Influence of Ethylene on Increased Susceptibility of Oranges to *Diplodia natalensis*

CHARLES R. BARMORE, Associate Professor, University of Florida, IFAS, and G. ELDON BROWN, Research Scientist III, Scientific Research Department, Florida Department of Citrus, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred 33850

ABSTRACT

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Incidence of stem-end rot (SER) (caused by *Diplodia natalensis*) of Valencia oranges increased with exposure to increasing concentrations of ethylene from 0 to 50 μl/L of air. Although penetration of *D. natalensis* into the fruit depends on abscission of the floral calyx and disc (button), increases in SER at higher ethylene concentrations could not be related to early abscission. Germination of latent propagules of *D. natalensis* on the button was not enhanced by ethylene. Hyphal penetration from latent infections on the button into the area of abscission occurred more rapidly after the fruit was exposed to ethylene at 50 than at 1 or 10 μl/L. Resistance to infection was associated with the accumulation of red pigment in cells of the abscission area of the fruit within 2 days of inoculation. No differences in pectin demethylation or accumulation of starch and lipids within the separation layer were observed by histochemical methods after ethylene treatment or inoculation with *D. natalensis*. Histochemical techniques indicated that cells beneath the separation layer contained phenolics, ligninlike materials, and lignin that accumulated after abscission. Ethylene at 50 μl/L suppressed the staining intensity for ligninlike materials, whereas the presence of the fungus at low ethylene concentrations enhanced staining.

Additional key words: *Citrus sinensis*

Stem-end rot (SER) (caused by *Diplodia natalensis* P. Evans) is a serious postharvest decay of citrus fruit in Florida, especially after degreening with ethylene (13). Fruit harvested early in the season are often treated with ethylene at 1–10 μl/L of air to hasten degradation of chlorophyll in the rind and thus produce a desirable orange or yellow color (7,15). *D. natalensis* is often present at the stem end of mature, harvested fruit in necrotic tissue on the surface of the floral calyx and disc (button). The fungus grows from the necrotic tissue into the rind through natural openings that occur when the button abscises (4). Degreening encourages development of SER because ethylene initiates abscission and degreening is conducted at 30 C, the optimal temperature for growth of *D. natalensis*.

During commercial degreening, the ethylene concentration is not always controlled accurately. Concentrations in excess of those needed for rapid chlorophyll degradation (>10 μl/L) enhance the incidence of SER caused by *D. natalensis* (7,13). Ethylene has been reported to increase the susceptibility of plant tissue to other pathogens (2,5,6).

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The purpose of this study was to identify further the factors responsible for the enhanced incidence of SER caused by *D. natalensis* in citrus fruit treated with ethylene at concentrations in excess of those needed for chlorophyll degradation.

MATERIALS AND METHODS

Valencia and Pineapple oranges (*Citrus sinensis* (L.) Osbeck) were harvested by clipping and leaving the button attached. Experiments were initiated within 4 hr of harvest. Ethylene treatments were conducted with a continuous flow-through system (1) at 30 C and 94–96% relative humidity (RH). Ethylene concentrations were maintained within ±10% of the concentration desired.

Abscission vs. ethylene concentration. Pineapple oranges with stems about 5 cm long were treated with ethylene at 0, 1, 10, or 50 μl/L of air. The bonding force of the button to each of 20 fruits from each treatment was determined at the beginning of the experiment and at intervals of 12 or 24 hr with a Chatillon pull tester (9).

Stem-end rot vs. ethylene concentration. To evaluate the effect of ethylene on the incidence of SER, Valencia oranges were graded and randomized into lots of 75 fruits and treated with ethylene at 0, 1, 10, or 50 μl/L of air for 96 hr. To evaluate the rate of fungal penetration, Valencia oranges were treated with ethylene at 0, 10, or 50 μl/L for 48, 72, or 96 hr, then dipped in a thiabendazole solution (1 mg/ml of water) for 15 sec. Each treatment, consisting of three replicates of 55 fruits each, was stored and evaluated weekly for incidence of SER. Incidence of hyphal penetration was also determined after treating 100 Valencia oranges with ethylene at 1, 10, or 50 μl/L for 48 hr. Buttons in one-half of the fruit in each ethylene treatment were removed and the buttons in the remaining fruit were left intact; fruit were then placed in storage. Hyphal penetration of tissue beneath the button was studied using an additional series of 75 Valencia oranges for each of the same ethylene treatments. After ethylene treatment for 48 hr, buttons were removed and fruit inoculated by placing 1 ml of an aqueous mycelial suspension (70% transmittance at 600 nm) of *D. natalensis* in the stem cavity. Mycelia were removed from a 1-wk-old culture growing on Difco potato-dextrose agar. After treatment, fruit in all experiments were stored at 29–30 C and 94–96% RH (conditions favoring rapid development of SER), and decay was evaluated after 2 wk.

