Effect of Ethanol on the Accumulation of Antifungal Compounds and Resistance of Tomato to *Fusarium oxysporum* f. sp. *lycopersici*

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The authors gratefully acknowledge the technical assistance of Helen Gehring.
Technical Paper 5906, Oregon Agricultural Experiment Station.
Accepted for publication 7 August 1984.

ABSTRACT

Danko, S. J., and Corden, M. E. 1984. Effect of ethanol on the accumulation of antifungal compounds and resistance of tomato to *Fusarium oxysporum* f. sp. *lycopersici*. Phytopathology 74:1475-1479.

The application of 1.0% ethanol to the roots of wilt resistant tomato plants (cultivar Jefferson) negated the effect of the *I*-gene for resistance to race 1 of *Fusarium oxysporum* f. sp. *lycopersici*. Vascular discoloration and foliar symptoms developed in ethanol-treated, inoculated plants just as in a wilt-susceptible cultivar (Bonny Best). The pathogen population in the xylem of the Jefferson plants increased rapidly when ethanol was applied, but remained low in inoculated, untreated plants. The antifungal activity of acetone extracts from the xylem of inoculated, ethanol-treated Jefferson plants remained low as it did in tomato plants of the same cultivar inoculated with race 2 of the pathogen, a race to which Jefferson plants are susceptible. By day 5 after inoculation, the fungitoxicity of xylem extracts was low in ethanol-treated plants inoculated with race 1 of the pathogen and in untreated plants inoculated with race 2, but was high in untreated, Jefferson plants inoculated with race 1. In vitro, ethanol neither stimulated nor acted as a substrate for ethylene biosynthesis by the pathogen, indicating that ethanol probably does not negate resistance through increased ethylene production by the pathogen. The fact that ethanol treatments that negate the resistance conferred by the *I*-gene also prevent the increase in antifungal compounds in the xylem supports the hypothesis that these antifungal compounds contribute to wilt disease resistance in tomato.

Additional key words: *Lycopersicon esculentum*, phytoalexin theory.

A range of chemical compounds including ethanol (12), ethylene (3), 2,4-dinitrophenol, sodium fluoride, sodium diethyldithiocarbamate, and thiourea (8) have been reported to induce susceptibility to disease in otherwise resistant plants. The mechanisms by which these compounds alter the response of plants to potential pathogens is not known, but the effect of these substances is generally assumed to be an alteration of the host metabolism rather than a direct effect on the pathogen (12). Scheffer and Walker (12) first reported that ethanol "breaks" single-gene resistance of tomato plants (*Lycopersicon esculentum* Mill.) inoculated with an incompatible race of *Fusarium oxysporum* Schlect. f. sp. *lycopersici* (Sacc.) Snyd. and Hans. Further experiments suggested that ethanol and other compounds may act as respiratory inhibitors, preventing the formation of a labile, oxidative phosphorylation-dependent substance that inhibits the advance of the fungus (8). Alternatively, ethanol may affect the permeability of host cell membranes, thereby altering host metabolism and host response to a potential pathogen (3,13).

While Scheffer and Walker provided evidence that ethanol induces the development of Fusarium wilt symptoms in tomato plants inoculated with an incompatible race of the pathogen (12), they did not indicate whether the pathogen population increased in the plants as in a normally compatible interaction. Conceivably, the pathogen could develop only to the extent that it normally develops in a resistant host, but could be induced by ethanol to produce metabolites such as ethylene that are known to influence disease development. In fact, ethanol has been reported to act as a precursor for ethylene biosynthesis by fungi (1). Ethylene can reproduce some of the symptoms observed in tomato plants infected by *Fusarium* and it has been implicated as a component of the normal compatible interaction (6). Stimulation of production of ethylene and other metabolites could account for the development of Fusarium wilt symptoms in ethanol-treated tomato plants even in the absence of extensive pathogen colonization of the host. A determination of whether the pathogen proliferates in incompatible hosts treated with ethanol is certainly an important aspect of understanding the mechanism of ethanol-induced susceptibility and, in a broader sense, the mechanism of host resistance in which antifungal compounds are thought to play a role (2).

The present study was undertaken to evaluate further the role of ethanol in induced susceptibility and the role of antifungal compounds in the resistance of tomato to Fusarium wilt.

