DC. Esquerre-Tugay et al (15) found that pretreating cantaloupe (*Cucumis melo* L.) seedlings with ethylene for 5 days caused a reduction in the disease development by *Colletotrichum lagenarium* (Pass.) Ellis & Halst. measured as an increase of glucosamine content of melon stem tissue. Ethylene treatment also increased the hydroxyproline-rich glycoprotein (HRGP) content of cantaloupe stems. Decreasing the HRGP content of cantaloupe tissue by incubating it with hydroxyproline resulted in increased infection. Stermer and Hammerschmidt (28) noted that a 50 C heat shock for 40 s resulted in increase in ethylene production, peroxidase activity, and HRGP in cucumber. Twelve hours after heat shock treatment, the seedlings also became resistant to *Cladosporium cucumerinum* Ellis & Arth. These observations have been used as evidence that ethylene-induced proteins such as peroxidase and HRGP may play a role in regulating disease development in cucurbits and other plants.

Abeles et al (1,2) reported that ethylene treatment of cucumber

seedlings results in the induction of a number of proteins, of which peroxidase isoenzymes form a major part. However, the physiological function of these age- and ethylene-induced peroxidases remains unknown. The experiments performed here were designed to evaluate the role of ethylene and ethylene-induced peroxidases in the susceptibility of cucumber seedlings to *C. lagenarium*. Two approaches were used in this evaluation. The first was to increase the levels of peroxidase and other ethylene-induced polypeptides by pretreating the leaves with ethylene and then challenging the leaves with *C. lagenarium*. If peroxidase or the consequences of its action provide the plant with resistance to the pathogen, then lesion development should be reduced on ethylene-treated plants. The second approach was to take advantage of the ability of silver thiosulfate to block ethylene action. Since silver thiosulfate was shown earlier to block the ability of ethylene to induce peroxidase (2), the pretreatment of leaves with this reagent should make them more susceptible to infection by *C. lagenarium* if ethylene-induced proteins play a role in disease resistance.

### MATERIALS AND METHODS

**Plant material.** Seedlings of *Cucumis sativus* L. 'Straight Eight' and 'Poinsett-76' were grown as described earlier (1). Plants were treated with ethylene (100  $\mu$ l/L) in Plexiglas containers (1) for 24 hr before inoculation with the pathogen. Silver thiosulfate was prepared by rapidly mixing equal volumes of 8 mM  $\text{Na}_2\text{S}_2\text{O}_3$  and 2 mM  $\text{AgNO}_3$  dissolved in water. Both 1 mM and 0.1 mM silver thiosulfate were mixed with 0.05% Triton X-100 (a surfactant) and sprayed until runoff on the leaf tissue 24 hr before the seedlings were inoculated with the pathogen. Water and sodium thiosulfate (4 mM) with 0.05% Triton X-100 were the controls.

**Fungal material.** A culture of *C. lagenarium* race 1 was obtained from Dr. R. Hammerschmidt and maintained on potato dextrose agar (PDA) or green bean agar. Suspensions of  $10^6$  spores per milliliter were prepared and fifteen 10  $\mu$ l drops applied to the upper surface of primary leaves of 3-wk-old plants. Inoculated plants were placed in plastic bags for 24 hr to increase humidity and promote infection development. Lesions were counted after

6 and 8 days. The primary leaf of each seedling was considered a sample. There were five samples per replication and four to five replications per treatment. Duncan's multiple range test was used to determine significant differences ( $P = 0.05$ ) in all experiments. Lesion size was measured on water and 0.1 mM of silver thiosulfate treatments 6 days after inoculation. Each leaf was considered a replication. There were 10 leaves per treatment and 3–12 lesions per leaf.

In order to determine whether ethylene increased lesion size, Straight 8 cucumber seedlings were inoculated with *C. lagenarium* and 24 hr later fumigated with ethylene. Lesions were counted and measured 6 days later. Air-treated seedlings were used as controls. There were six replications with 8–12 lesions per leaf. These experiments were repeated twice.