Isolation of *D. natalensis* from buttons. Valencia oranges were treated with ethylene at 1 or 50 μl/L of air for 48 hr. Buttons were removed, surface-sterilized with 1% sodium hypochlorite for 1 min, and plated on Difco potato-dextrose agar containing 50 mg of rose bengal per milliliter. After incubation at 30 C for 5 days, buttons from 40 fruits from each treatment were examined for mycelia of *D. natalensis*. The experiment was repeated twice.

Histological and histochemical studies. Fresh and fixed-embedded tissues of Valencia oranges were sectioned for viewing as described previously (3). Samples were prepared from tissue of the abscission zone before and after removal of the button from freshly harvested fruit. Buttons were loosened by treating the oranges with ethylene at 1 or 50 μl/L of air for 48 hr. Buttons were then removed, samples of the stem cavity taken for sectioning, and one-half of the remaining fruit inoculated with *D. natalensis*. All fruit were then stored at 29–30 C and 94–96% RH. Inoculated and uninoculated fruit were removed after 1, 2, and 3 days of storage and the tissue was examined histologically. Tests for phenolics, lignin and ligninlike materials (3), and chlorogenic acid (12) were done as reported previously. Other staining tests were used to detect demethylated pectin (14), starch (11), lipids, tannins, and

callose (10). Each staining procedure was evaluated on sections from three fruits.

RESULTS AND DISCUSSION

Incidence of SER in Valencia oranges treated with ethylene at 0, 1, 10, or 50 μl/L of air for 96 hr was 4, 24, 47, or 65%, respectively, after 4 wk of storage at 29–30 C (Fig. 1). These results supported the findings of Grierson and Newhall (7) and McCornack (13). Incidence of SER on fruit treated with ethylene at 10 or 50 μl/L increased rapidly during the second week of storage. More than 70% of the total SER developed during that period.

Because entry of *D. natalensis* depends on natural openings that occur at the button (4), faster abscission at 50 than at 10 μl of ethylene per liter of air might explain the increase in SER. However, no difference in the abscission rate, as measured by pull force, could be detected between the two treatments (Fig. 2). Abscission was complete within 48 hr. At 1 μl of ethylene per liter of air, the fruit bonding force did not approach 0 until after 72 hr. Development of SER in fruit treated at this low ethylene concentration could have been delayed because of the slower abscission rate. Even after 4 wk of storage, however, incidence of SER was much less in fruit treated with ethylene at 1 than at 10 or 50 μl/L (Fig. 1). Thus, abscission of the button does not ensure that decay will develop.

Penetration of rind by *D. natalensis* from the button was more rapid after ethylene treatment at 50 than at 10 or 1 μl/L of air. By removing the buttons from fruit after a 48-hr ethylene treatment, SER was prevented because latently infected buttons were separated from the fruit before hyphae penetrated the abscission zone. Removing the buttons from fruit treated with ethylene at 1 or 10 μl/L reduced SER by 90 and 91%, respectively, but only reduced SER by 65% in fruit exposed to ethylene at 50 μl/L (Table 1). More rapid penetration by hyphae of fruit exposed to an ethylene concentration of 50 μl/L was also demonstrated by treating the fruit with thiabendazole immediately after the ethylene treatment. Because thiabendazole is a relatively nonsystemic fungicide on citrus fruit (8), it controls surface mycelia but not those that have penetrated more deeply into the tissue. Incidence of SER in fruit treated with ethylene at 50 μl/L was three times greater than in fruit treated with 10 μl/L for 96 hr (Fig. 3).

Germination and growth of latent propagules of *D. natalensis* did not appear to be stimulated by high ethylene concentrations that encouraged high incidences of SER. Treatment of fruit with ethylene at 50 μl/L of air did not increase the frequency of isolation of *D. natalensis* from the buttons. In fact, recovery of *D. natalensis* from buttons of fruit treated with ethylene at 50 μl/L was less than that from buttons of fruit treated with 1 μl/L (72 vs. 88%).