MATERIALS AND METHODS

Jefferson (race 1 resistant, race 2 susceptible) and Bonny Best (race 1 and 2 susceptible) tomato plants were grown in washed silica sand and were watered daily with Hoagland's nutrient solution. Plants were grown in a controlled environment room at 30 C, a relative humidity of about 45%, and a 16-hr photoperiod with a light intensity of about 400 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Microconidia of *F. oxysporum* f. sp. *lycopersici* for plant inoculations were obtained by growing the pathogen in potato-dextrose broth in shake culture for 4 days. Cultures were filtered through Whatman #4 filter paper, the filtrate was centrifuged at 7,500 *g* for 10 min, the pellet was resuspended in sterile water, and the spore suspension was recentrifuged. The microconidial concentration was adjusted to 10^5 spores per milliliter with the aid of a hemacytometer.

Forty-day-old plants were inoculated by severing the tap root under water and placing the plants individually in 250-ml Erlenmeyer flasks containing 100 ml of the spore suspension. The plants were allowed to take up the inoculum for 4 hr in the growth room. Plants were inoculated with either race 1 spores in water, race 2 spores in water, or race 1 spores in 1.0% (v/v) ethanol. Uninoculated plants with tap roots severed and treated with 1.0% ethanol or with sterile water served as controls. Following

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inoculation, the plants were maintained in Hoagland's solution, except for the ethanol-treated plants which were transferred to flasks containing 1.0% ethanol for 4 hr each day. In early experiments we attempted to supply the plants with Hoagland's solution plus 1.0% ethanol for 24 hr each day as Scheffer and Walker (12) did, but found that the solution became heavily populated with bacteria in a few hours.

Pathogen populations in plants were estimated by excising 2.5-cm stem sections immediately below the cotyledonary node, surface sterilizing the sections with 1.3% sodium hypochlorite solution (25% Clorox, [v/v]), rinsing with sterile water, chopping the sections transversely into 35–40 pieces with a sterile razor blade, and then grinding the tissue in 10 ml of sterile distilled water for 1 min in a Sorval Omnimixer. The stem slurry was diluted and plated on potato-dextrose agar adjusted to pH 4.0 with lactic acid. The population of *Fusarium* was estimated by counting the number of colonies obtained from each of four replicates after 5 days at 22 C.

Daily after inoculation, xylem extracts were obtained from four 2.5-cm internode sections from the epicotyl of each of three plants per treatment by drawing 5.0 ml of absolute acetone through each by vacuum. Extracts were dried at 35 C under vacuum and were reconstituted with 0.2 ml of acetone per gram of stem section. The acetone extracts were evaluated for antifungal activity by spotting aliquots of 100 to 1,000 μl in deep-well slides, allowing the solvent to evaporate, and then adding 150 μl of a suspension of 10^5 microconidia of Fusarium per milliliter. The microconidial suspension was prepared in a medium containing NH_4NO_3, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 3.5 g; sucrose, 1.0 g; distilled water to 1.0 L; and adjusted to pH 4.5. The slide cultures were incubated at 30 C for either 24 or 40 hr, and then spore germination, colony growth, and sporulation were rated on a scale from 1 to 7: 1 = no conidial germination, 2 = conidial germination, 3 = small colonies formed, 4 = small to moderate-sized colonies with a trace of sporulation, 5 = moderate-sized colonies with trace to light sporulation, 6 = moderate to large colonies with a moderate amount of sporulation, and 7 = large colonies with heavy sporulation (14). In some experiments, antifungal activity was also estimated after 24 hr of growth by adding 10 μl of cotton blue in lactophenol to each bioassay slide, and counting the number of germinated spores in a 25-μl aliquot of the germination suspension. As discussed by Stromberg and Corden (14) the toxicant-to-fungus ratios in the bioassays approximated the ratios in inoculated plants.