**Ethylene production.** Ethylene production from Straight 8 cucumber seedlings was measured by placing one seedling per replication (three replications per treatment) in a 250 ml Erlenmeyer flask sealed with a rubber stopper. The seedlings were incubated for 4 hr at room temperature. A sample of the gas phase was withdrawn through a syringe needle and injected into a gas chromatograph (Varian Walnut Creek Instrument Div., Walnut Creek, CA, Model 3700) equipped with an alumina column and flame ionization detector.

**Peroxidase activity.** Peroxidase was extracted from Straight 8 cucumber leaf tissue by homogenizing 1 leaf per sample with a mortar and pestle in 100 mg of sand, 50 mg of insoluble polyvinylpyrrolidone, and 1 ml of Carlson's extraction buffer (0.1 M Tris, 0.1 M KCl, 0.04 M 2-mercaptoethanol, and 0.1 M sucrose, pH 7.5). The activity of peroxidase was measured with 1 mM guaiacol and 1 mM hydrogen peroxide as substrates and by monitoring the change in absorbance at 470 nm (2). Protein was determined according to Bradford (8) with bovine serum albumin as the standard. Specific activity was determined as change in absorbance per minute per milligram of protein.

**Gel electrophoresis.** Isoelectric focusing (IEF) was performed with the Pharmacia Phastsystem and precast Pharmacia IEF 3–9 gels. Protein samples (4  $\mu$ g per sample) were applied to the anode and middle portion of the gel. The following procedure was used for separation: 1) a prerun consisting of 2,000 volts (V), 25 milliamps (mA), and 3.5 watts (W) for 75 accumulated volt hr (AVhr); 2) a loading run consisting of 200 V, 2.5 mA, and 3.5 W for 15 AVhr; and 3) a separation phase consisting of 2,000 V, 2.5 mA, and 3.5 W for 410 AVhr. Separation bed temperature was maintained at 15 C. All electrophoretic techniques conformed to those recommended by Pharmacia. Isoelectric points were determined with the Pharmacia Isoelectric focusing Calibration Kit (Pharmacia, Inc., Piscataway, NJ). Activity gels were stained for peroxidase isozymes with 10 mM guaiacol and 10 mM peroxide as substrates.

**Silver thiosulfate activity.** In order to confirm that silver thiosulfate blocks ethylene action in cucumber seedlings, seedlings were pretreated with 0.1 mM silver thiosulfate or water as previously described. After 24 hr, the seedlings were exposed to either ethylene (100  $\mu$ l/l) or air. The plants were kept in the chambers for 24 hr at ambient temperatures. At 0, 24, and 48 hr after removal from the fumigation chamber, chlorophyll content and peroxidase activity were determined as described previously (2).

**Fungicide activity of silver thiosulfate.** Tests were conducted to determine whether silver thiosulfate was fungicidal. The first test consisted of cutting 7-mm diameter wells in PDA plates with a cork borer. The wells were filled with 100  $\mu$ l of water containing 0.05% Triton X-100 and 0.1 mM, 0.5 mM, or 1 mM silver thiosulfate. A plug of agar with *C. lagenarium* mycelium was placed in the middle of the plate and allowed to grow toward the wells. The plates were incubated at room temperature and observed for inhibition zones.

A second test assayed the ability of silver thiosulfate to inhibit germination of *C. lagenarium* conidia. A 100  $\mu$ l aliquot of silver thiosulfate (0, 0.1, or 1 mM) was spread over PDA plates. After drying, 50 conidia of *C. lagenarium* were spread evenly on the plate, and resultant colonies were counted after 3 days.

The ability of silver thiosulfate to inhibit sporulation also was tested. Cucumber leaves with equal numbers of *C. lagenarium* lesions were sterilized in 10% NaOCl for 3 min and placed in sterile humidity chambers. The leaves were sprayed until runoff with either sterile water or filter-sterilized 1 mM silver thiosulfate. After three days, each leaf was placed in a test tube with 2 ml of water and vortexed for 60 sec. The number of spores released from the tissue was counted with a hemacytometer. Experiments were conducted three times with four replications per treatment.

