Interactions of Ethylene with Citrus Stem-End Rot Caused by *Diplodia natalensis*

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ABSTRACT

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Ethylene treatment of citrus fruit to improve rind color (degreening) significantly enhances the incidence of stem-end rot caused by *Diplodia natalensis* and stimulates disease development. In an effort to identify the role of excessive exogenous ethylene in pathogenesis, tissue exposed to different ethylene and air atmospheres was taken from the base of citrus fruit adjacent to the abscission area at the point of fungal ingress and examined for the presence of fungal inhibitors and lignin. The major inhibitor, identified as scoparone (6,7-dimethoxycoumarin), accumulated most extensively in tissue taken from asymptomatic fruit receiving either high ethylene treatments (55 μl L^{-1}) followed by low inoculum levels, or low ethylene treatments (2 μl L^{-1}) followed by high inoculum levels. Accumulation of lignin, extracted from the same tissue and measured as lignothioglycolic acid, followed a response pattern similar to that observed for scoparone. Evidence for some role of scoparone and lignin in fruit resistance was indicated by the correlation between enhanced accumulation of these inhibitors and the lack of decay. However, other factors affected by high ethylene play a role in disease development since levels of inoculum that induced little disease at low ethylene were capable of causing decay at high ethylene. Sensitivity of *D. natalensis* to scoparone was reduced and its growth rate was increased when the fungus was grown in high-ethylene atmospheres. One role of such ethylene atmospheres in inducing extensive *Diplodia* stem-end rot could be that of stimulating more rapid growth of the fungus and invasion of tissue in spite of the presence of threshold inhibitory levels of scoparone.

Additional keywords: Postharvest pathology, scoparone, lignin

Treatment of Florida citrus fruit with ethylene is a commercial practice used to improve the rind color of early-season fruit. Upon degradation of chlorophyll by the ethylene, natural yellow, red, and orange pigments of the rind are exposed, to enhance cosmetic appeal of the fruit. The incidence of stem-end rot, caused by *Diplodia natalensis* Pole-Evans, is substantially greater on degreened (ethylene-treated) than on nondegreened fruit (5,17,23). Furthermore, ethylene concentrations in excess of the 5–10 μl L^{-1} needed to obtain a maximum rate of degreening enhance the disease to an even greater extent. Incidences of 50–65% stem-

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end rot may develop in naturally infected fruit degreened for 3–4 days with 50 μl L^{-1} of ethylene (5).

Stem-end rot originates from quiescent infections of *D. natalensis* established in necrotic tissue on the surface of the calyx and nectary (disk) of immature citrus fruit (12). Spores of the fungus produced by pycnidia formed on dead wood are dispersed in water to the immature fruit during rainfall in the summer months (11). At fruit maturity, *D. natalensis* can normally be isolated from the calyx and nectary of most Florida fruit. Entry of the fungus into the base of the fruit occurs during abscission of the calyx and nectary (12). Natural fissures that develop in the separation layer during abscission extend through necrotic tissue at the nectary surface where quiescent mycelia are established. Hyphae grow through these openings and quickly colonize starch-enriched cells of the deteriorating separation layer before growing into the base of the fruit, where they then rapidly invade the core and rind tissues. In the later stages of abscission, lignin is synthesized and accumulates in cells on the fruit side of the separation layer in both the pith and cortical regions (5,29). Lignification continues after abscission is complete, forming a protective layer at the fruit base. No protective layer is formed on the nectary surface, and once the calyx and nectary are removed, they quickly desiccate and die (29).

Stimulation of enhanced rates of abscission or germination of quiescent propagules of *D. natalensis* by high ethylene concentrations were excluded in a previous study as possible causes for increased disease susceptibility (5). Therefore, increased susceptibility appears to be related to the ease with which hyphae are able to penetrate cells at the surface of the fruit base. More rapid penetration at high ethylene was discernable as early as 2 days after the degreening process was initiated (5). Observations with histochemical techniques showed that cells of the fruit base contained phenolic compounds, ligninlike materials, and lignin. It was suggested that high ethylene concentrations may suppress the synthesis of fungitoxic compound(s) and/or the formation of physical barriers that confer resistance (5).