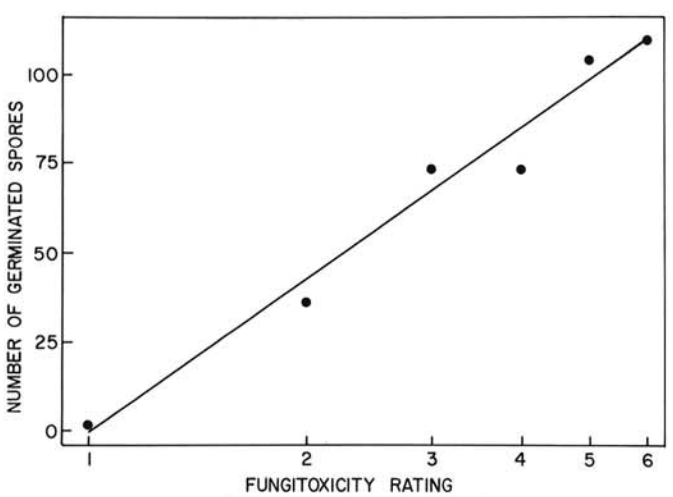

Fig. 1. The relationship between the spore germination assay and the slide bioassay. The number of germinated spores is linear with the log of the slide bioassay rating, and the linear least-squares-fitted curve has a correlation coefficient of 0.985. Slide bioassay ratings are a measure of fungitoxicity estimated by spore germination, colony development, and resporulation from about 1,500 microconidia of *Fusarium oxysporum* f. sp. *lycopersici*, race 1, incubated in 150 μl of nutrient medium containing tomato xylem extract. Fungitoxicity values are expressed by the following index: 1 = no conidial germination, 2 = conidial germination, 3 = small colonies formed, 4 = small to moderate-sized colonies with a trace of sporulation, 5 = moderate-sized colonies with trace to light sporulation, 6 = moderate to large colonies with a moderate amount of sporulation, and 7 = large colonies with heavy sporulation. Each point represents the average of 10–30 samples.

Disease development was evaluated by the severity of vascular and foliar symptoms according to Dimond et al (5). A vascular disease index was obtained by totaling the number of major vascular bundles discolored at each internode and dividing this number by the total number of bundles examined (ie, three per internode). Leaf grades were obtained by first classifying each leaf on a scale from 0 to 4: 0 = no disease symptoms; 1 = slight symptoms, including slight yellowing; 2 = moderate symptoms, including as much as 50% of the leaflet area yellow or flaccid, but no necrosis; 3 = severe symptoms, involving complete yellowing and/or flaccidity, but necrosis not complete; and 4 = leaf missing or completely nonfunctional. An average leaf grade was obtained for each plant, and the average of four replicate plants was used to evaluate treatments.

To evaluate ethylene production by the fungus in vitro, a medium was prepared that consisted of: glucose, 25 g; KNO_3, 2.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; K_2HPO_4, 0.31 g; KH_2PO_4, 0.94 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; H_2BO_3, 1.25 mg; $CaCl_2 \cdot 2H_2O$, 1.0 mg; $MnCl_2 \cdot 4H_2O$, 0.9 mg; $CuCl_2 \cdot 2H_2O$, 0.275 mg; MoO_3, 0.01 mg; and distilled water to 1 L. The initial pH was 6.1. The following amendments were made to the sterilized, cooled medium: 10 mM methionine, 10 mM methionine + 1.0% (v/v) ethanol, 1.0% ethanol, and no amendment. Methionine was sterilized by passing a 3.0% (w/v) solution of D-L methionine through a 0.4-μm filter. The media (20 ml in 125-ml cotton-stoppered flasks) were each inoculated with 4.6×10^5 microconidia of *F. oxysporum* f. sp. *lycopersici* race 1. Flasks were incubated at 30 C under constant light (400 $\mu E \cdot m^{-2} \cdot sec^{-1}$).

When ethylene measurements were made, the flasks were unplugged for 15 min in a laminar flow hood to allow exchange of the atmosphere in the flasks. The flasks were then stoppered with sterile serum caps and returned to the growth chamber. After 1 hr of incubation, a 1.0-ml sample of the atmosphere in each flask (three flasks per treatment) was collected and analyzed for ethylene content by gas chromatography. Chromatography was performed on a Hewlett-Packard 5830A gas chromatograph with a 1.8 m $\times$ 0.31 cm Poropak column. Injector temperature was 100 C; oven temperature, 50 C; and flame ionization detector temperature, 100 C. Ethylene production is reported as nanoliters of ethylene produced per milliliter of atmosphere per hour.