Fruit were inoculated by adding an aqueous mycelial suspension of *D. natalensis* to the stem cavity of the fruit after promoting abscission of the button with a 48-hr ethylene treatment. Removal of the button minimized the influence of abscission, inoculum concentration, and rate of hyphal growth on the development of SER. Ethylene treatment still influenced SER development, however. Fruit treated with ethylene for 48 hr at 1, 10, or 50 μl/L and stored for 2 wk in air at 29–30 C had 27, 44, and 89% SER, respectively.

Fruit that resisted infection at any ethylene concentration developed red pigment in the surface cells of the abscission zone within 2 days of inoculation. Intensity of pigment increased during the following 3 days and was usually greater on nondecayed fruit treated with ethylene at 1 μl/L than on nondecayed fruit treated at 50 μl/L. Surface mycelial growth of *D. natalensis* was less extensive on fruit that developed pigment and resisted infection than on infected fruit. Pigment did not develop within the cavity of uninoculated fruit held at any ethylene concentration. The chemical nature of the red pigment and its role, if any, in resistance is not known.

Histochemical studies on tissue taken from the site of fungal penetration at the abscission zone indicated several chemical changes (Table 2), some of which were similar to those reported by Wilson and Hendershott (16). At abscission, the five to eight layers of cells constituting the separation layer of the abscission zone had accumulated starch and lipids but no tannins or callose. Pectin of cell walls within these five to eight layers was demethylated. Differences in the accumulation of starch and lipid or in demethylation of pectin were not observed in tissue removed after treating the fruit with ethylene for 48 hr or during the 3 days following inoculation with *D. natalensis*. As reported previously (16), phenolic and ligninlike materials were detected at abscission in five to 15 layers of cells beneath the separation layer of

Table 1. Stem-end rot (caused by *Diplodia natalensis*) of Valencia oranges treated with three concentrations of ethylene as affected by removal of the floral calyx and disc (button) before 4 wk of storage[a]

Ethylene (μl/L of air)	Incidence stem-end rot (%)	
	Button intact	Button removed
1	20	2
10	46	4
50	74	26

[a] Fruit were treated with ethylene for 48 hr at 30 C, then stored at 29–30 C and 94–96% relative humidity.

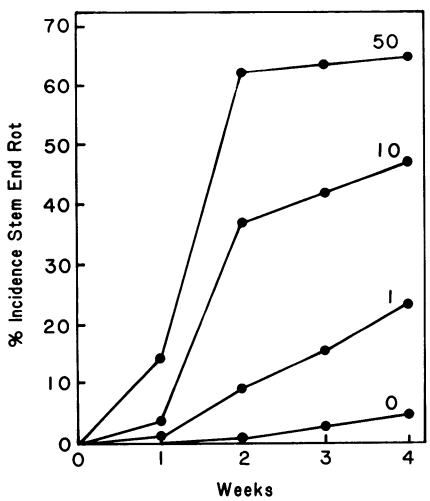

Fig. 1. Natural incidence of stem-end rot caused by *Diplodia natalensis* in Valencia oranges exposed to four concentrations of ethylene (0, 1, 10, or 50 μl/L of air) at 30 C for 4 days and stored for 4 wk at 29–30 C and 94–96% relative humidity.

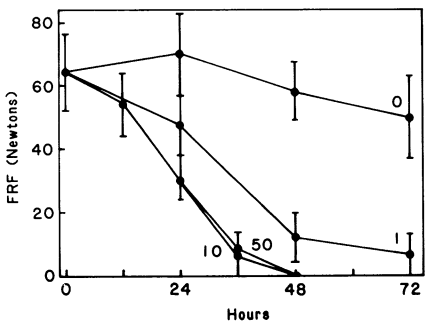

Fig. 2. Influence of ethylene at four concentrations (0, 1, 10, or 50 μl/L of air) on fruit abscission of Pineapple oranges treated at 30 C and 94–96% relative humidity. Abscission measured as force required to remove fruit from stem (fruit release force, in Newtons). Bars represent standard errors of the mean of 20 observations.