In studies reported herein, scoparone was identified as the most fungitoxic compound present in tissue of the fruit base. The studies measured changes in its accumulation and that of lignin in relation to ethylene concentration, inoculum density, and host susceptibility. Growth of *D. natalensis* was observed in response to scoparone and ethylene.

MATERIALS AND METHODS

Inoculum. An isolate of *D. natalensis* originally recovered from an infected Temple orange was grown on Difco potato dextrose agar at 30 C. Mycelium for inoculum was removed from the surface of 5- to 7-day-old cultures with a sterile cotton swab. Mycelium was weighed, macerated in sterile distilled water for 20–30 sec with a Sorvall omnimixer (Newtown, CT), and adjusted to concentration with additional water. Each fruit was inoculated by placing 75 μl of mycelial suspension in the stem cavity of the fruit base formed by the removal of the calyx and nectary. The inoculation site was allowed to dry about 1 h, and fruit were then stored at 30 C and 94–96% RH for 4 days, except when reported otherwise, while disease development was observed.

Fruit preparation. Valencia oranges (*Citrus sinensis* (L.) Osbeck) were harvested from local commercial groves. Fruit were clipped from the stem so that the intact calyx and nectary remained attached to the fruit at harvest. Fruit were randomized into the various treatments and placed in atmospheres of air or of ethylene in air at 30 C and 94–96% RH. Ethylene concentrations of 55 (high) and 2 (low) μl L^{-1} were maintained at ±10%, and levels were monitored with gas chromatography using flame ionization detection (7). Fruit were removed from the ethylene treatments after 2 days (except when reported otherwise), washed with water and a commercial-grade detergent using a pilot-scale citrus washer, and rinsed with potable water. The calyx and nectary were then removed from the fruit with a scalpel by forcing the nectary to separate from the fruit base along the separation layer of the abscission zone. To minimize natural inoculum, the stem cavity and surrounding area at the fruit base were sprayed with sterile distilled water and scrubbed using a cotton swab.

Effect of ethylene treatments on fruit susceptibility to stem-end rot was evaluated by randomly dividing a 250-fruit sample into five separate lots. In an attempt to precisely measure susceptibility, each lot of 50 fruit was inoculated with a different amount of inoculum. Amounts of inoculum ranging from 0.3 μg to 9.75 mg were selected, depending upon ethylene concentration and time of inoculation, to give a concentration that could cause 50% of the individual fruit to become infected (EC_{50}).

Tissue removal and extraction for inhibitors. Tissue was removed from fruit by inserting a cork borer (4–6 mm diameter) through the cells of the separation layer and partially into the fruit core. The borer was withdrawn, and the base of the fruit was then thinly sliced and removed. The central core of fruit base tissue, 4–6 mm in diameter and of about 2 mm thickness, was stored at −22 C until extraction. Tissue for each sample was collected from 30–50 fruit following the 2-day ethylene treatment and each day thereafter for 4 days.

For extraction, the tissue was thawed, infiltrated with 40% ethanol or ethyl acetate under vacuum for about 35 min, and shaken with 35–50 ml of additional solvent on a wrist action shaker for about 4 hr (19). The extract was filtered with Whatman No. 42 filter paper to remove debris and was concentrated to 10–15 ml by vacuum evaporation at 40 C. The extract was evaporated to dryness with nitrogen gas at room temperature, and the remaining residue was redissolved in 2 ml of equal-volume ethyl acetate/methanol. The sample was then filtered through a 0.45-μm filter and stored at 4 C in the dark until analysis.

