

AN ABSTRACT OF THE THESIS OF

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Title: PRODUCTION AND CHARACTERIZATION OF ANTIFUNGAL
COMPOUNDS PRODUCED BY TOMATO PLANTS INOCULATED
WITH FUSARIUM OXYSPORUM F. SP. LYCOPERSICI

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Malcolm E. Corden

The fungitoxicity of acetone extracts from the xylem of Fusarium wilt disease resistant tomato plants (cv. Jefferson) inoculated with Fusarium oxysporum f. sp. lycopersici was greatly influenced by environmental factors. Extracts obtained from plants 1 h after watering were 4-times more toxic than extracts from plants subjected to water stress for 5 h before extraction, and 10-times more toxic than those from plants after 23.5 h of water stress. Materials from plants extracted at various times during the day exhibited a striking diurnal pattern of antifungal activity. Material from plants extracted at the end of the dark period were the most toxic, and extracts obtained at the end of the light period were the least toxic. The extracts were most toxic to the pathogen in vitro at pH 4.5 and were completely ineffective at pH 6.0 and above.

Monogenic resistance of tomato to race 1 of the wilt pathogen was negated by the application of 1% ethanol through the roots. Vascular and foliar disease symptoms were pronounced 15 days after inoculation in ethanol-treated plants, but were absent in ethanol-treated uninoculated

plants and in untreated inoculated plants. The pathogen population increased dramatically in inoculated plants treated with ethanol, but remained low and constant in inoculated, untreated plants. By 5 days after inoculation, the fungitoxicity of xylem extracts was very low in ethanol-treated, inoculated plants, but was very high in the untreated, inoculated plants. Similar results were obtained with isolated stem sections treated with ethanol and inoculated with the pathogen.

The antifungal compounds in xylem extracts were partially purified and characterized. Chromatography on silica gel, Sephadex LH-20, and Sephadex G-10 resolved four highly antifungal compounds. Sequential thin layer chromatography on silica gel was used to isolate one compound which formed hexagonal orange crystals in cold methanol and melted at 96 C. The isolated compound had strong absorption bands in the visible and ultraviolet spectra, indicating the presence of a π - π conjugated system. No α -tomatine or rishitin could be detected in any extracts, suggesting that antifungal compounds other than these two substances may be involved in wilt disease resistance in tomato.

Production and Characterization of Antifungal Compounds
Produced by Tomato Plants Inoculated with
Fusarium oxysporum f. sp. lycopersici

by

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This thesis is dedicated to my mother, Jane Ann Niedzialkowski Danko. Sorrow is not forever; love is.

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PRODUCTION AND CHARACTERIZATION OF ANTIFUNGAL COMPOUNDS
PRODUCED BY TOMATO PLANTS INOCULATED WITH
FUSARIUM OXYSPOURUM F. SP. LYCOPERSICI

CHAPTER 1

INTRODUCTION

The resistance of plants to potential pathogens has been a subject of considerable interest to plant pathologists since the birth of phytopathology as a science. The observation that some cultivars of a given species may be resistant to a pathogenic organism while others are susceptible led plant pathologists, first, to select disease resistant plants for cultivation and, more recently, to investigate the physiological nature of disease resistance in those plants.

OVERVIEW OF DISEASE RESISTANCE MECHANISMS IN PLANTS

Physiological reactions in plants challenged by a potential pathogen can manifest themselves in a variety of ways. First, mechanical barriers to the pathogen may develop. These barriers may serve to shield susceptible tissue from the pathogen or to physically contain the pathogen within a limited space. The papillae that form in plants resistant to powdery mildews (1) and the tyloses that form in xylem vessels of plants resistant to wilt diseases (6) have both been implicated as resistance conferring barriers. A second resistance mechanism involves the production of fungitoxic chemicals in the host. The toxicants may be constitutive in the

plant or induced by the pathogen. Constitutive antifungal compounds include the materials found in the heartwood and bark of many tree species (28). Induced antifungal compounds or "phytoalexins" have been reported in many plant species (35): e.g. hemigossypol in cotton (53), phaseollin in bean (14), and phytuberin in potato (51). With the exception of safynol (2) and wyerone acid (37), most phytoalexins are polyphenolic compounds.

Other mechanisms of resistance include the "hypersensitive response" in which a rapid necrosis of host cells in the vicinity of the pathogen limits its growth (29), agglutination of bacterial pathogens by "lectins" (24), and resistance of the host to phytotoxins produced by the pathogen (42).

RESISTANCE OF TOMATO PLANTS TO FUSARIUM WILT

In the wilt disease of tomato caused by Fusarium oxysporum f. sp. lycopersici, the mechanism of resistance most often ascribed to the host is physical containment of the pathogen in the xylem vessels by tyloses, gel plugs, and gums. Tyloses are generally more abundant in the roots and hypocotyl of resistant cultivars than they are in susceptible cultivars inoculated with the pathogen (7). Beckman and his associates (6, 7) suggest that tyloses, as well as gel plugs and gums, accumulate in the xylem vessels of wilt resistant plants and occlude the vessels, thereby restricting the pathogen to a limited portion of the xylem. This proposed mechanism assumes that the host produced structures develop rapidly to localize the pathogen before it can spread further, and that the occluding structures are not degraded by the pathogen. In some cases, however, tyloses may not

form in sufficient quantity to completely occlude infected vessels (48) and, in a similar wilt disease of tomato caused by Verticillium albo-atrum, tyloses occur more abundantly in compatible host-pathogen interactions than in incompatible interactions (18).

A number of fungitoxic substances have been detected that may contribute to disease resistance in tomato plants by inhibiting the growth and development of the pathogen in wilt resistant cultivars (31, 32, 43). In early studies, antifungal substances could not be consistently isolated from resistant tomato plants (30, 45), and some later studies failed to find higher levels of antifungal substances in resistant cultivars than in susceptible cultivars (23, 36).

ANTIFUNGAL COMPOUNDS IN TOMATO PLANTS

The earliest attempt to detect antifungal compounds in wilt resistant tomato plants was made by Fisher in 1935 (21). He detected a substance in the intermediate-resistant cultivar Marglobe that was toxic to Fusarium and was not detectable in the susceptible cultivar Bonny Best. This material lost its toxicity when autoclaved, when passed through a negatively charged filter, or when allowed to stand for 4 to 6 days.

Gottlieb (26), in 1943, used sterile, expressed sap from tomato plants as a growth medium for the wilt pathogen. Growth was greatest in sap from the highly susceptible Bonny Best plants, intermediate in sap from the intermediate-resistant Marglobe plants, and least in sap from the highly-resistant Pan America cultivar plants. The inhibitory substance in the

resistant plants was stable at 100 C for 2 h, could be adsorbed on charcoal, and could be distilled at 95 C and reduced pressure without loss of activity.

Heinze and Andrus (30) detected no difference, however, in toxicity between extracts from the highly-resistant cultivar Pan America and the susceptible cultivar Bonny Best. They conceded that a toxic material may have been lost or destroyed during the filtration or autoclaving procedures.

Snyder, Baker and Hansen (45) were unable to detect differences in growth of the wilt pathogen in xylem fluid forced by root pressure from the cut ends of resistant or susceptible plants truncated at the soil level. They concluded that resistance to Fusarium wilt in tomato does not operate in the xylem.

Expressed juice from the stems of both resistant and susceptible tomato plants stimulated the growth of the pathogen in a culture medium, and no difference in growth on the preparations from either plant was noted, whether autoclaved or filter sterilized (25). However, from this data, Gothoskar et al. (25) did not dismiss the possibility for involvement of antifungal chemicals in Fusarium wilt resistance in tomato.

In tomato, the steroidal glycoside α -tomatine (Fig. 1) and the nor-sesquiterpenoid rishitin (Fig. 2) have received considerable attention in proposed resistance mechanisms. In some cases, however, these substances either did not accumulate to fungitoxic levels in the plants (3) or they failed to accumulate to higher levels in resistant than in susceptible cultivars (23, 36).

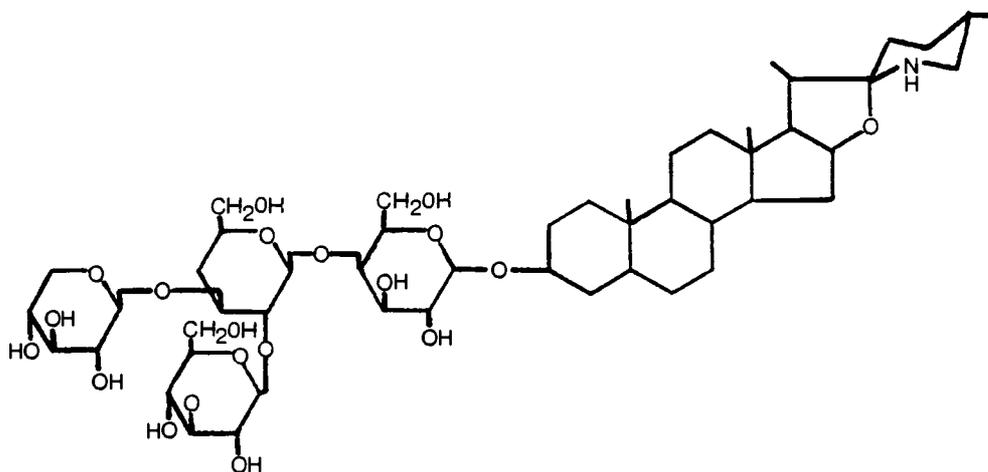


Fig. 1. Structure of α -tomatine: O- β -D-xylopyranosyl-(1 \rightarrow 3)-[O- β -D-glucopyranosyl-(1 \rightarrow 2)]-O- β -D-glucopyranosyl-(1 \rightarrow 4)-O- β -D-galactopyranosyl-tomatidine.

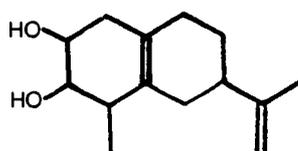


Fig. 2. Structure of rishitin.

α -Tomatine. Irving, Fontaine, and Doolittle (32) detected an antifungal substance in expressed juice of tomato plants that was stable at 100 C for at least 1 h, could be adsorbed on charcoal, and was dialyzable. They named this preparation "lycopersicin", a term subsequently replaced by α -tomatine (34). α -Tomatine occurred in all tomato cultivars tested, regardless of their resistance to Fusarium wilt (32).

The presence of α -tomatine in tomato plants has been documented several times since the work of Irving, Fontaine, and Doolittle (32) but generally there was little evidence for the involvement of this compound in Fusarium wilt resistance in tomato. Kern (34) found no correlation between α -tomatine concentration and resistance in a number of tomato cultivars, and Langcake, Drysdale, and Smith (36) found that, although the α -tomatine concentration increased after inoculation with Fusarium, the increase was similar in cultivars of high and low wilt disease resistance. Similar results were obtained when α -tomatine was extracted from isolated vascular tissue and tap roots (39) but no α -tomatine was detected in xylem sap (36, 39).

Hammerschlag and Mace (27) found more α -tomatine in methanol extracts of roots from resistant than from susceptible tomato cultivars. The antifungal activity of extracts from resistant roots was almost twice as great as that from susceptible roots, whether or not the plants were infected with Fusarium. They ascribed the differences between their results and the results of others to differences in cultivars or age of the plants used.