**Scanning electron microscopy.** Inoculated leaf sections approximately 1 cm<sup>2</sup> were removed and fixed in formalin-acetic acid for 7 days at 4 C. Specimens then were brought to room temperature and dehydrated in a graded ethanol series. Following three exchanges with absolute ethanol, specimens were critical-point-dried and sputter coated with gold palladium. Prepared specimens were viewed with a Cambridge S-120 scanning electron microscope (Cambridge Industries, Deerfield, IL) at 10 kV. Duplicates were run on each treatment.

## RESULTS

The development of *C. lagenarium* lesions on ethylene-treated cucumber leaves 6 and 8 days after inoculation is summarized in Table 1. Pretreatment of cucumber seedlings with 100  $\mu$ l of ethylene/l for 24 hr did not limit lesion development; and in Poinsett-76, ethylene appeared to enhance lesion formation. In contrast, both 0.1 and 1.0 mM silver thiosulfate reduced the number of lesions formed in leaf tissues. The water and sodium thiosulfate controls of Poinsett-76 had fewer lesions than did Straight 8 at 6 days. However, the numbers of lesions on the ethylene-treated plants of the two cultivars were similar. The 0.1-mM silver thiosulfate treatment did not significantly reduce lesion number in Poinsett-76 when compared to the water and sodium thiosulfate controls; however, it was significantly less than the ethylene-treated plants. Lesion size diameter was less ( $P = 0.05$ ) in the 0.1-mM silver thiosulfate treatment (2.9 mm) than in the water control (3.8 mm). Cucumber seedlings fumigated with ethylene 24 hr after inoculation had larger lesions (5.5 mm) than the air controls (3.6 mm).

Rates of ethylene production from infected leaves are shown in Figure 1. Compared to water controls, *C. lagenarium* infection promoted stress ethylene production in cucumber leaves at 3 and 6 days after inoculation. Silver thiosulfate, which reduced lesion development (Table 1), also reduced stress ethylene production in tissue inoculated with *C. lagenarium* at 3 days. Significant differences were not detected between water and 0.1-mM silver thiosulfate treatments throughout the experiment.

The peroxidase activity of infected leaves was greater than control tissue at day 6 and 8 (Fig. 2). Silver thiosulfate, which reduced lesion development, also reduced peroxidase activity in these leaves. Peroxidase activity increased as the tissue naturally



Fig. 1. Stress ethylene production by Straight 8 cucumber seedlings at 1, 3, 6, and 8 days after treatment with 0.1 mM silver thiosulfate (STS) followed 24 hr later by inoculation with *Colletotrichum lagenarium* (CL) conidia ( $10^6$  conidia per milliliter). The error bar indicates the least significant difference ( $P = 0.05$ ) among treatments according to Duncan's multiple range test.

aged, as seen in the water controls. However, the peroxidase levels of the inoculated seedlings was threefold greater than the water controls at day 6. At 8 days after inoculation, peroxidase levels in the *C. lagenarium* treatment decreased compared to day 6. The 0.1-mM silver thiosulfate treatment had less peroxidase activity than the water controls, indicating that silver thiosulfate decreased the peroxidase activity that would normally increase due to aging. Peroxidase activity of the 0.1-mM silver thiosulfate treatment inoculated with *C. lagenarium* was not significantly different from the water controls throughout the experiment.

IEF gels indicated enhanced peroxidase isozymes at pI 4 and 6 (Fig. 3) in those plants inoculated with the pathogen alone. The pI 4 isozymes appear to account for the majority of increased total peroxidase activity observed in Figure 2. This particular IEF technique does not resolve the pI 9 isozyme. Plants treated with water or 0.1 mM silver thiosulfate did not have enhanced peroxidase isozymes.

Silver thiosulfate (0.1 mM) decreased chlorophyll degradation and peroxidase activity. Ethylene-treated plants at 48 hr had 30% less chlorophyll content than the ethylene-silver thiosulfate treatment (0.49 vs. 0.70  $\mu\text{g}$  of chlorophyll [663,645 nm] per gram of fresh weight). Although the ethylene-silver thiosulfate treatment was significantly higher in chlorophyll than the ethylene-water treatments, the ethylene-silver thiosulfate treatment was significantly lower (20%) than the water controls at 48 hr.