Inhibitor and lignin analysis. Identification and quantitation of inhibitory compounds was conducted with a high-performance liquid chromatography (HPLC) system comprised of a Waters 600 E system controller (Milford, MA), and Shimadzu (Columbia, MD) model SIL-6A autoinjector, model C-R3A integrator, and model RF-535 fluorescence detector (ex 340 nm/em 425 nm). Spectral acquisition was performed with a Waters 990+ photodiode array detection system. The analytical column was a Zorbax RX (Chadds Ford, PA) (4.6 mm × 25 cm) with a guard column. The injection volume was 10 μl, and the flow rate was 1.0 ml/min. Analysis was performed by binary gradient elution using 0.1% acetic acid in acetonitrile (solvent A) and 0.1% acetic acid in water (solvent B). The initial mobile phase was composed of 10% A to a final composition of 50% A over a period of 65 min at a flow rate of 1.0 ml min^{-1}. Three identified coumarins, umbelliferone, scopoletin, and scoparone, were eluted at 25.4 ± 0.22, 25.8 ± 0.19, and 32.2 ± 0.14 min, respectively. These constituents and their on-line spectra obtained by photodiode array detection were compared to known standards for identification; each sample was analyzed in triplicate.

Tissue extracted with ethyl acetate was prepared into an acetone powder to measure lignothioglycolic acid (LTGA), using the thioglycolic acid method (18,28). A 50-mg subsample was analyzed, and concentrations of lignin were determined in triplicate using a standard curve derived with lignin extracted from orange rind (10).

Bioassay. Thin-layer chromatography (TLC) was used to bioassay for inhibitory compounds and for identification by comparison to known standards. Extracts were spotted on silica gel plates (250 μm thick) and developed in a solvent system of ethyl acetate and chloroform (60:40, v/v). The plates were dried after development to remove the solvents by exposing plates to 54 C forced air, 70 C in a drying oven, and ambient air in successive 10-min intervals. Mycelium of *D. natalensis* was collected from cultures, and 0.31 g was macerated in 50 ml of sterile distilled water as described previously. Mycelium suspension-media was prepared by mixing 37.5 ml of the mycelium suspension with autoclaved and cooled (40 C) 1.5% (w/v) Difco potato dextrose agar (24). Bioassay plates were sprayed with sterile surfactant solution (0.04% v/v Triton X-100) to wet the silica gel and minimize the thickness of the layer of agar required for bioassay. Excess water was removed with 54 C forced air, and mycelium suspension-media was sprayed on the plate to a glossy finish.

The plates were incubated at 30 C and 100% RH for about 18 h and treated with activated charcoal to contrast the areas of inhibition with those of growth (21). Silica gel in areas of inhibition was removed and extracted with ethyl acetate. The extract was concentrated and filtered, and the identity of the inhibitor was identified using TLC and HPLC.

Effect of ethylene and scoparone on the growth of *Diplodia natalensis*. Cultures of *D. natalensis* were grown for 1 day at 30 C in petri dishes (100 × 15 mm) containing 12 ml of Difco potato dextrose agar. A disk (4 mm diameter) of inoculum was aseptically removed from the culture and added to 50 ml of sterile orange juice medium in a cotton-stoppered 250-ml Erlenmeyer flask. The medium was prepared with a single source of concentrated orange juice as previously described (8), except that juice was clarified by centrifugation and filtration and used at twice the concentration. In trials with scoparone, ethanol was added to the initial concentrated scoparone solution to enhance solubility before dilution to test concentrations. Final media solutions contained 1% (v/v) ethanol. Growth at each scoparone concentration was measured after 3 days in six replicated flasks. Growth of *D. natalensis* in air or ethylene was evaluated after 1, 2, and 3 days using 10 replicated flasks for each day.

Flasks of the cultures were placed on a reciprocating water bath shaker at 29.3 ± 0.2 C. The shaker was positioned in a cabinet, and the air or ethylene atmospheres were maintained as described previously. The mycelium was harvested on dried, preweighed No. 41 Whatman filter paper with a Buchner funnel, washed twice with distilled water, and dried for 6 days at 60 C to constant weight.