Arneson and Durbin (3) calculated that the concentration of α -tomatine

in tomato leaves was 0.001 M, if they assumed a uniform distribution within the tissue. They also suggested that since α -tomatine is more likely confined to specific tissues and perhaps to specific intracellular sites, the localized concentration may approach the 0.1 M value necessary for complete inhibition of growth by the wilt disease pathogen. An especially interesting observation was that tomato pathogens were much less sensitive to α -tomatine than were non-pathogens of tomato.

Rishitin. Rishitin was first isolated from potato tubers infected by Phytophthora infestans (33), and is present in tomato fruits and stems (50). In tomato plants inoculated with Fusarium oxysporum f. sp. lycopersici, Gentile and Matta in 1976 (23) isolated rishitin from the xylem of susceptible cultivars in advanced stages of the disease. They also noted that the presence of rishitin was correlated with the occurrence of vascular browning.

McCance and Drysdale (39) found higher levels of rishitin in inoculated stem segments and fruits of a highly resistant tomato cultivar than in a low resistance cultivar. However, they noted that the rishitin content following tap root inoculations was relatively low and probably not adequate to inhibit the pathogen to any great extent, particularly if the rishitin was uniformly distributed throughout the tissues. However, they suggested that rishitin could achieve antifungal levels in localized cells if it was not uniformly distributed.

Hutson and Smith (31) detected two antifungal compounds from tomato plants inoculated with Fusarium, and one was identified as rishitin. The

rishitin content was greatest in roots of the resistant cultivar, but was barely detectable in extracts of isolated xylem from the stems of this cultivar. Further, the rishitin concentration in isolated xylem of a susceptible cultivar was much higher than that in the resistant cultivar.

Other antifungal compounds. Antifungal compounds have been detected and implicated in resistance mechanisms in other tomato diseases. deWit and Flach (16) detected two phytoalexins in tomato leaves, and five phytoalexins, including rishitin, in tomato fruits inoculated with Cladosporium fulvum.

Tomato plants inoculated with Verticillium albo-atrum accumulate rishitin and other antifungal compounds. Tjamos and Smith (50) detected up to seven fungitoxic materials, including rishitin, in extracts from wounded or Verticillium inoculated tomato stems and roots, and Elgersma (20) detected five antifungal compounds, including rishitin, in xylem extracts of tomato plants inoculated with Verticillium. Rishitin accumulation, however, was never greater in resistant than in susceptible cultivars. The unidentified compounds were not identical with the antifungal terpenoids found in potato. Hutson and Smith (31) detected six antifungal materials in Verticillium inoculated tomato plants, and again rishitin was among the compounds detected and the unidentified compounds were not identical with the terpenoid phytoalexins from potato.

Recently, Stromberg and Corden (47) observed that acetone extracts from the xylem of inoculated Fusarium resistant tomato plants were

significantly more fungitoxic than were similar extracts from Fusarium susceptible plants, but the antifungal compounds were not identified.

JUSTIFICATION FOR THE PRESENT STUDY

To fully understand the basis for resistance in tomato to Fusarium wilt, the antifungal materials in xylem extracts from inoculated tomato plants must be purified and characterized. The fact that several antifungal substances other than α -tomatine and rishitin are produced by tomato plants permits the speculation that these yet unidentified substances may be important in wilt disease resistance in tomato.

The somewhat contradictory results of previous studies on the levels of antifungal compounds in tomato suggests that some effort must be made to evaluate the environmental factors that may influence the production or availability of these antifungal materials. Finally, methods are needed to positively implicate antifungal compounds in the wilt resistance mechanisms in tomato plants.

To achieve these goals, the present study was undertaken. First, an evaluation was made of environmental factors that influence the production of antifungal compounds by Fusarium wilt resistant tomato plants after inoculation. Second, resistance in tomato was modified with ethanol to determine if induced susceptibility was accompanied by a reduction in the levels of antifungal compounds normally present in the xylem of the resistant cultivar after inoculation. Third, an attempt was made to purify and characterize the antifungal compounds produced by Fusarium resistant

tomato plants after inoculation.

CHAPTER 2

THE INFLUENCE OF WATER STRESS AND PHOTOPERIOD ON THE ANTIFUNGAL SUBSTANCES IN THE XYLEM OF TOMATO PLANTS INOCULATED WITH FUSARIUM OXYSPORUM F. SP. LYCOPERSICI

ABSTRACT

Xylem extracts from Jefferson cultivar (wilt disease resistant) tomato plants inoculated with Fusarium oxysporum f. sp. lycopersici were most toxic to the pathogen at pH 4.5 and were completely ineffective at pH 6.0 or above. Plants subjected to water stress before extraction yielded extracts that were as much as ten-fold less toxic than extracts from plants receiving adequate water. A diurnal pattern in the concentration of xylem toxicants was noted. The most toxic extracts were obtained at the end of an 8 h dark period, while the least toxic extracts were those obtained after a 16 h light period. Environmental influences on the xylem toxicants may help to explain the variation in previous reports on the occurrence of antifungal materials as a factor in Fusarium wilt disease resistance.

INTRODUCTION

Antifungal compounds have long been recognized as potential factors in disease resistance in plants, and, in many species of plants, the concentrations of these compounds increase substantially after inoculation with a non-pathogen (35). In general, the compounds most often associated with disease resistance are polyphenolics (35), as are the compounds α -tomatine (4) and rishitin (27). These two substances (4, 23), and a number of other

antifungal compounds (11), have been detected in tomato plants (Lycopersicon esculentum Mill.) inoculated with Fusarium oxysporum Schlecht. f. sp. lycopersici (Sacc.) Sny. and Hans. However, these compounds have not been isolated consistently by different workers (23, 25, 31, 34, 45), and, in some cases, their role in disease resistance was discounted because greater concentrations were not always found in the more resistant cultivars (23, 36).

Investigations of polyphenolic compounds in plants have revealed striking effects of environmental factors on their concentrations. A diurnal concentration cycle was observed for the leuco-anthocyanins in Sedum album (13). The level of leuco-anthocyanins increases to a maximum at the end of a dark period and reaches a minimum at midday, when the light intensity was greatest. The production of a yellow flavanoid pigment in tomato fruit is also influenced by light (40). In this case, a red-far red, phytochrome mediated reaction exists, in which red light promotes yellow cuticle coloration, and far-red light blocks pigment production.

The enzyme phenylalanine-ammonia lyase (PAL) has been implicated as the primary controller of polyphenol biosynthesis in plants (11, 41). PAL is an extremely labile enzyme with a half-life of about 6 h (19, 55). The synthesis of PAL is dependent on light, while the degradation of the enzyme is photo-independent (55), and, thus, the net PAL concentration increases during the day and decreases at night.

Since both plant polyphenols and the enzymes involved in their

synthesis are affected by light, antifungal polyphenols may also be influenced by light. Since the Fusarium wilt disease pathogen lives longer in shaded than in non-shaded disease resistant tomato plants (44), light may also control the synthesis of antifungal polyphenols in these plants.

Water stress in plants is known to decrease the level of PAL, probably due to an inhibition of protein synthesis (5). A labile enzyme, such as PAL, would naturally be expected to decrease in amount as water stress increases and protein synthesis decreases. Decreasing amounts of PAL in plants should reduce polyphenol production, and since water stress increases the susceptibility of intermediate-resistant tomato plants to Fusarium wilt (49) water stress may play a role in the decrease of antifungal polyphenols in Fusarium inoculated tomato plants.

In addition to these effects on the production of polyphenolic compounds in vivo, the pH of the bioassay medium can influence the effectiveness of antifungal compounds in vitro. For example, α -tomatine is about 300 times more fungitoxic at pH 8.0 than it is at pH 3.0, suggesting that the unprotonated form is the effective antifungal species (4).

The objectives of this investigation were to determine the effect of pH on the in vitro antifungal activity of tomato xylem extracts, and to evaluate the effects of photoperiod and water stress on the production of antifungal substances by tomato plants inoculated with the wilt disease fungus.

MATERIALS AND METHODS

Single-gene-resistant Jefferson tomato plants were grown in washed silica sand and were watered daily with Hoagland's nutrient solution. Plants were maintained in a controlled environment room at 30 C, a relative humidity of about 45%, and a 16 h photoperiod with light intensity of about $400 \mu\text{E m}^{-2}\text{s}^{-1}$.

Microconidia of Fusarium oxysporum f. sp. lycopersici for plant inoculations were obtained by growing the pathogen for 4 days in shake culture in potato dextrose broth. Cultures were filtered through Whatman #4 filter paper, the filtrate was centrifuged at 7500 g for 10 min, the pellet was resuspended in sterile water, and the spore suspension was re-centrifuged. The microconidial concentration was adjusted to 10^4 spores ml^{-1} with the aid of a hemocytometer.

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 h in the growth room under conditions favoring a high transpiration rate. Plants were then replanted in washed silica sand.

Xylem extracts were obtained from internode sections from the epicotyl of plants by drawing 5.0 ml of absolute acetone through each section by vacuum. Extracts were dried at 35 C under vacuum, and the residue was triturated with 0.2 ml absolute acetone g^{-1} plant material. The supernatant solution was decanted from a pink residue and stored at -15 C.

The extracts were evaluated for antifungal activity by spotting aliquots in deep well slides, allowing the solvent to evaporate, and then adding 150 μl of a suspension of 10^4 Fusarium microconidia ml^{-1} . The microconidial suspension was prepared in a medium containing: NH_4NO_3 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, KH_2PO_4 3.5 g, sucrose 1.0 g, and distilled water to 1 L (10). The slide cultures were incubated at 30 C for 40 h in the dark, and then spore germination, colony growth, and sporulation were graded on a scale from 1 to 7: 1 = no conidial germination, 2 = conidial germination, 3 = small colonies formed, 4 = small to moderate 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 (47).

RESULTS AND DISCUSSION

Influence of pH on Antifungal Activity of Xylem Extracts. The pH of separate aliquots of germination medium was adjusted with 0.1 N HCl or NaOH to pH 3.0, 4.0, 4.5, 5.0, and 6.0. Because components of this medium precipitated out of solution at higher pH, the bioassay medium for pH 7.0 and 9.0 consisted of distilled water. Aliquots of tomato xylem extracts obtained from four 1.0 cm internode sections from the epicotyl of each of 24 tomato plants 6 days after inoculation were added to the deep well slides. Aliquots ranged from 100 μl to 1000 μl , and controls consisted of slides at each pH without extract.

The fungitoxicity indices obtained were converted to a relative toxicity

value, where relative toxicity equals $1 - \left[\frac{\text{(Fungitoxicity index for } 1000 \mu\text{l of xylem extract)}}{\text{(Fungitoxicity index for the control)}} \right]$. Relative toxicity ratings were used to compensate for differences in growth of Fusarium due to the pH of the medium.

The extracts were most fungitoxic at pH 4.5, and were completely nontoxic at pH 6.0 and above (Fig. 1). The effectiveness of these extracts at low pH suggests that the antifungal materials are more available to the fungus in this pH range, possibly because they are unionized and, thus, more easily taken up through fungal cell membranes. Polyphenols, the compounds most often implicated as phytoalexins, would be unionized at low pH.

Xylem sap of Jefferson tomato plants was collected by excising the stems of 40 day old plants at the cotyledonary node, attaching a piece of rubber tubing to the stump, and collecting the exuded xylem sap with a syringe. The pH of the sap was 5.6, which approaches the upper limit for fungitoxicity of the xylem extracts in vitro, but is nevertheless still within the toxic region.