All experiments were repeated at least once.

RESULTS

To evaluate the validity of the slide bioassay based on spore germination, colony growth, and resporulation used in these studies, a comparison was made of the slide bioassay with a more quantitative assay of spore germination. Both assays were performed on a total of 96 tomato xylem extract samples. Because extensive hyphal growth in some samples after 40 hr of incubation precluded a measurement of the number of germinated spores, spore germination and slide bioassay rating were determined after 24, rather than 40, hr of incubation. No slide cultures reached the large-colony, heavy-resporulation stage (rating 7) after only 24 hr of incubation, and no ungerminated spores were observed in any slide cultures, possibly because of spore lysis.

The results (Fig. 1) demonstrate the relationship between the slide bioassay and the spore germination assay. The plot of number of germinated spores versus the log of the bioassay rating resulted in a straight line with a correlation coefficient of 0.985. Some extracts inhibited colony growth and resporulation but not germination. Others inhibited germination but allowed good growth of a few spores that managed to germinate. These differences may be due to differences in concentration and/or composition of the extracts. The slide bioassay could distinguish qualitative as well as quantitative differences in toxicity and required only one-tenth the time for evaluation as the spore germination method.

Fifteen days after inoculation with the pathogen or treatment with ethanol, disease symptoms (as evaluated by the vascular index and leaf grade) were almost nonexistent in Jefferson tomato plants (Table 1). However, when inoculated plants were treated with ethanol, severe disease symptoms developed. Breaking the single-gene resistance in cultivar Jefferson plants with ethanol verifies the earlier report (12) on the action of ethanol in this disease. The transfer of inoculated plants from Hoagland's solution to water for 4 hr each day had no significant effect on subsequent symptom development in these plants.

The population of race 1 of the pathogen in Jefferson tomato plants remained low for the first 5 days after inoculation (Fig. 2), but in susceptible Bonny Best tomato plants, the pathogen population began to increase on day 3 and continued to increase through day 5 when it was about 10 times the population on day 1. In Jefferson plants inoculated with race 1 of the pathogen and treated daily with 1.0% ethanol, the pathogen population increased on day 4 and by day 5 was about 10 times the day 1 population (Fig. 2). Ethanol treatment induced an increase in disease symptoms accompanied by an increase in the pathogen population.

Antifungal activity of xylem extracts from Jefferson plants was high on days 1 and 2 after inoculation, but generally decreased thereafter and remained low in uninoculated control plants, in plants inoculated with race 2 of the pathogen, and in plants inoculated with race 1 and treated with ethanol (Fig. 2). The plants inoculated with race 2 and the ethanol-treated plants inoculated with race 1 eventually developed severe disease symptoms. In the Jefferson plants inoculated with race 1, the fungitoxicity of the xylem extracts dropped initially but then increased to the original high level by day 5. This group of plants remained free of disease symptoms. Notably, day 3 was the first time when a difference in the antifungal activity of extracts from plants in the three treatment groups was expressed, and this was also the first day when a difference in pathogen populations was apparent. In intact plants or in wounded, uninoculated control plants treated with either 1.0% ethanol or water, the fungitoxicity of xylem extracts followed essentially the same pattern as for the compatible and ethanol-induced compatible interactions except on day 1. Actual fungitoxicity values varied from experiment to experiment; however, the comparisons between treatments for each experiment and the resulting trends were strictly reproducible.

TABLE 1. The influence of ethanol on wilt disease development in cultivar Jefferson tomato plants 15 days after inoculation with *Fusarium oxysporum* f. sp. *lycopersici* race 1

Inoculation[v]	Treatment[w]	Disease symptoms[x]	
		Vascular index[y]	Leaf grade[z]
Inoculated-S	none	0.8 a	2.7 a
Inoculated-R	1.0% ethanol	0.7 a	2.7 a
Inoculated-R	none	0.1 b	0.1 b
Uninoculated-R	1.0% ethanol	0.0 b	0.2 b
Uninoculated-R	none	0.0 b	0.2 b

[v] Inoculated plants were allowed to take up a suspension of 10^4 microconidia of *Fusarium* per milliliter for 4 hr. Uninoculated plants were allowed to take up sterile distilled water under the same conditions. R indicates resistant Jefferson plants; S indicates susceptible Bonny Best plants.