Analysis of data. Data on the accumulation of scoparone and LTGA in tissue with time were transformed with logarithms to improve linearity before analysis with linear regression (26). Slopes of the regression lines were compared using described methods (22). The EC_{50} values for inoculum or scoparone levels were determined by the linear relationship between probit of percentage infection or growth inhibition and the logarithm of inoculum or scoparone concentration, respectively (1).

RESULTS

Fruit susceptibility. Susceptibility of oranges to *D. natalensis* was altered extensively by ethylene (Table 1). Fruit treated for 2 days with low ethylene (2 $\mu l\ L^{-1}$) required 16.0 mg ml^{-1} of inoculum to cause 50% infection. Only 0.03 mg ml^{-1} of inoculum was required for a comparable level of infection when fruit were treated for a similar period of time with high ethylene (55 $\mu l\ L^{-1}$). Fruit developed resistance to infection with time after treatment with either level of ethylene. Fruit inoculated 5 days after ethylene treatment were more resistant than fruit inoculated only after 2 days. Irrespective of inoculation time, however, fruit receiving the higher ethylene rate were always more susceptible to *D. natalensis* than fruit receiving the low rate. Resistance of fruit at high ethylene was less after 4 days of continuous treatment than after 2 days, but after 7 days of continuous high ethylene, it was greater than at either of the two shorter exposure periods.

TABLE 1. Effective concentration (EC_{50})[a] levels of inoculum of *Diplodia natalensis* required for infection of ethylene-treated Valencia oranges

Ethylene ($\mu l\ L^{-1}$)	Exposure before inoculation, days		Inoculum EC_{50} (mg ml^{-1})[a]
	Ethylene	Air	
2	2	0	16.0
	2	2	98.0
	2	5	130.0
55	2	0	0.030
	2	2	0.220
	2	5	9.28
	4	0	0.004
	7	0	0.414

[a]Quantity of mycelium required to cause 50% of the inoculated fruit to decay within 10 days after inoculation and storage at 30 C and 96% RH.

TABLE 2. Scoparone extracted from Valencia orange tissue after 2 days of ethylene treatment and inoculation with *Diplodia natalensis* and during the following 4 days of storage in air

Treatments: Ethylene ($\mu l\ L^{-1}$)	Treatments: Inoculum (mg ml^{-1})	Scoparone ($\mu g\ g^{-1}$ fwt) at time (days): 0	1	2	3	4	Symptomatic fruit at day 4: Percent	r[a]
0	...[b]	0.5	2.7	2.7	13.7	18.9	0	0.97**
2	...	2.2	0.9	3.3	6.5	12.8	0	0.89*
	0.084	2.2	2.8	15.5	71.1	57.2	0	0.94*
	30.000	2.2	48.8	291.2	595.5	1,264.1	68[c]	0.99**
55	...	2.6	1.4	71.1	279.8	1,070.3	24[c]	0.92*
	0.064	2.6	2.3	278.0	1,265.6	2,313.6	66[c]	0.93*
	3.000	0.0	17.2	229.2	348.7	276.1	100[d]	0.86[NS]

[a]Correlation coefficient between time and scoparone concentration. ** $P = 0.01$; * $P = 0.05$; NS = not significant.
[b]Fruit were not inoculated.
[c]Tissue extracted at day 4 came from asymptomatic fruit.
[d]Tissue extracted at day 4 came from symptomatic fruit.