Influence of Water Stress on Xylem Antifungal Compounds. Water was withheld from Jefferson tomato plants for 1 to 23.5 h before xylem extraction, and then all plants were extracted within a 3 h period on the 6th day after inoculation to minimize any possible effect that the photoperiod might have on the antifungal activity of the extracts. Extracts were obtained from four epicotyl internode sections from each of four plants per treatment,

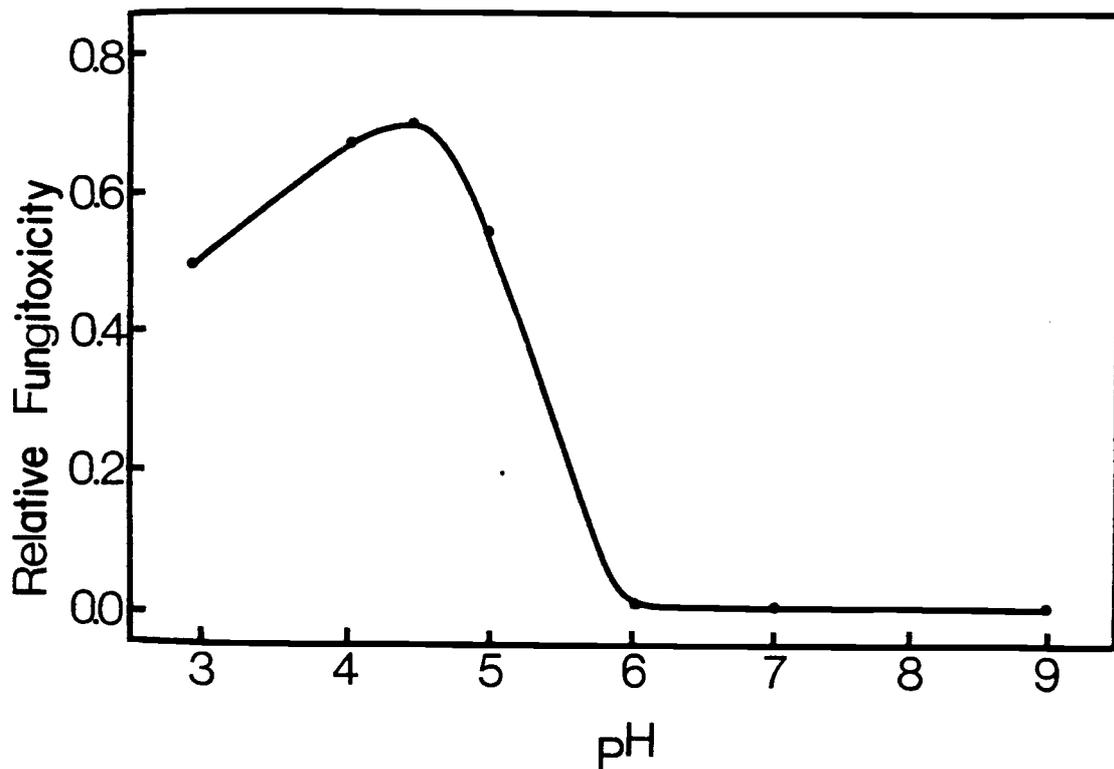


Fig. 1. The effect of pH on in vitro antifungal activity of acetone extracts from the xylem of Jefferson cultivar tomato plants inoculated with Fusarium oxysporum f. sp. lycopersici. Fungitoxicity indices were determined by estimating spore germination, colony development, and resporulation from about 1500 microconidia of the pathogen incubated 40 h in 150 μ l of nutrient medium containing the residue from 1000 μ l of the xylem extract. Fungitoxicity was first 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. Relative fungitoxicity was used to eliminate growth differences due to pH and was equal to $1 - [(\text{Fungitoxicity index for } 1000 \mu\text{l of xylem extract}) / (\text{Fungitoxicity index for the control})]$.

and were bioassayed in amounts ranging from 250 μl to 750 μl . Permanent wilting percentage, corresponding to a water potential of about -15 bars (22), was reached at about 48 h after watering.

The toxicity of the extracts decreased with increasing water stress in the plants (Fig. 2). The extracts were four-fold less toxic at 4.5 h after watering than at 1 h after last watering, and by 23.5 h after last watering, the extracts were ten-fold less toxic than the initial extracts.

Reduced antifungal activity following water stress could be due to a decrease in the levels of the antifungal materials within the xylem, or to a decrease in the extractability of these materials. In any case, the decrease in the level of these antifungal substances corresponds to a decrease in the amount of PAL that would be expected and to a decrease in the resistance of intermediate-resistant tomato plants following water stress (49).

Influence of Photoperiod on Xylem Fungitoxicants. Jefferson tomato plants were extracted at 4 h intervals for a 24 h period on the 8th and 9th days after inoculation. Plants were watered every 4 h to minimize the effect of water stress on antifungal activity of the extracts. The extracts were obtained from four epicotyl internode sections from each of four plants and were bioassayed at amounts ranging from 50 μl to 450 μl .

The antifungal activity of xylem extracts decreased throughout the light period, then increased during the dark period (Fig. 3) in a manner similar to that of leuco-anthocyanins in Sedum (13). These results suggest that the antifungal materials are produced continuously and broken down or

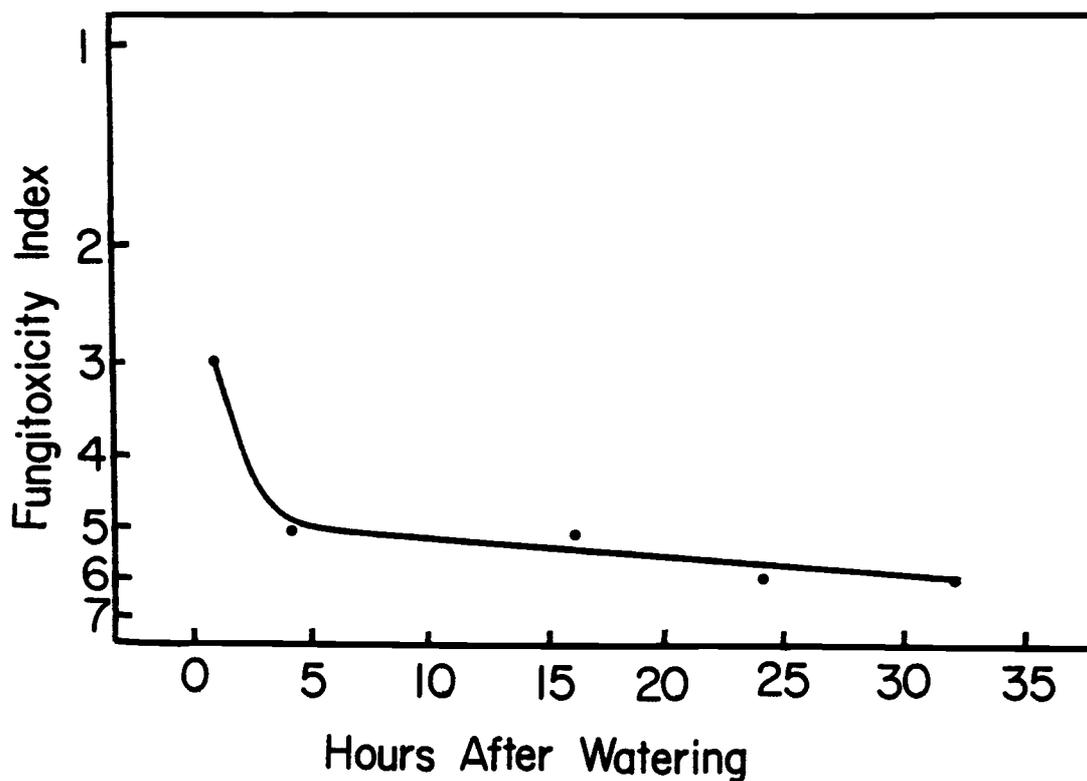


Fig. 2. The influence of water stress (expressed as time after last watering) on xylem fungitoxicants from Jefferson cultivar tomato plants inoculated with *Fusarium oxysporum* f. sp. *lycopersici*. Fungitoxicity was estimated by spore germination, colony development, and resporulation from about 1500 microconidia of the pathogen incubated 40 h in 150 μ l of nutrient medium containing the residue from 1000 μ l of the xylem extract. Fungitoxicity values are the average of bioassays of extracts from four plants in two separate experiments and are expressed by the index in the legend for Fig. 1.

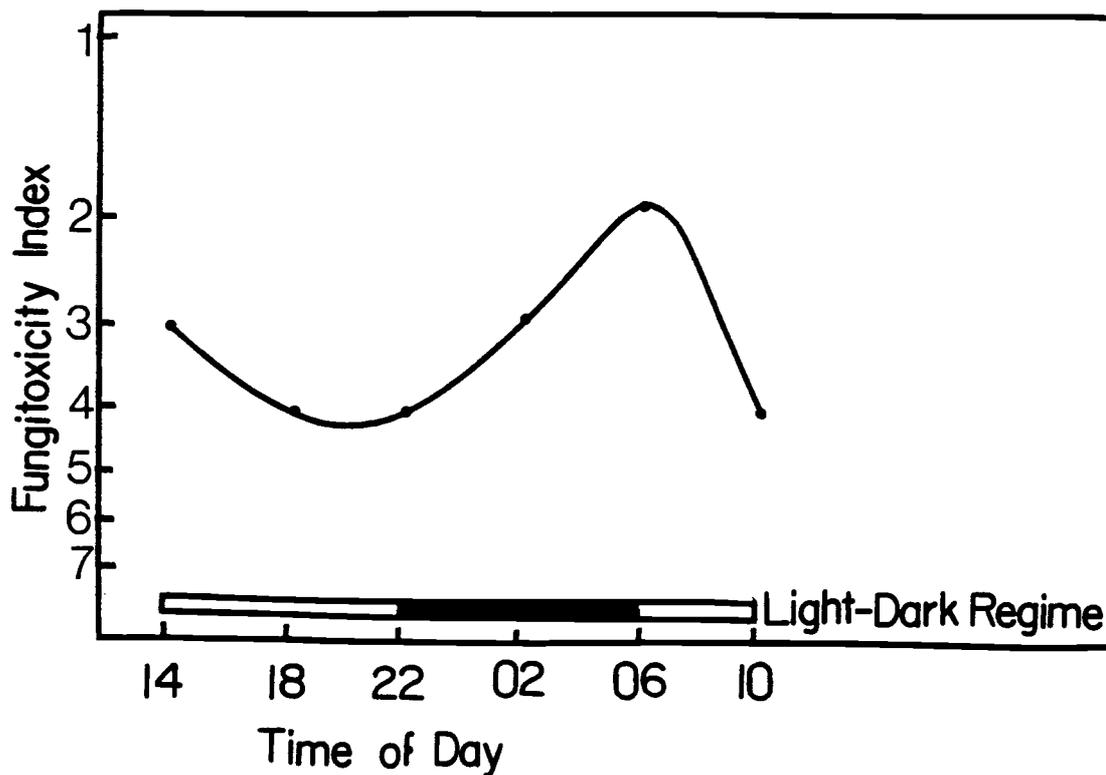


Fig. 3. The influence of photoperiod on xylem fungitoxicants from Jefferson cultivar tomato plants inoculated with *Fusarium oxysporum* f. sp. *lycopersici*. Fungitoxicity was estimated by spore germination, colony development, and resporulation from about 1500 microconidia of the pathogen incubated 40 h in 150 μ l of nutrient medium containing the residue from 450 μ l of the xylem extract. Fungitoxicity indices are the average of bioassays of extracts from four plants and are expressed by the index in the legend for Fig. 1. Xylem extractions were initiated at 14 h on the 5th day following inoculation and ended at 10 h on day 6.

modified by light. The experiments of Scheffer and Walker (44) and of Gothoskar et al. (25) led these workers to suggest that resistance of tomato to Fusarium wilt is closely associated with the metabolism of the host and is probably derived from a very labile substance formed continuously by the plant. The data here suggest the presence of a photo-labile substance such as that in Sedum (13) and are in agreement with the conclusions of Gothoskar et al. (25).