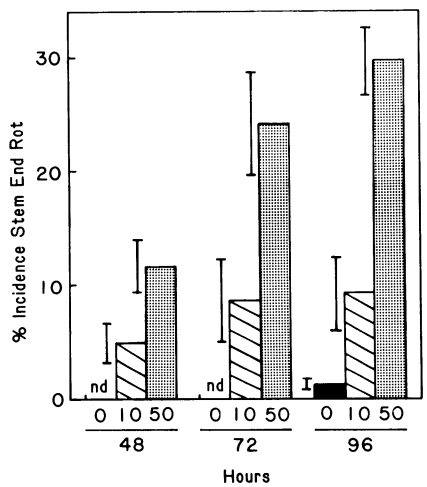

Fig. 3. Incidence of stem-end rot caused by *Diplodia natalensis* in Valencia oranges treated with thiabendazole (1 mg/ml of water) after exposure to ethylene (0, 10, or 50 μl/L of air) for 48, 72, or 96 hr and stored for 4 wk at 29–30 C and 94–96% relative humidity. Bars represent standard deviation of the mean; nd = no decay.

Table 2. Staining response within the abscission zone of Valencia oranges 3 days after treatment with ethylene at 1 or 50 μl/L of air in the presence or absence of *Diplodia natalensis*

		Staining intensity[a]			
		NI[b]		I	
Stain	Test	1	50	1	50
$FeCl_3$-$K_3Fe(CN)_6$	Phenolics	++	++	++	++
Fast red B salt	Phenolics	++	++	++	++
Phloroglucinol-HCl	Lignin	++	+	+++	+
Chlorine sulfite	Lignin	++	+	++	+
Toluidine blue	Lignin	++	+	+++	+
Safranin-fast green	Lignin	+	+	+	+
Crystal violet-erythrosin B	Lignin	+	+	+	+
Potassium hydroxide	Chlorogenic acid	++	+	++	+
Hydroxylamine-ferric chloride	Demethylated Pectin	++	++	++	++
Iodine-potassium iodide	Starch	++	++	++	++
Sudan-IV	Lipids	++	++	++	++
Ferric sulfate	Tannins	0	0	0	0
Aniline blue	Callose	0	0	0	0

[a] Rating scale based on intensity of staining: 0 = no staining, + = slight, ++ = moderate, and +++ = intense staining.
[b] NI = not inoculated and I = inoculated with an aqueous suspension of mycelium of *D. natalensis*.

the rind. The quantity of phenolics and ligninlike materials, as indicated by intensity of staining, increased during the 3 days following abscission. Changes in staining intensities in response to ethylene and the fungus, however, were not consistent among all staining procedures. Ethylene at 50 μl/L reduced the accumulation of chlorogenic acid detected with KOH and also reduced syringyl-containing, ligninlike materials detected with chlorine-sulfite when compared with tissue exposed to ethylene at 1 μl/L. In the presence of the fungus, staining with phloroglucinol-HCl and toluidine blue for cinnamaldehyde-containing, ligninlike materials was more intense in tissue from fruit treated with ethylene at 1 than at 50 μl/L. Staining of tissue for phenolics with $FeCl_3$-$K_3Fe(CN)_6$ and fast red B salt during the 3 days after abscission was not affected by the ethylene treatment or by inoculation with *D. natalensis*. Lignin detected with crystal violet-erythrosin B and safranin-fast green was limited to scattered areas within the three to four layers of cells immediately below the separation layer. Staining occurred 2 days after abscission and was not influenced by the fungus or ethylene.

We were not able to relate the enhanced infection of citrus fruit by *D. natalensis* at high ethylene treatments to a more rapid rate of abcission or to the stimulation of latent propagules. Rather, increased susceptibility appeared associated with changes in the cells at the abscission zone. Evidence for the effects of ethylene on some chemical changes within these tissues was indicated by the pigmentation response and the histological studies. Ethylene may be altering processes that affect fungal penetration. The suppression by ethylene of synthesis of a fungitoxic compound by the host tissue in response to fungal infection would partially explain why SER developed less extensively at the lower than at the higher ethylene concentration. Development of *Fusarium* in tomato plants and roots of Douglas-fir is increased by low concentrations of ethylene (5,6). Graham and Linderman (6) concluded that ethylene influenced the host response to infection rather than growth of the fungus directly. Beijersbergen and Bergman (2) suggested that ethylene may increase the susceptibility of the tissue to *Fusarium* by inhibiting the formation of a fungitoxic substance by the host tissue. The presence of phenolics and ligninlike materials indicates that these materials may have a role in resistance similar to that observed in the resistance of injured exocarp to *Penicillium digitatum* (3).

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