Silver thiosulfate did not inhibit germination, mycelial growth, or sporulation of *C. lagenarium*. Mycelial growth was not inhibited when silver thiosulfate (0.1, 0.5, 1.0 mM) was incorporated into PDA (data not shown). Inhibition of conidial germination

TABLE 1. Lesions developed by *Colletotrichum lagenarium* on cucumber leaves after treatment with either water, 4mM sodium thiosulfate (NaTS), 0.1 mM silver thiosulfate (STS), or 1 mM STS

Pretreatment	Lesions per leaf <sup>w</sup>			
	DAY 6 <sup>x</sup>		DAY 8	
	S-8 <sup>y</sup>	P-76 <sup>z</sup>	S-8	P-76
Water	13.65 A	11.45 B	14.62 A	12.07 CD
NaTS	14.60 A	10.90 BC	14.55 A	12.65 BC
Ethylene	14.45 A	13.85 A	14.45 A	13.75 AB
0.1 mM STS	4.82 D	9.75 C	6.23 E	11.00 D
1.0 mM STS	0.92 E	2.20 E	1.46 G	4.01 F

<sup>w</sup>Fifteen drops (10  $\mu\text{l}$ /drop) of inoculum ( $10^6$  spores per milliliter) were placed on each leaf.

<sup>x</sup>Significant interactions were observed between day 6 and 8. Therefore, differences between treatments were determined within days. Numbers followed by the same letter are not significantly different ( $P = 0.05$ ). LSD = 1.4 for day 6 and 1.12 for day 8.

<sup>y</sup>Straight-8 cultivar.

<sup>z</sup>Poinsett-76 cultivar.

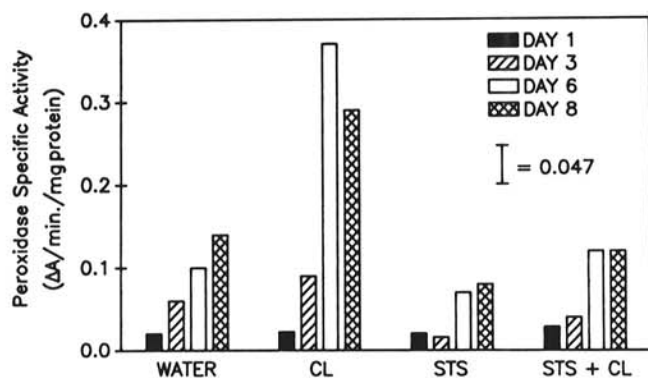


Fig. 2. Induction of total peroxidase specific activity (change in absorbance per min per mg of protein) after 1, 3, 6, and 8 days in Straight 8 cucumber leaves as affected by treatment with 0.1 mM silver thiosulfate (STS) followed by inoculation with *Colletotrichum lagenarium* (CL) conidia ( $10^6$  conidia per milliliter) 24 hr later. The error bar indicates the least significant difference ( $P = 0.05$ ) among treatments according to Duncan's multiple range test.

also was not observed, and sporulation capacity was not inhibited by silver thiosulfate. Scanning electron microscopy of the leaf surface showed no difference between the water and 0.1-mM silver thiosulfate treatments on germination and growth of *C. lagenarium*. However, the 1-mM silver thiosulfate treatment inhibited germination of *C. lagenarium* (Fig. 4). In experiments in which the inoculated leaf was surface sterilized (3 min in 10% NaOCl) after 24 hr, the pathogen was observed growing out of leaf tissue at both 0.1-mM and 1-mM silver thiosulfate levels.

## DISCUSSION

Henstrand and Handa (20) showed that less than 15% of mRNA species induced in wounded tissue are affected by inhibitors of ethylene and that HRGP mRNA species were not a result of ethylene stimulation. Their results suggest that most of the stress-induced changes in gene expression are not regulated by ethylene. Our results also support the hypothesis that ethylene-induced proteins, of which peroxidases represent a major portion, do not contribute to the resistance of cucumber to *C. lagenarium*. However, production of stress ethylene during infection appears to enhance apparent formation of lesions by promoting senescence. The resulting necrotic tissue in the lesion contributes to the stress ethylene produced during the disease process. As shown in Table 1, silver thiosulfate reduced the formation of lesions, and may have done so because it blocked the ability of ethylene to induce senescence. The results also suggest that when endogenous ethylene was blocked by silver thiosulfate, lesion size was decreased. In contrast, exogenous ethylene applied after inoculation was found to increase lesion size. Recently, ethylene production in rose was found to increase Grey mold (*B. cinerea*) severity (13). Sprays with silver thiosulfate, aminoxyacetic acid, and aminoethoxyvinylglycine decreased disease severity. Silver thiosulfate blocked ethylene action and, thereby, reduced disease severity.