These findings, however, may appear to contradict the results of Scheffer and Walker (44) who found that Fusarium survived longer in shaded plants resistant to the pathogen than in unshaded plants. Although the present study suggests that the antifungal compounds in tomato are light sensitive, the fact that PAL levels increase in the light indicates that the synthesis of photo-labile polyphenolics will be stimulated by light.

The effect of water stress and photoperiod on the toxicity of xylem extracts from tomato plants may provide some understanding to account for the variation in previous reports on the occurrence of antifungal materials as a factor in Fusarium wilt resistance. Indeed, other factors, such as light intensity (44) and decreased temperature (12) tend to increase resistance of tomato plants to the disease.

In spite of the fact that different levels of antifungal materials may be present at different times during the day and at different times after watering, the pathogen within the xylem is exposed to the highest concentrations at some time during the day. Even if the period of exposure to

high concentrations of these antifungal compounds is short, a brief exposure may be enough for continued inhibition of the pathogen.

CHAPTER 3

THE EFFECT OF ETHANOL ON THE ACCUMULATION OF
ANTIFUNGAL COMPOUNDS AND RESISTANCE OF TOMATO TO
FUSARIUM OXYSPORUM F. SP. LYCOPERSICI

ABSTRACT

The application of 1% ethanol to the roots of wilt disease resistant tomato plants (cv. Jefferson) negates 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 rapidly increased when ethanol was applied, but remained low in inoculated, untreated plants. The fungitoxicity of acetone extracts from the xylem of inoculated, ethanol treated plants remained low as it did in Jefferson plants inoculated with race 2 of the pathogen, a race to which Jefferson plants are susceptible. By the 5th day 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, race 1 inoculated Jefferson plants. Similar results were obtained when excised stem sections rather than whole plants were used for these experiments. The fact that ethanol treatments that negate the resistance conferred by the I gene also prevents the increase in antifungal compounds in the xylem, lends credence to the hypothesis that these fungitoxic materials contribute to wilt disease resistance in tomato.

INTRODUCTION

The nature of resistance in tomato (Lycopersicon esculentum Mill.) to the wilt disease pathogen Fusarium oxysporum Schlecht. f. sp. lycopersici (Sacc.) Sny. and Hans. has been studied intensively since the introduction of the I gene for resistance from Lycopersicon pimpinellifolium (9). Resistance generally has been ascribed to the production of gums, gel plugs, and tyloses that physically contain the pathogen in the xylem of the roots and hypocotyl of the plants (7). However, experiments with stem cuttings (44) in which resistance mechanisms in the roots and hypocotyl are bypassed have demonstrated that resistance is not localized in any one portion of the plant. Moreover, recent scanning electron microscopy (48) suggests that gums, gel plugs, and tyloses are not produced in sufficient quantity in the stems to physically contain the pathogen.

Early attempts to demonstrate antifungal compounds in tomato plants as the basis for resistance were, for the most part, unsuccessful. Generally, no difference in fungitoxicity of plant extracts or homogenates could be detected between susceptible and resistant cultivars (52). Although rishitin and α -tomatine have been implicated as phytoalexins in tomato (32, 39), the amounts of these materials were often greater in susceptible than in resistant cultivars inoculated with Fusarium (23, 36).

After the development of a simple method to obtain extracts exclusively from the xylem of plants (10), Stromberg and Corden (47) demonstrated that acetone extracts of the xylem of wilt disease resistant tomato plants became

increasingly fungitoxic after inoculation with the pathogen, while extracts from wilt susceptible plants maintained their relatively weak fungitoxicity. These experiments demonstrated that fungitoxic materials may play a role in wilt disease resistance.

Plants of wilt resistant tomato cultivars lose their resistance when fed 1% solutions of ethanol through the cut ends of the stem (44). Induction of susceptibility in the normally wilt resistant tomato plants with ethanol offers the opportunity to determine if the altered disease reaction is accompanied by a corresponding reduction in the amount of fungitoxic materials in the xylem. The present study was undertaken to seek this experimental verification of the role of fungitoxic materials in resistance to Fusarium wilt in tomato.

Studies on wilt diseases of tomato sometimes have utilized isolated stem sections to investigate the early phases of the diseases (39, 50). Studies on the pathogen population in the host indicate that resistance or susceptibility in tomato is determined within about 5 days after inoculation (38, 47). To verify claims that isolated stem sections provide a reliable system for studying the early reactions in wilt disease resistance, we compared the action of ethanol in isolated stem sections and in whole plants.

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 h photoperiod with light intensity of about $400 \mu\text{E m}^{-2}\text{s}^{-1}$.

Microconidia of Fusarium oxysporum f. sp. lycopersici for plant inoculations were obtained by growing the pathogen in shake culture in potato dextrose broth for 4 days. Cultures were filtered through Whatman #4 filter paper, the filtrate was centrifuged at 7500 g for 10 min, the pellet was resuspended in sterile water, and the spore suspension was recentrifuged. The microconidia concentration was adjusted to 10^4 spores ml^{-1} with the aid of a hemocytometer.

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 h in the growth room under conditions causing a high transpiration rate. Plants were inoculated with either race 1, race 2, or race 1 spores in 1.0% (v/v) ethanol. Uninoculated plants treated with 1.0% ethanol or with sterile water served as controls. Following 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 h each day.

In experiments with excised tomato stem sections, 15.0 cm long stem segments were cut from the epicotyl of 60-day-old plants. The leaves were removed, and the sections were surface sterilized by washing with 1.3% sodium hypochlorite solution and rinsing in sterile distilled water. The inoculum suspension was introduced into the xylem by vacuum infiltration

for 1 min. The stem sections were then maintained in humid chambers at 30 C and a 16 h photoperiod with light intensity of about $400 \mu\text{E m}^{-2}\text{s}^{-1}$ for the course of the experiment.

Pathogen populations in plants were estimated by excising 2.5 cm stem pieces immediately below the cotyledons, surface sterilizing the sections with 1.3% sodium hypochlorite solution, 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 sterile distilled water for 1 min in a Sorvall omnimixer. The stem slurry was diluted and plated in potato dextrose agar adjusted to pH 4.0 with lactic acid. The Fusarium population was estimated by counting the number of colonies obtained from each of four replicates.

Daily after inoculation, xylem extracts were obtained from four 2.5 cm internode sections from the epicotyl of each plant by drawing 5.0 ml of absolute acetone through each of four replicates by vacuum. Extracts were dried at 35 C under vacuum and were reconstituted with 0.2 ml acetone g^{-1} stem section.

The acetone extracts were evaluated for antifungal activity by spotting aliquots of 50 to 1000 μl in deep well slides, allowing the solvent to evaporate, and then adding 150 μl of a suspension of 10^4 Fusarium microconidia ml^{-1} . The microconidia suspension was prepared in a medium containing: NH_4NO_3 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, KH_2PO_4 3.5 g, sucrose 1.0 g, distilled water to 1.0 L (10), and was adjusted to pH 4.5. The slide cultures were incubated at 30 C for 40 h in the dark, and then spore germination, colony growth, and

sporulation were graded 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 (47).

Disease development was evaluated by the severity of vascular and foliar symptoms according to Dimond et al. (17). A vascular index was obtained by totalling the number of major vascular bundles discolored at each internode and dividing this number by the total number of bundles examined (i.e. three times the number of internodes). 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 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, and then averaging the leaf grades for each plant. Results are reported as the average of four replicates.

RESULTS

Fifteen days after inoculation with the pathogen or treatment with ethanol, disease symptoms as evaluated by 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 the Jefferson cultivar

TABLE 1. The influence of ethanol on wilt disease development in Jefferson cultivar tomato plants 15 days after inoculation with Fusarium oxysporum f. sp. lycopersici race 1 (Fol R1)

Inoculation	Treatment ^w	Disease symptoms	
		Vascular index ^x	Leaf grade ^y
Fol R1	1% ethanol	0.70 a ^z	2.67 a
Fol R1	none	0.06 b	0.07 b
Uninoculated	1% ethanol	0.02 b	0.20 b
Uninoculated	none	0.01 b	0.23 b

^wEthanol treated plants were fed a 1.0% solution of ethanol through the roots for a 4 h period each day.

^xVascular 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.

^yLeaf grade is an estimate of foliar disease symptoms based on a rating scale where 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 leaf grades from four plants.

^zIn each column, values followed by a common letter do not differ significantly ($P = 0.05$) according to Student's t test.

with ethanol verifies the earlier report (44) on the action of ethanol in this disease.

The population of race 1 of the pathogen in Jefferson tomato plants remained low for the first 5 days after inoculation (Fig. 1-A) but, in Bonny Best plants, began to increase on day 3 and continued to increase through the 5th day when it was about ten times the population on day 1. In Jefferson plants inoculated with race 1 of the pathogen and treated daily with 1% ethanol, the pathogen population increased on the 4th day and by the 5th day was about 30 times that on day 1 (Fig. 1-A). Ethanol treatment induces not only an increase in disease symptoms, but allows the pathogen population to increase similarly to that in the susceptible cultivar.

Antifungal activity of xylem extracts from Jefferson plants was high on the 1st day after inoculation, but generally decreased thereafter and remained low in plants inoculated with race 2 of the pathogen or with race 1 and treated with ethanol (Fig. 1-B). Both groups of plants eventually developed disease symptoms. In the plants inoculated with race 1 and that remained disease free, the fungitoxicity of the xylem extracts initially dropped, but then increased to the original high toxicity by the 5th day. Notably, the 3rd day 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.

When excised Jefferson stem sections were used in place of whole plants, the pathogen population remained constant in sections inoculated

Fig. 1. A, 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% ethanol (EtOH), for the first 5 days following inoculation with microconidia of the pathogen. B, Fungitoxicity of xylem extracts from stems of Jefferson tomato plants inoculated with race 1 (R1), race 2 (R2), or R1 with 1% ethanol treatments for the first 5 days following inoculation with microconidia of the pathogen. Fungitoxicity was estimated by spore germination, colony development, and resporulation from about 1500 microconidia of the pathogen incubated 40 h in 150 μ l of nutrient medium containing the residue from 100 μ l of the xylem extract. Fungitoxicity values are the average of bioassays of extracts from four plants and 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.