[w] Ethanol-treated plants were fed a 1.0% solution of ethanol through the roots for 4 hr each day.

[x] In each column, values followed by a common letter do not differ significantly ($P = 0.05$) according to Student's t-test.

[y] Vascular index is the ratio of the number of major vascular bundles discolored at all internodes to the total number of bundles examined. The index ranges from 0 for a healthy plant to 1 for a severely diseased plant. Each vascular index is the mean from four plants.

[z] Leaf grade is an estimate of foliar disease symptoms based on a rating scale in which: 0 = no disease symptoms; 1 = slight symptoms, including slight yellowing; 2 = moderate symptoms, including as much as 50% of the leaflet area yellowed or flaccid, but no necrosis; 3 = severe symptoms, involving complete yellowing and/or flaccidity, but necrosis not complete; and 4 = leaf missing or completely nonfunctional. Each leaf grade is based on the average of leaf grades from four plants.

Purification of the antifungal compounds in tomato xylem extract revealed the presence of at least four compounds that were highly toxic to *F. oxysporum* f. sp. *lycopersici* race 1. However, no α-tomatine or rishitin could be detected in these extracts. Full details of purification and characterization of these antifungal compounds will be reported elsewhere.

Ethylene production by *F. oxysporum* f. sp. *lycopersici* was not apparent until about 65–86 hr after inoculation of the culture media (Fig. 3), when the cultures were in log growth phase. After the cultures reached stationary phase (about 120 hr after inoculation), the rate of ethylene production decreased. Ethylene production occurred only in the presence of methionine. Ethanol neither stimulated ethylene production nor served as a precursor for ethylene synthesis. In fact, the cultures did not produce as much ethylene in the presence of 1.0% ethanol as in its absence. The lower production of ethylene in the presence of ethanol may be due, in part, to a slightly decreased growth of the fungus in the presence of ethanol (*unpublished*). In a rich growth medium, ethanol does not stimulate growth or ethylene production by *F. oxysporum* f. sp. *lycopersici* in spite of reports that dehydration of ethanol to ethylene is a major pathway of ethylene biosynthesis in fungi (1).

DISCUSSION

Because disease symptoms and a significant increase in the pathogen population are evident in tomato plants by day 5 after inoculation, the susceptibility of the host plants to the wilt disease