Results of the in vitro test conducted in this study are similar to those of other reports (13) where silver thiosulfate did not affect the pathogen. Sporulation tests indicated that the pathogen could continue growing in the leaf tissue, sporulate, and germinate when reisolated on green bean agar or PDA. Further evidence that supports a nonfungicidal effect of silver thiosulfate on *C. lagenarium* can be observed in the 0.1-mM silver thiosulfate treatments between Straight 8 and Poinsett-76. Silver thiosulfate at 0.1 mM did not significantly reduce lesion number in Poinsett-76 leaves. If silver thiosulfate had fungicide activity, it should have reduced lesion number on both cultivars. These two cultivars respond to ethylene differently. Ethylene caused a more rapid

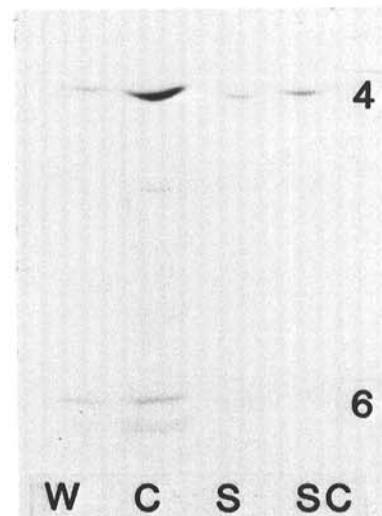


Fig. 3. Peroxidase isozymes of cucumber leaves treated with water (W), *Colletotrichum lagenarium* (C), silver thiosulfate (0.1 mM) (S), or silver thiosulfate with *C. lagenarium* (SC).



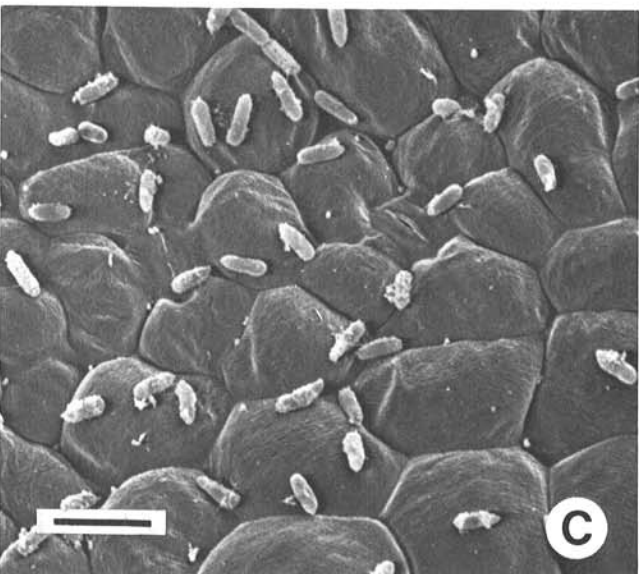
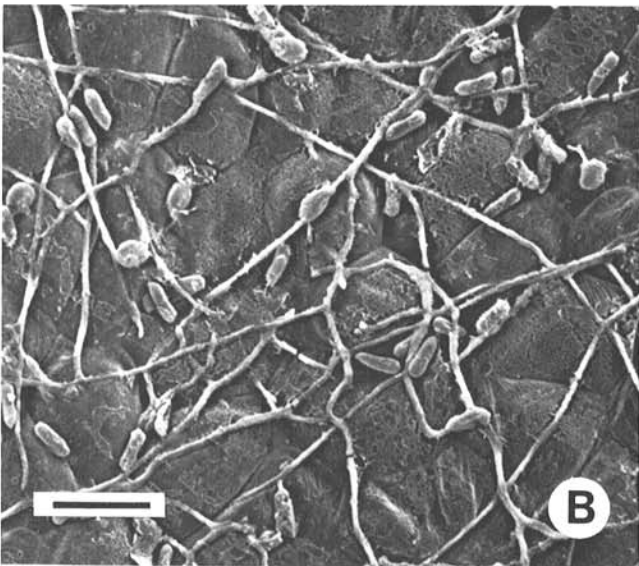
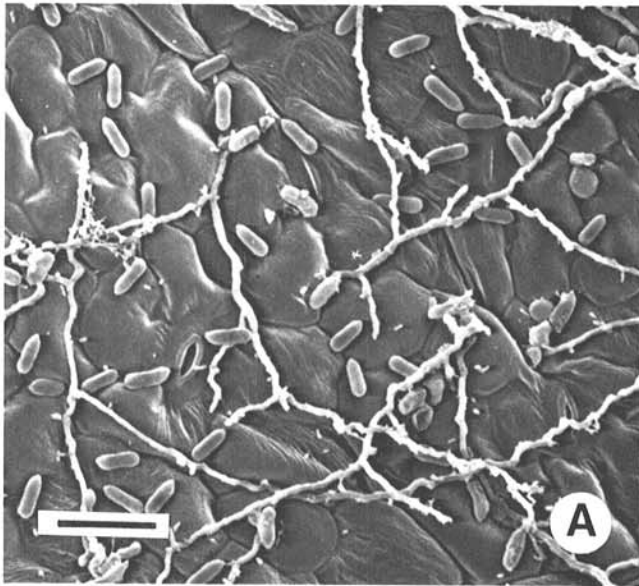