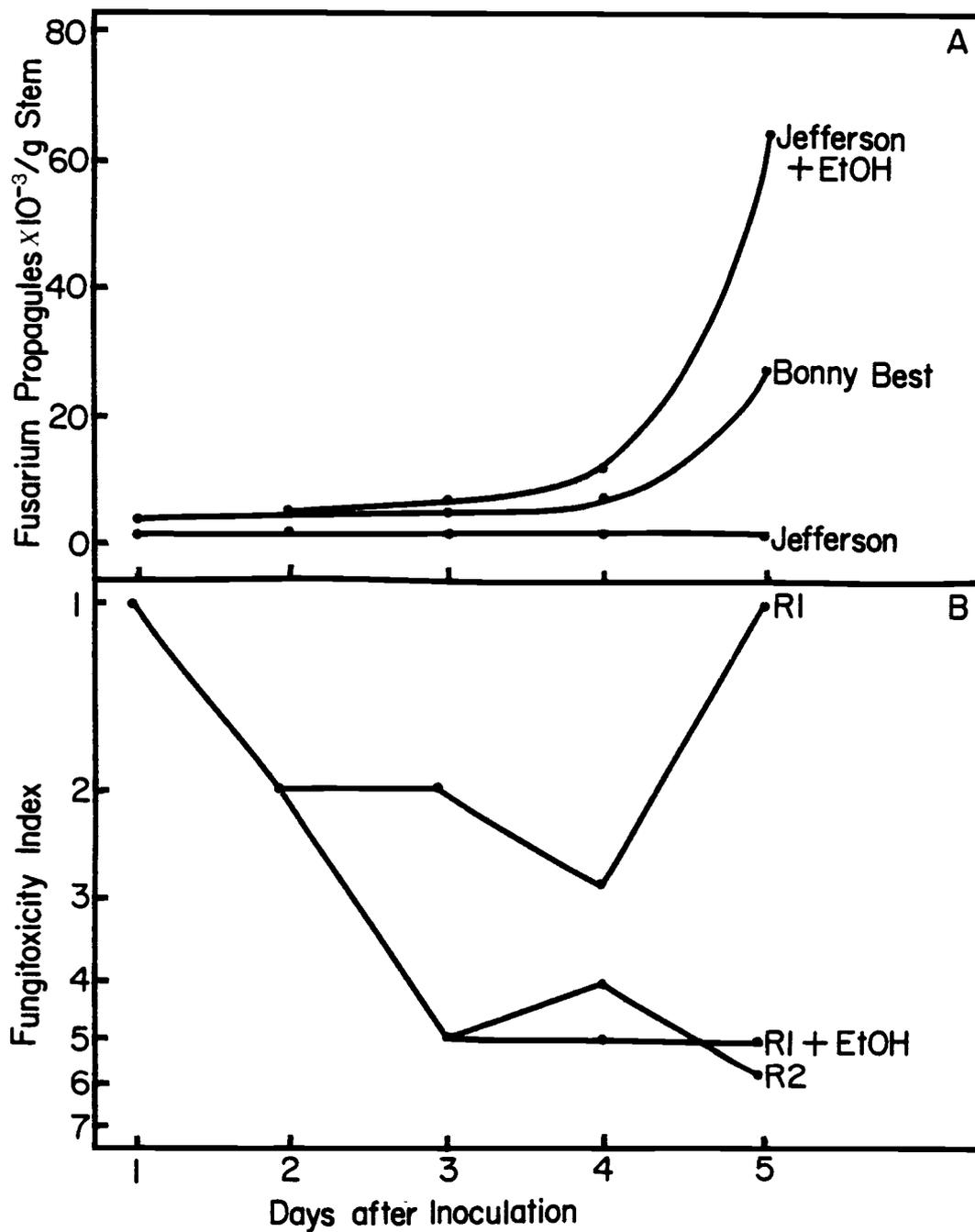


Fig. 1. The population of *Fusarium oxysporum* f. sp. *lycopersici* and the fungitoxicity of xylem extracts from tomato plants for the first 5 days after inoculation.

with race 1 of the wilt disease pathogen (Fig. 2-A). In stem sections where 1.0% ethanol was added to the inoculum, the pathogen population increased to about five times that in stem sections without ethanol. Just as in whole plants, the antifungal activity of xylem extracts from ethanol treated and untreated stem sections remained the same for the first 2 days after inoculation (Fig. 2-B). Thereafter, the fungitoxicity of extracts from ethanol treated sections decreased and remained lower than extracts from the untreated sections. As in the whole plants, the decrease in antifungal activity of extracts from ethanol treated stem sections paralleled the increase in the pathogen population.

DISCUSSION

Because symptom development and significant increases in the pathogen population are evident by the 5th day after inoculation, the susceptibility of the host plants to the wilt disease pathogen is clearly established by this time. Consequently, any resistance mechanism must also be set in motion by the 5th day after inoculation, and, thus, we concentrated our study on this early period of the host-pathogen interaction.

The use of excised stem sections in studying the early events in the wilt disease syndrome is a method which, in our case, yielded results similar to those obtained with whole plants. Thus, we confirm earlier reports on the suitability of excised stem sections for studies on this disease (32, 50). In fact, excised stem sections may, for some purposes, be more desirable than whole plants, because, for example, rishitin accumulation

Fig. 2. A, The population of Fusarium oxysporum f. sp. lycopersici race 1 (Fol R1) in excised stem sections of Jefferson tomato plants and in excised stem sections of Jefferson tomato plants treated with 1% ethanol (EtOH) for the first 6 days following inoculation with microconidia of the pathogen. B, Fungitoxicity of xylem extracts from excised stem sections of Jefferson tomato plants inoculated with Fol R1 and treated with EtOH, or inoculated with Fol R1 and untreated, for the first 5 days following inoculation with microconidia of the pathogen. Fungitoxicity was estimated by spore germination, colony development, and resporulation from about 1500 microconidia of the pathogen incubated 40 h in 150 μ l of nutrient medium containing the residue from 50 μ l of the xylem extract. Fungitoxicity values are the average of bioassays of extracts from three stem sections and are expressed by the index in the legend for Fig. 1-B.

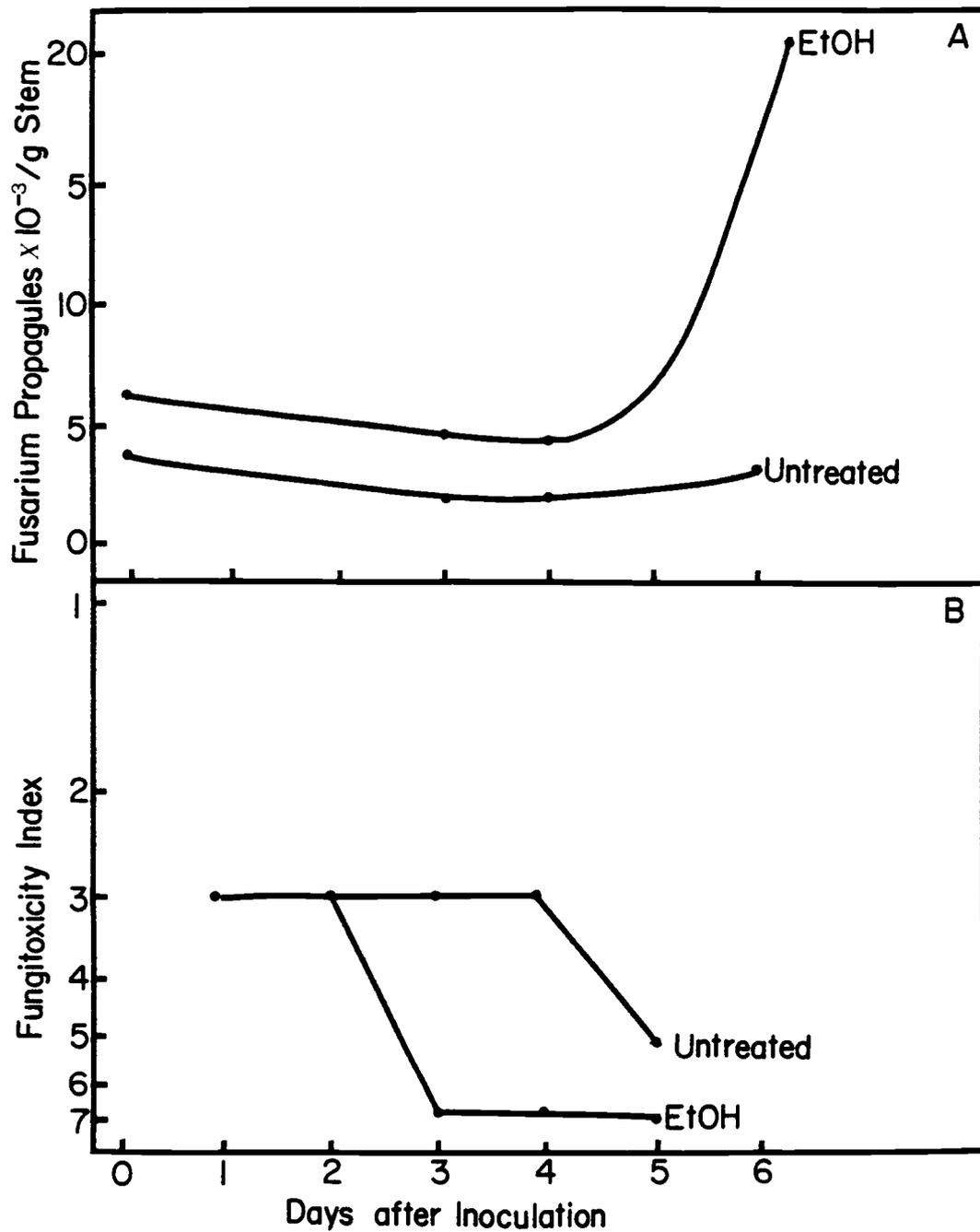


Fig. 2. The population of *Fusarium oxysporum* f. sp. *lycopersici* and the fungitoxicity of xylem extracts from tomato stem sections for the first 6 days after inoculation.

is greater in Fusarium infiltrated, excised stem sections than in tap root inoculated plants (39). Stem sections, however, could not be maintained for more than 5 days without dehydration of the pith and colonization by secondary microorganisms. Nevertheless, the use of excised stem sections provides a convenient, alternate method to study the early events in pathogenesis.

Ethanol negates the resistance to Fusarium wilt of tomato conferred by the I gene, but whether this is a direct effect on the host or on the pathogen is unknown. Ethanol affects plant respiration and the permeability of cell membranes (44), and at the levels used in this investigation could supply a carbon source for the pathogen. Isopropanol and methanol had no effect on the resistance of tomato to Fusarium wilt (44); thus, breaking resistance appears to be specific for ethanol, and is not a general response to treatment with alcohols. In addition, the transfer of inoculated plants from Hoagland's solution to water for a 4 h period each day has no effect on the resistance of tomato plants to Fusarium wilt (M. E. Corden, personal communication).

Induced susceptibility with ethanol in Jefferson tomato plants with a simultaneous increase in the pathogen population and a decrease in the level of xylem fungitoxicants suggests that these antifungal substances may, indeed, play a role in wilt disease resistance in tomato.

CHAPTER 4

CHARACTERIZATION OF ANTIFUNGAL COMPOUNDS FROM THE
XYLEM OF TOMATO PLANTS INOCULATED WITH
FUSARIUM OXYSPORUM F. SP. LYCOPERSICI

ABSTRACT

Antifungal compounds from the xylem of wilt disease resistant tomato plants (cv. Jefferson) inoculated with Fusarium oxysporum f. sp. lycopersici were partially purified and characterized. Four antifungal substances, including both hydrophilic and hydrophobic materials, were detected by various forms of chromatography. One of these antifungal compounds was purified to homogeneity by sequential thin layer chromatography and contained a conjugated unsaturated system. No rishitin or α -tomatine was detected in the xylem extracts, suggesting that compounds other than these two are involved in wilt disease resistance in tomato.

INTRODUCTION

Many studies on the nature of resistance in tomato (Lycopersicon esculentum Mill.) to the wilt disease pathogen Fusarium oxysporum Schlect. f. sp. lycopersici (Sacc.) Sny. and Hans. have concerned the involvement of α -tomatine and rishitin as fungitoxic agents that inhibit the growth of the pathogen in the host (27, 31, 39). It was generally concluded that neither substance could account for disease resistance, usually because the concentrations were not greater in resistant than in susceptible cultivars (23, 36). In studies of other tomato diseases, similar results were obtained (20)

and in some cases, rishitin could not be detected at all (16).