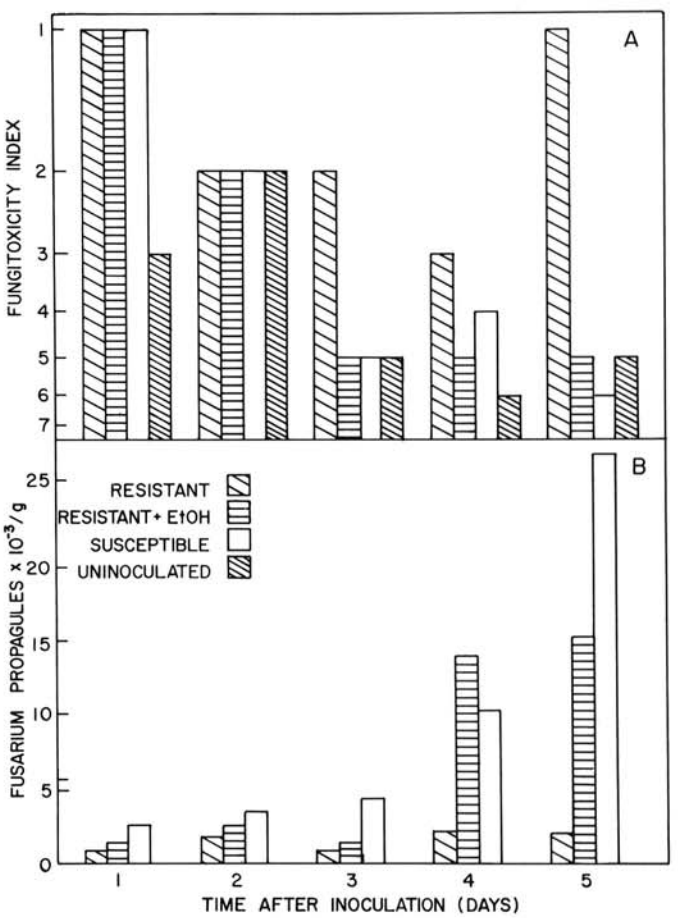

Fig. 2. A, Fungitoxicity of xylem extracts from stems of Jefferson tomato plants inoculated with race 1 (resistant), race 2 (susceptible), or race 1 with 1.0% EtOH treatments, for the first 5 days following inoculation with microconidia of the pathogen. Fungitoxicity was estimated by spore germination, colony development, and resporulation in the presence of 100 μl of tomato xylem extract as described in the legend to Fig. 1. **B,** The population of *Fusarium oxysporum* f. sp. *lycopersici* race 1 in stems of Bonny Best (Fusarium wilt susceptible), Jefferson (Fusarium wilt resistant), and Jefferson tomato plants treated with 1.0% ethanol (EtOH), for the first 5 days following inoculation with microconidia of the pathogen.

pathogen is clearly established by this time. Consequently, any resistance mechanism must also be set in motion by day 5 after inoculation. Thus, we concentrated our study on this early period of the host-pathogen interaction.

Ethanol negates the resistance to Fusarium wilt conferred by the *I*-gene, but whether this is through direct action on the host or on the pathogen is unknown. Ethanol can affect the permeability of cell membranes (13) and, at the level used in this investigation, could supply a carbon source for the pathogen. Scheffer and Walker (12) found that isopropanol and methanol had no effect on the resistance of tomato to Fusarium wilt. Thus, ethanol-induced susceptibility appears to be specific, and is not a general response to treatment with alcohols.

The effect of ethanol on resistance of tomato to Fusarium wilt could be interpreted as the repression of an active resistance response. Current theories on the mechanisms of resistance of tomato to Fusarium wilt involve a sequential, two-component process in which tyloses, gels, and gums initially wall off the pathogen in the roots and hypocotyls of resistant tomato plants, followed by the accumulation of antifungal compounds that reinforce the resistance process (11). Evidence for the involvement of tyloses, gels, and gums in resistance mechanisms of tomato to Fusarium wilt has been discussed by others (2,15).

Evidence for the involvement of antifungal compounds in resistance includes the fact that the level of antifungal compounds in the xylem of resistant tomato plants inoculated with *Fusarium* is consistently higher than in similarly inoculated susceptible plants (15). Even if these antifungal compounds are not responsible for resistance, their levels are correlated with resistance both in a natural and in a chemically modified situation. As such, these compounds may be associated with the resistance response either by directly inhibiting the pathogen or by modifying host metabolism. In addition, in the absence of repeated treatments of inoculated tomato plants with ethanol, the plants eventually recover and new growth appears to be free of disease symptoms (12). Similar recovery of diseased plants has also been reported for naturally compatible combinations of tomato and *F. oxysporum* f. sp. *lycopersici* (9). Moreover, preliminary results with isolated tomato stem sections indicate that in the absence of repeated ethanol treatments, the pathogen population drops to its original low levels after depletion of the ethanol supplied at the time of inoculation (M. E. Corden, *unpublished*). These results with isolated stem sections must be regarded with caution, however, since stem sections may behave differently than whole plants. These observations suggest, however, that some active defense mechanism may be involved in the resistance of tomato to *Fusarium*.