TABLE 3. Lignothioglycolic acid (LTGA) extracted from Valencia orange tissue after 2 days of ethylene treatment and inoculation with *Diplodia natalensis* and during the following 4 days of storage in air

Treatments: Ethylene ($\mu l\ L^{-1}$)	Treatments: Inoculum (mg ml^{-1})	LTGA (mg g^{-1} fwt) at time (days): 0	1	2	3	4	Symptomatic fruit at day 4: Percent	r[a]
0	...[b]	4.2	5.7	6.7	8.9	10.0	0	0.99**
2	...	4.5	5.0	7.6	9.7	10.5	0	0.97**
	0.084	4.5	6.1	9.0	11.5	14.0	0	0.99**
	30.000	4.5	5.7	11.9	12.4	17.7	68[c]	0.97**
55	...	5.5	7.8	10.5	11.8	15.7	24[c]	0.99**
	0.064	5.5	8.7	12.7	17.6	22.0	66[c]	0.99**
	3.000	6.2	9.0	11.7	15.1	11.4	100[d]	0.89*

[a]Correlation coefficient between time and LTGA concentration. ** $P = 0.01$; * $P = 0.05$.
[b]Fruit were not inoculated.
[c]Tissue extracted at day 4 came from asymptomatic fruit.
[d]Tissue extracted at day 4 came from symptomatic fruit.

The level of resistance, however, did not nearly approach that observed in fruit receiving any of the low-ethylene treatments.

Fungal inhibitors. Concentrated extracts from tissue were bioassayed on TLC plates to detect the presence of inhibitors of *D. natalensis*. Inhibitors were most evident in extracts taken from tissue removed at day 4 from inoculated asymptomatic fruit treated with high ethylene. The major inhibitor was discerned at an *Rf* value of 0.65 and identified as scoparone (6,7-dimethoxycoumarin) after comparison with authentic scoparone in TLC and HPLC analyses. Small quantities of umbelliferone (7-hydroxycoumarin) and scopoletin (6-methoxy-7-hydroxycoumarin) were also detected, but these compounds were not associated with inhibition of *D. natalensis* in the TLC bioassays.

Quantification of scoparone and lignin. Quantities of scoparone and lignin recovered from tissue of the base of the fruit at the end of the 2-day ethylene treatment and during the following 4 days are shown in Tables 2 and 3, respectively. Fruit used in these tests were harvested during March 19 to April 1, 1991, from the same group of trees. Inoculum was adjusted to low and high levels within each ethylene concentration in an attempt to induce noninvasive or invasive responses in the tissue at the time of sampling. Diseased and healthy fruit could be discerned only on the last sampling at day 4, when symptoms were expressed.

Scoparone content of the tissue increased with time, except in fruit treated with high ethylene and inoculum levels, which all succumbed to infection (Table 2). Most (74–98%) of the variation in scoparone content in the various treatments could be ascribed to the effect of time. In slope comparison tests, rates of scoparone accumulation in control, low-ethylene, and low ethylene with low inoculum-treated fruit did not differ significantly. Differences ($P = 0.0433$) in accumulation rates did occur between noninoculated low- and high-ethylene-treated fruit, but the effect of high ethylene may have included some effect on the fungus due to the activity of natural inoculum, which caused infection in 24% of the fruit. The greatest quantities of scoparone were recovered at day 4 from asymptomatic tissue of fruit receiving either low ethylene and high inoculum or high ethylene and low inoculum. These same treatments also contained the greatest quantities of scoparone at 2 days following inoculation. Accumulation of scoparone in tissue of high-ethylene-treated fruit was significantly less ($P = 0.0237$) at high then at low inoculum levels. At the high inoculum level, scoparone accumulated with time until day 4, when a substantial decrease in concentration was observed (Table 2).

Accumulations of LTGA increased with time (Table 3) similar to accumulations of scoparone. Correlation coefficients between LTGA and time were significant in all treatments. The poorest relationship was observed with tissue recovered from fruit receiving high ethylene and inoculum, where 79% of the variation in LTGA accumulation was accounted for by time. Rate of accumulation did not vary between the control and the low-ethylene-treated fruit ($P = 0.2675$), but addition of low or high levels of inoculum to low-ethylene-treated fruit significantly enhanced LTGA accumulations ($P = 0.0061$ and $P = 0.0006$, respectively). Addition of a low rate of inoculum to fruit treated with high ethylene significantly enhanced LTGA levels ($P = 0.0001$) above that observed in fruit receiving only high ethylene. This response occurred even though some natural inoculum was present on the high-ethylene-treated fruit. High inoculum applied to fruit receiving high ethylene caused 100% infection and less ($P = 0.0001$) accumulation of LTGA than in similar ethylene-treated fruit inoculated with less inoculum. Levels of LTGA at day 0 were higher in fruit treated with high than with low ethylene.