Recent evidence, however, suggests that α -tomatine and rishitin are not the only antifungal materials in tomato plants. For example, five different antifungal substances, including rishitin, were isolated from the fruits, and two antifungal compounds were obtained from the leaves of tomato plants inoculated with Cladosporium fulvum. Notably, rishitin was not detected in leaf extracts in the Cladosporium-tomato system. The two antifungal compounds from tomato leaves were vanillin positive, suggesting that they might be sesquiterpenes, but none of the antifungal substances isolated yielded a positive reaction for phenols with diazotized sulphanic acid (16).

Elgersma (20) found a higher concentration of rishitin in excised tomato xylem tissue from Verticillium wilt susceptible than from wilt resistant cultivars. However, at least four other antifungal compounds not identical with antifungal terpenoids from potatoes were detected by thin layer chromatography. In 1977, Stromberg and Corden (47) reported that acetone extracts from the xylem of a Fusarium wilt resistant cultivar of tomato were significantly more fungitoxic than corresponding extracts from a susceptible cultivar, but the chemical nature of these antifungal substances was not determined.

We have now partially purified and characterized the antifungal substances in a continuing effort to determine if resistance to Fusarium wilt in tomato is due to antifungal substances that limit the development of the

pathogen in the host.

MATERIALS AND METHODS

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

Microconidia of Fusarium oxysporum f. sp. lycopersici for plant inoculations were obtained by growing the pathogen in shake culture in potato dextrose broth for 4 days. Cultures were filtered through Whatman #4 filter paper, the filtrate was centrifuged at 7500 g for 10 min, the pellet was resuspended in sterile water, and the spore suspension was recentrifuged. The microconidial concentration was adjusted to 10^4 spores ml^{-1} with the aid of a hemocytometer.

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 h in the growth room under conditions favoring a high transpiration rate. Plants were then replanted in washed silica sand.

Five days after inoculation, xylem extracts were obtained from four 2.5 cm internode sections from the epicotyl of each plant by drawing 5.0 ml of absolute acetone through each by vacuum. Extracts were dried at 35 C under vacuum, and the residue was triturated with 0.2 ml absolute acetone

g^{-1} plant material. The supernatant solution was decanted from a pink residue and stored at -15 C . The rubber tubing and adapters used in extraction of internode sections were each soaked in 100 ml acetone before use. The extracts were evaporated to dryness and the residue was redissolved in 5.0 ml acetone.

The extracts were evaluated for antifungal activity by spotting aliquots in deep well slides, allowing the solvent to evaporate, and then adding 150 μl of a suspension of 10^4 Fusarium microconidia ml^{-1} . Controls consisted of 1000 μl acetone evaporated in the same manner as the xylem extracts. The microconidial suspension was prepared in a medium containing: NH_4NO_3 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, KH_2PO_4 3.5 g, sucrose 1.0 g, distilled water to 1 L (10), and was adjusted to pH 4.5. The slide cultures were incubated at 30 C for 40 h in the dark, and then spore germination, colony growth, and sporulation were graded 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 (47).

Gas-liquid chromatography was performed on a Hewlett-Packard 5830A gas chromatograph. Operational parameters were: oven temperature 170 C, injection temperature 210 C, flame ionization detector temperature 210 C, nitrogen carrier gas flow rate 50 ml min^{-1} . The column was a 152 cm X

4 mm stainless steel column packed with 3% OV-225 on 100/120 Supelcoport.

Absorption profiles and spectra were measured on a Beckman DB spectrophotometer. Absorption profiles of column effluents were measured for each fraction at the specified wavelength, and absorption spectra were measured by continuously scanning from 720 nm to 320 nm.

RESULTS

Crude acetone extracts from tomato xylem contained 20% water by volume, and when evaporated to dryness and the residue triturated with acetone, a large amount of a pink solid remained. This residue was soluble in water or 80% (v/v) acetone, but not absolute acetone. When bioassayed at high concentrations, this water soluble residue was not fungitoxic. Acetone controls were also not fungitoxic.

Silica Gel Column Chromatography. To separate the antifungal compounds in crude acetone extracts of tomato xylem, chromatography on silica gel was performed. Silica gel, 60-200 mesh (J. T. Baker Chemical Co.), was washed twice with a solution of 30% (v/v) ethyl acetate in cyclohexane, dried under suction, and then dried at 100 C for 1 h. The gel was then slurried with the same solvent and poured into a column 2.5 cm in diameter to a bed height of 29 cm. The extract from about 50 g of tissue was evaporated to dryness, dissolved in 2.0 ml of 30% (v/v) ethyl acetate in cyclohexane, and applied to the column. Fractions (50 ml) were collected as the column was eluted with a stepwise gradient. Steps consisted of 100 ml aliquots of solvent with 10% (v/v) changes in solvent composition

from 30% (v/v) ethyl acetate in cyclohexane to 100% ethyl acetate, and then from 10% (v/v) methanol in ethyl acetate to 50% (v/v) methanol in ethyl acetate. Each collected fraction was evaporated to dryness, and the residue was dissolved in 5.0 ml acetone. The procedure was repeated three times with similar results each time.

Column chromatography on silica gel separated four major peaks of antifungal activity (Fig. 1), which were designated I, II, III, and IV in order of elution from the column. Peaks I and II were eluted before any methanol appeared in the column effluent. Peaks I, II, and IV contained a number of pigmented substances, including chlorophylls, and peak III contained no pigmented compounds.

Sephadex LH-20 Column Chromatography. Sephadex LH-20 (Pharmacia Fine Chemicals) suspended in methanol was poured into a column 1.5 cm in diameter and 48 cm in bed height. The extract from about 50 g of tomato stems was evaporated to dryness and triturated with 2.0 ml methanol. The methanol soluble supernatant was applied to the column and eluted with methanol in 3.0 ml fractions. Each fraction was evaporated to dryness and dissolved in 2.0 ml methanol before further analysis. The procedure was repeated five times, with similar results each time.

Two major peaks and four minor peaks of antifungal activity were detected in the effluent from this column (Fig. 2-A). The first major peak was so highly fungitoxic that the relative toxicity of the other peaks was overestimated, but when a five-fold dilution of the column fractions was

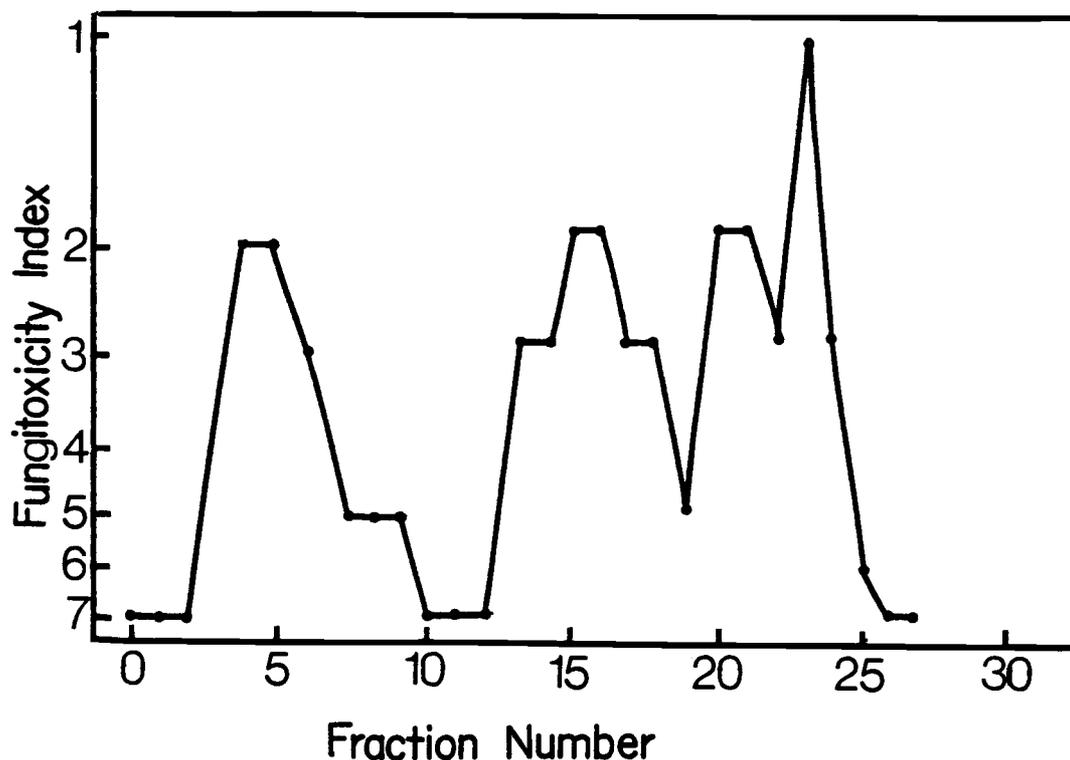


Fig. 1. Silica gel column chromatography of xylem extracts from Jefferson tomato plants obtained 5 days after inoculation with *Fusarium oxysporum* f. sp. *lycopersici*. The column was eluted with a stepwise gradient from 30% (v/v) ethyl acetate in cyclohexane to 100% ethyl acetate and then from 10% (v/v) methanol in ethyl acetate to 50% (v/v) methanol in ethyl acetate in steps consisting of 10% (v/v) changes in solvent composition. Prior to bioassay, each 50 ml fraction was evaporated to dryness, and the residue dissolved in 5.0 ml acetone. Fungitoxicity was estimated by spore germination, colony development, and resporulation from about 1500 microconidia of the pathogen incubated 40 h in 150 μ l of nutrient medium containing the residue from 450 μ l of the condensed column fractions. 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.

Fig. 2. Sephadex LH-20 column chromatography of xylem extracts from Jefferson tomato plants obtained 5 days after inoculation with Fusarium oxysporum f. sp. lycopersici. The column was eluted with methanol, and 3.0 ml fractions were collected. A, Fungitoxicity of column fractions, after condensation to 2.0 ml in methanol. Fungitoxicity was estimated by spore germination, colony development, and resporulation from about 1500 microconidia of the pathogen incubated 40 h in 150 μ l of nutrient medium containing the residue from 1000 μ l of the condensed column fractions. Fungitoxicity values are expressed by the index in the legend for Fig. 1. B, Absorption profile for the Sephadex LH-20 column effluent, measured by individual fractions in 2.0 ml methanol at 280 nm.