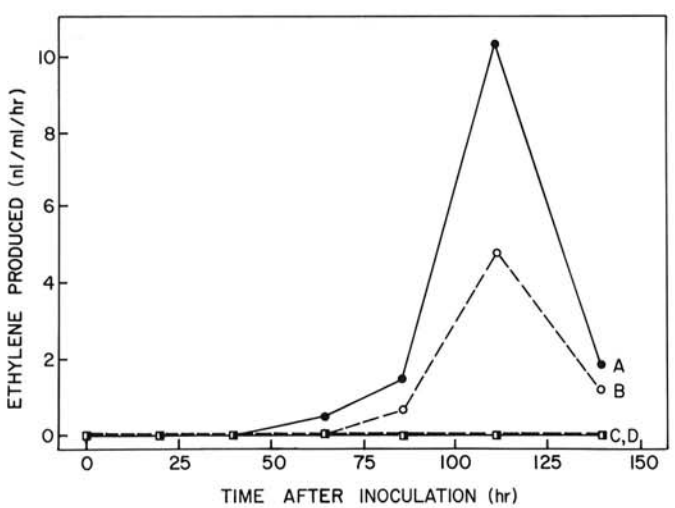

Fig. 3. The amount of ethylene generated by *Fusarium oxysporum* f. sp. *lycopersici* race 1 in vitro at various times after inoculation of a rich synthetic culture medium. Treatments consisted of: curve A, 10 mM methionine; curve B, 10 mM methionine + 1.0% (v/v) ethanol; curve C, 1.0% ethanol; and curve D, no amendment. Flasks were incubated at 30 C under constant light.

If the induced active response to infection is resistance, one would expect that the pathogen population in both compatible and incompatible combinations would increase until the resistance response is triggered. At this point, the pathogen should continue to grow in the susceptible host, but should be inhibited in the resistant host. If susceptibility is the active, induced response, the pathogen population in both compatible and incompatible hosts should remain low until susceptibility is induced. At that time, the pathogen population in the susceptible host should increase while that in the resistant host should not. The pathogen population curves in Fig. 2 indicate that the latter may occur in Fusarium wilt of tomato. If induced susceptibility is the process that determines compatibility, the direct involvement of antifungal compounds in resistance need not be invoked unless induction of susceptibility involves the reduction of fungitoxic levels of preformed antifungal compounds. Beckman and MacHardy (11) have interpreted the data of Stromberg and Corden (14) as supporting this hypothesis. Our data and that of Stromberg and Corden (14) indicate that the levels of antifungal compounds in the xylem of tomato cultivars susceptible or resistant to Fusarium wilt are initially moderately high and drop off after inoculation. The high fungitoxicity of xylem extracts that we observed on day 1 in all inoculated tomato plants regardless of their compatibility with *Fusarium* was not observed by Stromberg and Corden (14). The apparent nonspecific induction of antifungal compounds in tomato plants may have been missed by these researchers because of differences in extraction techniques which have consistently given us extracts two-to-three times more toxic than those reported earlier (14). After 5 days, however, the antifungal activity of the resistant cultivar increases to levels much higher than the initial constitutive levels, while that in the susceptible cultivar remains low (14). Thus, evidence exists for the presence of constitutive antifungal compounds in tomato xylem, but the levels induced in resistant plants 5 days or more after inoculation are much higher than the constitutive levels (14). The antifungal activity of these compounds may be trivial, however, and the actual role of these substances may be, for example, as effectors of allosteric host enzymes (7) or substances formed as the consequence, rather than the cause, of resistance (4).

Research similar to the present study has been conducted to determine whether antimicrobial compounds (phytoalexins) are the cause or consequence of disease resistance in other plant species. Resistance or susceptibility was modified either with chemicals (10) or with temperature changes (16) and plants either became resistant in the absence of antimicrobial levels of phytoalexins (10) or became susceptible even in the presence of antimicrobial concentrations of phytoalexins (16). Such evidence sheds doubt on the role of antimicrobial compounds in these diseases. The present study on Fusarium wilt of tomato, however, shows a consistent correlation of high levels of antifungal compounds with resistance. Moreover, in susceptible Bonny Best tomato plants inoculated with *F. oxysporum* f. sp. *lycopersici*, race 1, severely diseased plants can produce apparently healthy shoots (9) concomitant with a decrease in pathogen population and an increase in the concentration of antifungal compounds to toxic levels in the xylem (14).

Ethanol clearly "breaks" single-gene resistance of tomato to *F. oxysporum* f. sp. *lycopersici*, but whether ethanol will cause a nonhost species (eg, carnation or banana) to become susceptible to *F. oxysporum* f. sp. *lycopersici* is unknown. Likewise, whether ethanol will cause other formae speciales of *F. oxysporum* (eg, *F. oxysporum* f. sp. *dianthi* or *F. oxysporum* f. sp. *cubense*) to become pathogenic on tomato is unknown. Further research on the mode of action of ethanol as a susceptibility-inducing agent could yield many clues on mechanisms of specificity and resistance in plant disease.

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