Growth responses of *D. natalensis* to ethylene and scoparone. Growth of *D. natalensis* was significantly enhanced in atmospheres containing high ethylene (Table 4). Growth at the low ethylene concentration was less than in air for the first 2 days of culture but greater after 3 days. Maximum growth occurred at the high ethylene level and was significantly greater than growth at the other two atmospheres at both 2 and 3 days.

The concentration of scoparone causing 50% reduction in growth of *D. natalensis* in air was 116.4 $\mu g\ ml^{-1}$ (Fig. 1). When grown in an atmosphere containing 55 $\mu l\ L^{-1}$ of ethylene, *D. natalensis* was less sensitive to scoparone, and 142.0 $\mu g\ ml^{-1}$ of scoparone was required for a similar reduction in growth. Comparisons of growth at the various concentrations showed that differences were significant at the lower scoparone levels of 200 and 100 $\mu g\ ml^{-1}$ ($P = 0.0054$ and 0.0007, respectively).

TABLE 4. Growth of *Diplodia natalensis* at 29.3 C in atmospheres containing ethylene at 0, 2, or 55 $\mu l\ L^{-1}$

Ethylene ($\mu l\ L^{-1}$)	Amount of mycelium (mg dwt) at time (days)		
	1	2[a]	3[b]
0	68.3	400.4	508.3
2	40.5	378.9	547.6
55	55.3	463.0	612.4

[a] $P > F$ for 0 vs. 2, 0 vs. 55, and 2 vs. 55 were 0.0357, 0.0001, and 0.0001, respectively.
[b] $P > F$ for 0 vs. 2, 0 vs. 55, and 2 vs. 55 were 0.0002, 0.0001, and 0.0001, respectively.

DISCUSSION

Scoparone was the major inhibitory compound associated with Diplodia stem-end rot in studies reported herein, and it also has been identified as the phytoalexin responsible for resistance to citrus diseases caused by *Phytophthora citrophthora* (2,3,27), *Penicillium digitatum* (20), and *Diaporthe citri* (4). Levels of scoparone recovered from asymptomatic fruit in samples with some symptomatic fruit were equal to or greater than those reported for tissues infected by these other citrus pathogens. Accumulations of scoparone in citrus tissue appeared to occur in those instances where there was enough disease pressure to induce some decay. Scoparone concentrations increased even though samples collected before symptom expression at day 4 obviously contained some tissue that was destined to decay. At low ethylene and disease pressures where no decay developed, accumulations of scoparone were not substantial. Quantities that may be inhibitory to infection, particularly at 2 days after inoculation, were observed to accumulate in fruit treated with low ethylene and high inoculum or high ethylene and low inoculum (Table 2). Less scoparone accumulated when ethylene and inoculum concentrations were such as to cause 100% of the fruit to decay.