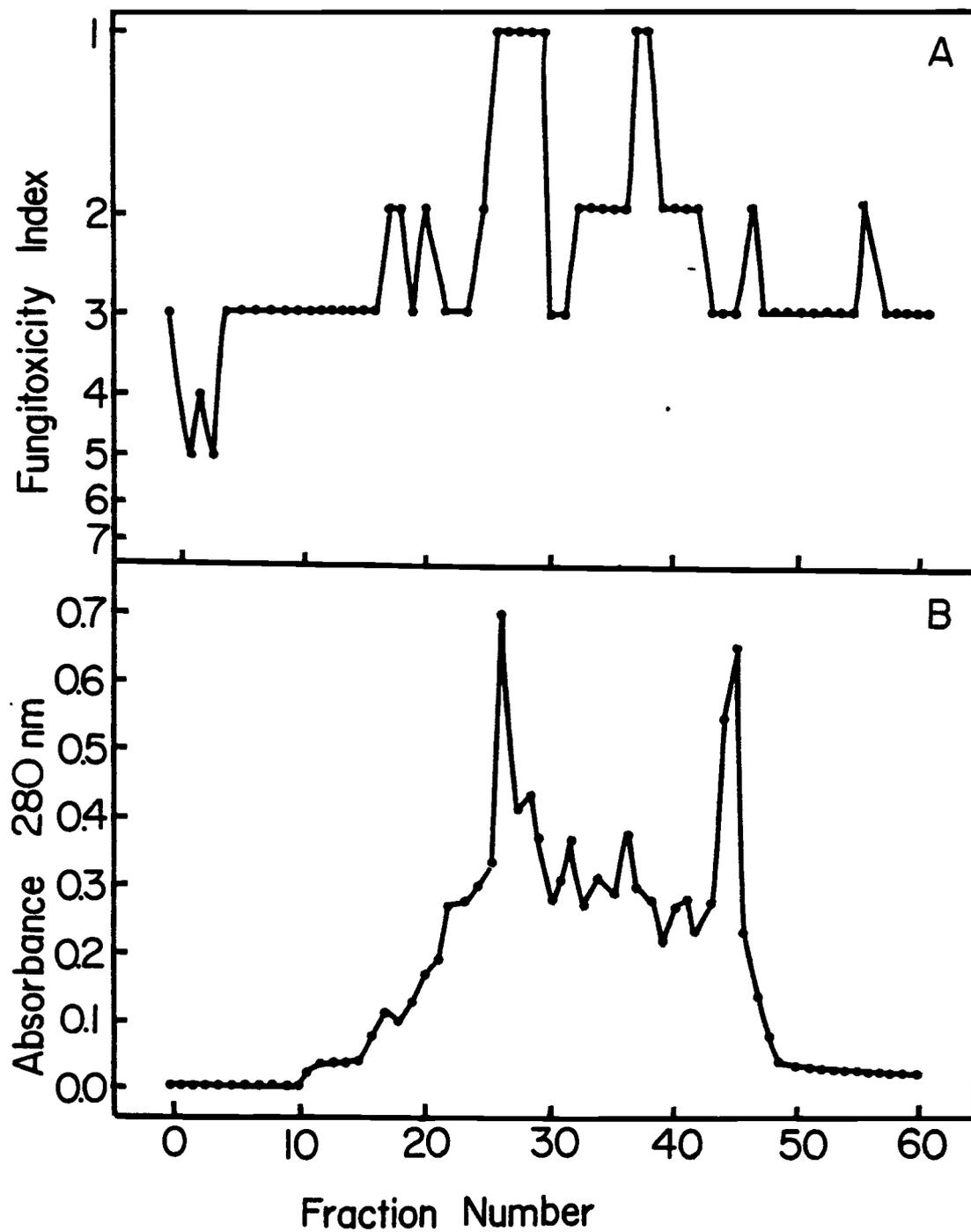


Fig. 2. Sephadex LH-20 column chromatography of xylem extracts.

bioassayed, only those fractions containing the first major peak were fungitoxic.

When the absorbance of individual fractions from the LH-20 column was measured at 280 nm, two major peaks in the resulting absorption profile appeared. The first of these peaks appeared in the same fractions as the major peak of antifungal activity, and the second appeared after most of the fungitoxic substances had been eluted (Fig. 2-B).

Sephadex G-10 Column Chromatography. Sephadex G-10 (Pharmacia Fine Chemicals) in distilled water was used to prepare a column 1.5 cm in diameter with a bed height of 48 cm. The extract from about 50 g of stem tissue was evaporated to dryness, the residue was suspended in 50 ml of hot water, and the suspension was filtered through diatomaceous earth (Celite analytical filter aid; Johns Manville Products). The filtrate was extracted three times with 50 ml of ethyl acetate, the ethyl acetate was evaporated off under reduced pressure, and the residue was dissolved in 1.0 ml distilled water. This sample was applied to the column which was then eluted with distilled water. One-hundred-fifty 5.0 ml fractions were collected, the solvent was evaporated off at 40 C under reduced pressure, and the residue from each fraction was dissolved in 1.0 ml acetone for bioassay. The procedure was repeated twice with similar results each time.

The procedure used to prepare a sample for chromatography on Sephadex G-10 removed most of the more hydrophobic materials in the extract, some of which were fungitoxic. The hydrophilic, fungitoxic materials that remain-

Fig. 3. Sephadex G-10 column chromatography of xylem extracts from Jefferson tomato plants obtained 5 days after inoculation with Fusarium oxysporum f. sp. lycopersici. Prior to chromatography, the extracts were suspended in water, filtered through celite, and extracted with ethyl acetate. Extracts were then dissolved in water, the column was eluted with water, and 5.0 ml fractions were collected. A, Fungitoxicity of column fractions, after evaporation of fractions to dryness and dissolution of the residue in 1.0 ml acetone. Fungitoxicity was estimated by spore germination, colony development, and resporulation from about 1500 microconidia of the pathogen incubated 40 h in 150 μ l of nutrient medium containing the residue from 600 μ l of the condensed column fractions. Fungitoxicity values are expressed by the index in the legend for Fig. 1. B, Absorption profile for the Sephadex G-10 column effluent, measured by individual fractions in 5.0 ml water at 254 nm.

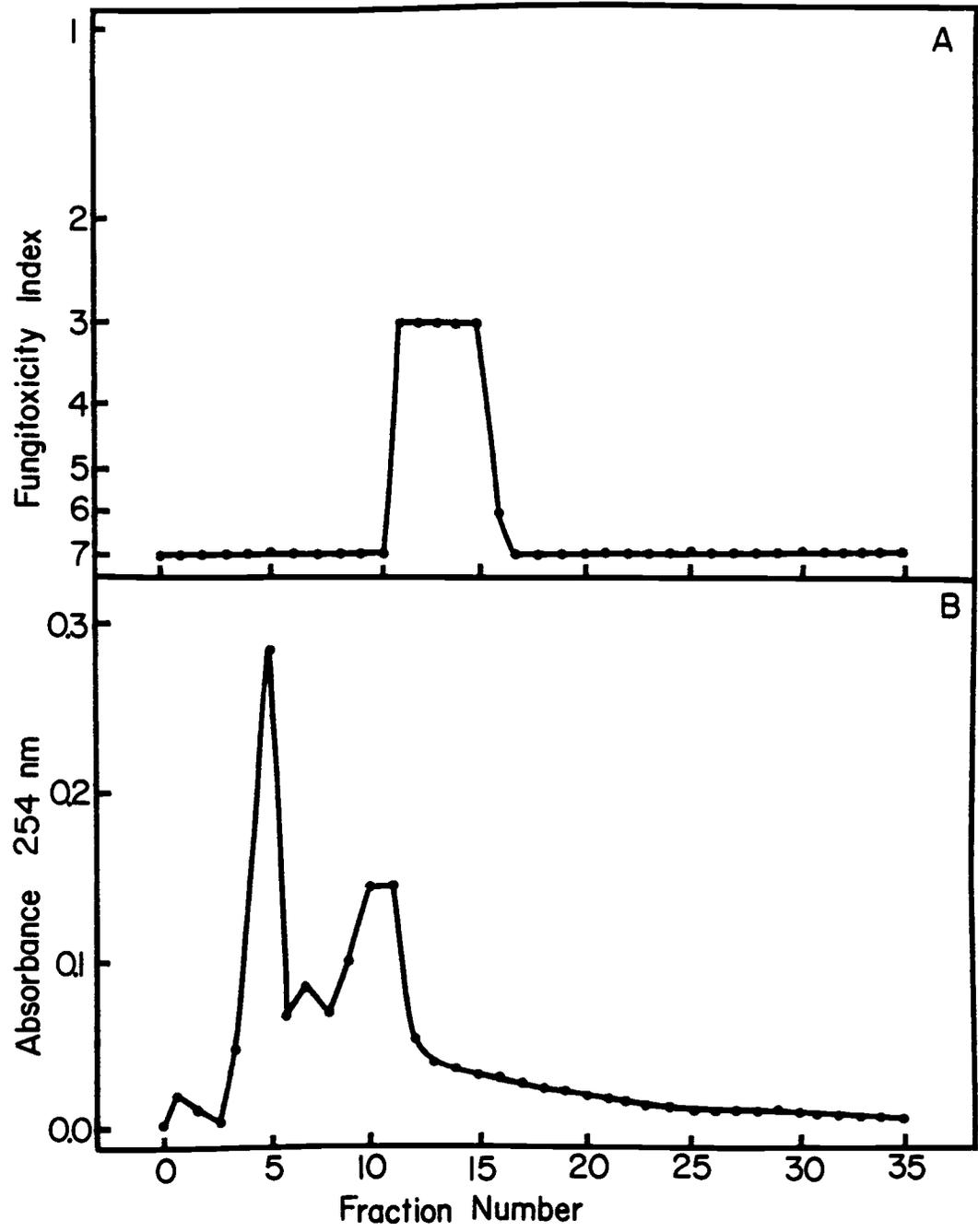


Fig. 3. Sephadex G-10 column chromatography of xylem extracts.

ed were eluted as a single peak in fractions 11 to 15 (Fig. 3-A). Most of the 254 nm absorbing materials in this extract were eluted before the antifungal materials (Fig. 3-B).

Thin Layer Chromatography. Glass plates (20 X 20 cm) were spread to a thickness of 0.25 mm with silica gel "G" (particle size 10-40 μm , 13% CaSO_4 binder; Sigma Chemical Co.) and were dried and activated at 110 C for 1 h before use. The solvent systems used, on a volume basis, were: (i) CHCl_3 , (ii) CHCl_3 : MeOH (96 : 4), (iii) cyclohexane : ethyl acetate (95 : 5), (iv) cyclohexane : ethyl acetate (90 : 10), (v) cyclohexane : ethyl acetate (50 : 50), (vi) ethyl acetate : MeOH : acetic acid : water (30 : 20 : 10 : 1), (vii) isopropanol : formic acid : water (73 : 3 : 24), and (viii) cyclohexane. For analysis of separated materials, the silica gel from developed plates was scraped off in 1.0 cm increments, beginning at the origin (fraction 1) and ending at the solvent front (fraction 16). The gel was then extracted three times with 10 ml acetone and the slurry was filtered through a fine porosity sintered glass filter. Filtrates were concentrated at 35 C and reduced pressure before bioassay.

Sequential thin layer chromatography on silica gel with various solvent systems provided the most convenient purification procedure for the anti-fungal compounds from tomato. As a rule, solvent (ii) was used to obtain a preliminary separation of the materials in the crude extracts. As with silica gel column chromatography, four major bands of antifungal activity were present, i.e. fractions 2, 5, 10, and 15.

The material in fraction 15, when rechromatographed successively in solvents (i), (iii), and (iv), yielded one major peak of antifungal activity in each solvent. R_f values in solvents (i), (ii), (iii), and (iv) were, respectively, 0.67, 0.97, 0.35, and 0.57. This substance was easily detected by its bright orange color in visible light.

The fungitoxic material in fraction 5 was rechromatographed in solvent (v). R_f values for this substance in solvents (ii) and (v) respectively were 0.22 and 0.10. This substance could be detected visually by the appearance of a hydrophobic spot after the plate was sprayed with distilled water, but did not react with vanillin, antimony trichloride, Nile blue A, or alkaline hydroxylamine. Fractions 2 and 10 were not subjected to further chromatography.