Fig. 4. Scanning electron micrographs of the leaf surfaces treated with water (A), 0.1 mM silver thiosulfate (B), or 1 mM silver thiosulfate (C) and 24 hr later inoculated with *Colletotrichum lagenarium*. The scale bars equal 25  $\mu$ m.

chlorosis of Straight 8, as compared with Poinsett-76, which appears delayed (unpublished observation). Since these cultivars respond to ethylene differently, differential responses to silver thiosulfate would be expected. Scanning electron microscopy showed similar germination between the water and 0.1-mM silver thiosulfate treatments (Fig. 4). However, silver thiosulfate at higher concentrations (1 mM) inhibits germination.

Ethylene production was promoted during the development of lesions by *C. lagenarium* (Fig. 1). Silver thiosulfate, which suppressed lesion formation, also reduced the onset and the amount of stress ethylene production. These results show that stress ethylene is produced in proportion to the amount of damaged tissue and that silver thiosulfate blocks the ability of ethylene to induce senescence.

Additional support for this hypothesis is shown in Figure 2. In this case, an increase in peroxidase followed the rise in ethylene production in *C. lagenarium* lesions. The enhanced acidic peroxidase isozymes were similar to the ethylene-stimulated isozymes reported previously (1) and correspond to the acidic peroxidases reported by Smith and Hammerschmidt (26). Since silver thiosulfate blocks ethylene action, it had the dual effect of blocking the induction of peroxidases and reducing senescence and the stress ethylene produced by that process. The ability of silver to block ethylene-induced senescence has been observed in many systems (2,3,17,22). *Cercospora arachidicola* S. Hori stimulated ethylene production which induces abscission in peanuts (22). Silver ( $\text{AgNO}_3$ ) was found to block abscission, but not ethylene production, in contrast to our results and those found in fruit (25). Silver thiosulfate also induced resistance in melon seedlings to *Fusarium oxysporum* Schlecht. f. sp. *melonis* Snyder and Hansen race 0 (10). In contrast, silver thiosulfate increased disease incidence to the soilborne pathogen *Pythium ultimum* Trow (18). These varied results indicate that ethylene action must be investigated for each individual species. A general role of ethylene in disease resistance cannot be assumed.

In summary, we conclude that ethylene-induced proteins, of which peroxidase is a major component, do not play a role in disease resistance in the cucumber-*C. lagenarium* system. It is still possible that they are important for other disease systems, such as systemic infections or root infections. However, our results do indicate that ethylene is important for lesion formation. When ethylene action was blocked by silver thiosulfate, lesion formation was delayed and reduced. The pathogen stimulates endogenous ethylene production, an essential step in lesion development.

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