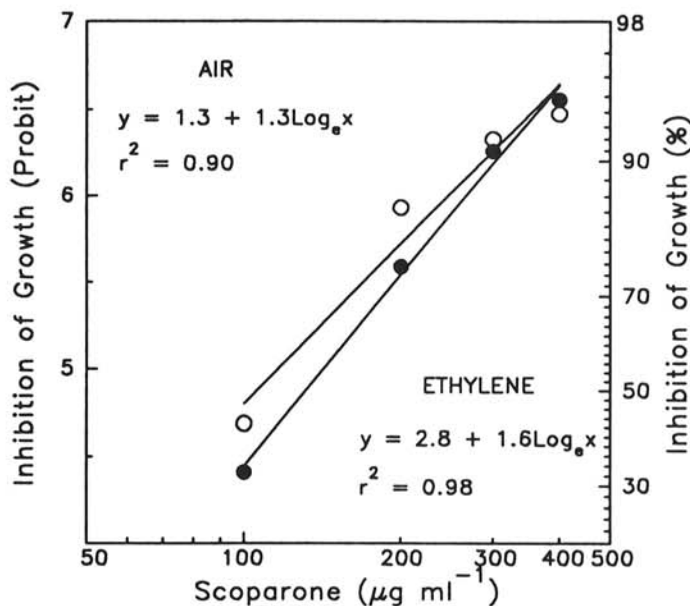

Fig. 1. Relationship between log concentration of scoparone and inhibition of growth (mycelial weight) of *Diplodia natalensis* after 3 days in atmospheres containing ethylene at levels of 0 (○) or 55 (●) $\mu l\ L^{-1}$.

Still, the exact role of scoparone in resistance is difficult to assess from the results of this study. Fruit treated at low ethylene with a noninvasive level of inoculum did not decay even in the relative absence of scoparone. In this situation, the accumulation of lignin could be a factor conferring resistance. Lignin accumulation may also have played a role in the increased resistance observed when inoculations were delayed following the ethylene treatment (Table 1).

The premise that high ethylene increases susceptibility of citrus fruit to *D. natalensis* by suppressing fungal inhibitors and/or physical barrier formation was not supported by our results. In fact, resistant tissue removed from high-ethylene-treated fruit consistently contained higher levels of scoparone and lignin than resistant tissue from low-ethylene-treated fruit. Interestingly though, accumulations of scoparone and lignin in high-ethylene-treated fruit were suppressed and the extent of disease was increased by much lower levels of inoculum than were required with fruit treated at low ethylene concentrations. Obviously, other factors associated with responses to high ethylene apparently stimulate even small amounts of inoculum to cause infection.

Growth of *D. natalensis* at low scoparone concentrations under high ethylene may have been impeded to a lesser extent than growth at low ethylene because of the reduced sensitivity of the fungus to scoparone. This would favor more extensive invasion and disruption of the synthesis of scoparone or lignin by the infected tissue. Accumulation of scoparone and lignin was disrupted in tissue taken only from decayed fruit (Tables 2 and 3). Stimulation of hyphal growth by the presence of high ethylene concentrations would also encourage colonization of the tissue before scoparone and lignin could reach inhibitory levels. Responses of *D. natalensis* to ethylene atmospheres have been studied previously (9,15).

Analysis of tissue measures only gross changes in inhibitory compounds and does not identify the timing and areas of localized accumulation. Localized distribution of phytoalexin compounds in the host tissue at the site of hyphal penetration has been shown to be significant to an incompatible host response (30). In citrus fruit tissue, scoparone and lignin may be highly localized in distribution, possibly in association with the vascular bundles, particularly the xylem, where metabolic pathways for coumarin and lignin synthesis are functional. Penetration by *D. natalensis* could occur through parenchyma cells spatially removed from the xylem, where such accumulations may be much lower.

Ethylene may affect cell wall integrity, causing the tissue to more easily succumb to degradative enzymes of the pathogen, as suggested in the response of tomato to *Verticillium* (13). Increased cell permeability resulting from the action of ethylene may make nutrients more readily available for fungal growth, and increases in respiratory activity may enhance senescence and predisposition of the tissue to fungal attack. Ethylene reportedly increases gray mold of rose and carnation flowers by enhancing senescence of the host tissue (14). Increased activity of polygalacturonase and cellulase is associated with the abscission process in citrus fruit (16,25). Activity of polygalacturonase was positively correlated with increasingly higher ethylene concentrations (25). Degradation of middle lamella of fruit base cells by polygalacturonase may favor intercellular penetration by *D. natalensis* (6). Increased activity of polygalacturonase due to the effect of high ethylene may also impede the deposition of lignin in the middle lamella region and therefore interfere with host resistance.

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