A comparison of the antifungal materials in crude extracts was made with rishitin and α -tomatine. Rishitin, when chromatographed in solvents (ii) and (v), yielded a red to violet colored spot when sprayed with a solution containing 1 g vanillin, 30 ml MeOH, 0.2 ml conc. H_2SO_4 , and then heated at 105 C for 3 min. R_f values for rishitin in solvents (ii) and (v) were 0.31 and 0.43 respectively. No spot corresponding to rishitin could be detected in crude extracts.

α -Tomatine, when chromatographed in solvents (vi) and (vii) produced a violet color when sprayed with sulfuric acid (50% v/v H_2SO_4) and then heated at 110 C for 5 min. R_f values for α -tomatine in solvents (vi) and (vii) were, respectively, 0.27 and 0.86. No spot corresponding to α -tomatine

could be detected in crude extracts.

Materials toxic to Fusarium were extracted from the rubber tubing and adapters used in the extraction of tomato xylem, but these materials, when chromatographed in solvent (viii), were not identical with any of the materials isolated from tomato xylem.

Analytical Procedures. The material from silica gel thin layer chromatography fraction 15 in solvent (ii), after subsequent chromatography in solvents (i), (iii), and (iv), yielded a single peak in gas liquid chromatography with a retention time of 61.26 min. This same material produced a UV and visible absorption spectrum with bands centered at 255 nm (K band) and 440 nm, with a shoulder at 320 nm (Fig. 4). The presence of the K band at 255 nm is indicative of a π - π conjugated system in this compound. No bathochromic or hypsochromic solvent effects were noted in the spectrum of this substance; the spectrum was the same in both methanol and hexane. The compound appeared as an orange oil when dried under vacuum, but tended to form hexagonal crystals when allowed to stand in methanol at -15 C. These crystals readily reverted to an oil when handled, and melted at 96 C.

DISCUSSION

Chromatography on silica gel yielded at least four fungitoxic substances extractable from tomato xylem. The fact that chlorophylls and other pigments were present in the extract suggests that xylem parenchyma, and possibly other adjacent cells as well, are extracted along with the xylem

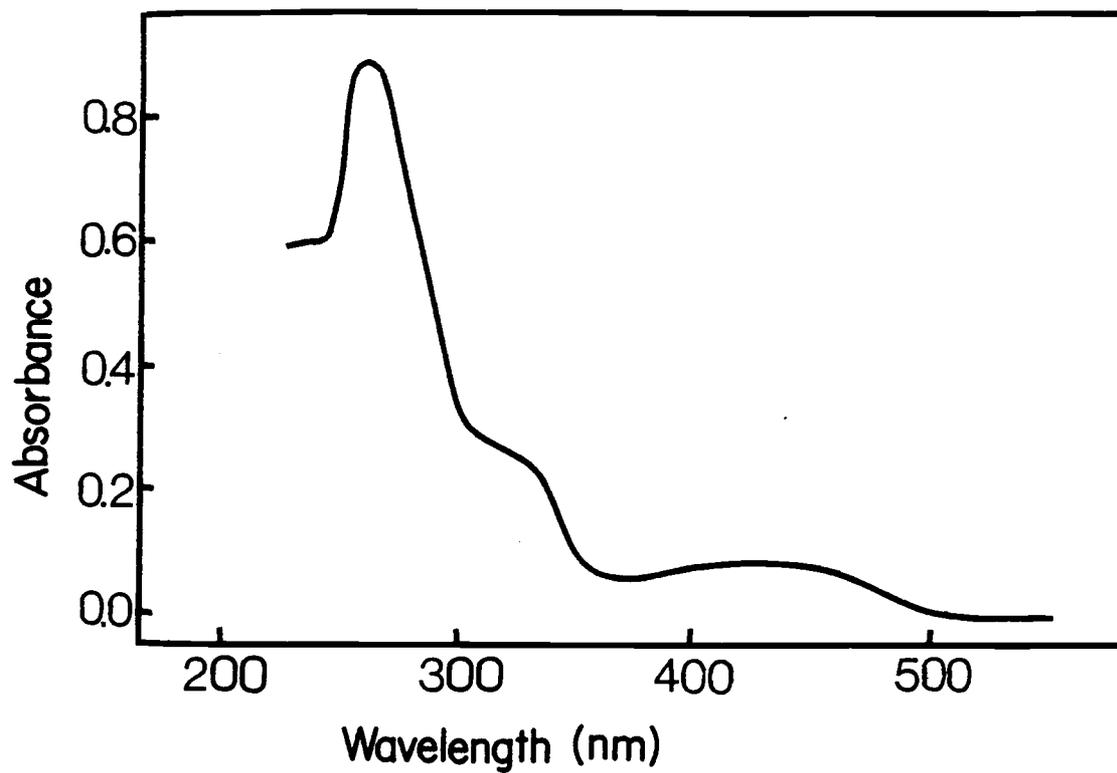


Fig. 4. Absorption spectrum in methanol of a purified antifungal compound isolated from the xylem of Jefferson tomato plants 5 days after inoculation with Fusarium oxysporum f. sp. lycopersici. Absorbance was scanned from 220 nm to 720 nm. The absorption spectrum was featureless above 550 nm.

vessels. Good evidence exists to demonstrate that the gross toxicity of xylem extracts from tomato markedly changes after inoculation with Fusarium oxysporum f. sp. lycopersici (47), and the ability to separate individual antifungal compounds from crude extracts provides a means to monitor the levels of each antifungal compound independently. Such a study will provide additional insight into the possible role of these antifungal substances in resistance of tomato plants to Fusarium wilt.

The antifungal compounds in these extracts encompass a wide range of solubilities, with at least one compound soluble in hexane, and at least one other soluble in water. Of the materials with high antifungal activity, only one occurred in sufficient quantity to be conveniently analysed. This substance (fraction 15 from thin layer chromatography in solvent (ii)) is pigmented and possesses a π - π conjugated system, as disclosed by the ultraviolet absorption K band at 255 nm. The compound may be no more than a chloroplast pigment which fortuitously possesses antifungal activity. However, the fact that a fungitoxic substance is pigmented does not automatically exclude it from consideration as a plant defense chemical, as some researchers have assumed (31). Indeed, gossypol is a yellow substance from cotton that has been implicated as a plant defense agent (8). Judging, however, from the relative amounts and toxicities of the substances isolated from tomato xylem, fraction 15 is far less toxic to Fusarium g^{-1} than are the other antifungal materials in the extract.

Significantly, no α -tomatine or rishitin was detected in the tomato

xylem extracts, suggesting that compounds other than these may be involved in wilt disease resistance in tomato.

CHAPTER 5

DISCUSSION AND CONCLUSIONS

Results concerning the levels of antifungal materials, especially α -tomatine (27) and rishitin (23), produced by tomato plants inoculated with Fusarium oxysporum f. sp. lycopersici have provided apparently conflicting results (30, 31, 36, 39). These discrepancies suggest that growing conditions may, in part, affect the levels of xylem fungitoxicants in tomato and the compounds studied to date may not play a primary role in the resistance reactions of tomato cultivars to Fusarium wilt.

The present study demonstrates that the antifungal activity in vitro of xylem extracts from tomato is profoundly affected by pH, with maximum antifungal activity at pH 4.5, and no antifungal activity at pH 6.0 or above. These results, however, demonstrate the effect of pH on the antifungal activity of crude extracts; the pH response of purified individual fungitoxic compounds may be quite different from the response of crude extracts. The amount of the toxicants in tomato xylem decreases with increasing water stress of the plants, a decline which parallels the decrease of phenylalanine ammonia lyase in water stressed plants (5). The results further demonstrate that the levels of these fungitoxic materials reaches a peak at the end of a dark period and wanes throughout a light period. This suggests that the antifungal compounds are either produced in higher quantities in the absence of light and are continuously modified to non-fungitoxic substances, or they

are continuously produced and converted to non-fungitoxic substances in the light. The effects of these and yet untested factors may explain not only the varying results from different laboratories, but may also provide clues to the nature of wilt disease resistance in tomato. The results reported here support the hypothesis that resistance may, at least in part, be due to the production of labile antifungal compounds by the host (25).

The application of ethanol to wilt disease resistant tomato plants inoculated with Fusarium not only negates resistance (44), but also prevents the increase of antifungal substances in the xylem of those plants. In fact, the pathogen population remains the same in both ethanol treated and untreated Jefferson tomato plants as long as the level of antifungal compounds in the xylem remains the same. Only after the antifungal activity of xylem extracts from ethanol treated plants is lower than that from untreated plants does the pathogen population increase in the ethanol treated plants. These results confirm the hypothesis that the mere ability to produce antifungal compounds is not the factor which ultimately confers resistance to a tomato cultivar inoculated with the wilt disease pathogen (46). Indeed, the difference between resistance and susceptibility may reside in the rapid accumulation of antifungal compounds. Such a phenomenon has been demonstrated in the wilt susceptible cultivar, Bonny Best, where the pathogen population in stems decreased after 8 days following inoculation and where the concentration of antifungal compounds begins to accumulate at that same time (47). However, by this time, the susceptible plants are already severely diseased.

In extracts obtained from the xylem of inoculated tomato plants, no rishitin or α -tomatine was detected. Since reliable evidence exists that these compounds are produced by tomato plants (23, 27), their absence in acetone extracts of the xylem may be a result of the extraction procedure. Since both rishitin and α -tomatine are soluble in 80% (v/v) acetone, the solvent used should have extracted these two compounds. The extraction method itself, however, differs greatly from the methods used by other researchers who generally used homogenates of either tomato stems, leaves, and roots (36, 39) or of frozen "predominantly xylem" tissue (39). These extracts would contain more materials from tissues not associated with xylem vessels than does the method used in the present study. The detection of antifungal compounds other than rishitin and α -tomatine in our study and the studies of other researchers (16, 20, 31, 50) suggests that compounds other than rishitin and α -tomatine may be involved in resistance of tomato to Fusarium wilt.

The results of this and other studies support further speculations on the reactions of tomato to infection by Fusarium, especially concerning the mechanism of wilting in susceptible cultivars and the development of vascular discoloration.

Tyloses, gel plugs, and gums have been implicated as resistance conferring structures which physically contain the pathogen within a limited portion of the tomato xylem vessels (6, 7). This hypothesis stems from reliable observations that these structures occur more frequently in

inoculated resistant than in susceptible cultivars of tomato (7, 31), but significant occlusion of vessels by tyloses occurs only after antifungal compounds have accumulated (31). Zimmerman (54), however, suggests that tyloses may serve to plug vessels containing air emboli or water vapor blocks which form during pathogenesis. In Fusarium wilt of tomato, tyloses may block off emboli after the advance of the pathogen has been halted by host produced antifungal compounds, and the wilting in susceptible cultivars may be due to the production of extensive emboli (54).

Vascular browning is generally considered to be an indication of a susceptible interaction between the tomato plant and the Fusarium wilt pathogen, but browning has been observed sooner and more intensely in resistant than in susceptible hosts (31). Since vascular browning is probably due to the oxidation and polymerization of polyphenols to melanins (15), one may speculate that the same compounds responsible for resistance are those responsible for vascular discoloration. If the determinant of resistance is the timing of accumulation of labile antifungal compounds, then the condensation products of these toxicants (i. e. melanin pigments) would accumulate sooner in resistant than in susceptible host-pathogen combinations. Furthermore, if the synthesis and accumulation of these substances are elicited by the pathogen, then browning would eventually develop more extensively, albeit at a later time, in the susceptible than in the resistant host.

Our studies suggest that the fundamental difference between susceptibil-

ity and resistance of tomato to Fusarium wilt is due to the timing of accumulation of labile antifungal compounds in the host, and that some recognition event is the ultimate determinant of susceptibility or resistance.

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