Fungitoxicity of Xylem Extracts From Tomato Plants Resistant or Susceptible to Fusarium Wilt

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ABSTRACT

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Acetone extracts of xylem vessels in the stems of wilt-resistant (Jefferson) and -susceptible (Bonny Best) tomato cultivars contained fungitoxic materials at concentrations that suppressed the growth of *Fusarium oxysporum* f. sp. *lycopersici* race 1 in vivo. Following inoculations in which spores of the pathogen were distributed throughout the major vessels of the stem, the population of viable fungal cells in both tomato cultivars rapidly decreased. In the susceptible cultivar, about 3 days after inoculation, the fungal population began to increase, while the fungitoxicity of the xylem extract decreased. In the resistant cultivar during this period, the *Fusarium* population remained low and the xylem extracts became highly fungitoxic. The fungitoxic material persisted in the resistant cultivar at a level that suppressed the growth of the pathogen; it may, in part, have been responsible for containment of the pathogen and thereby contributed to wilt resistance in tomato. In stems of noninoculated control plants, a high bacterial population developed that can confound many experiments with this disease.

Monogenic resistance in tomato (*Lycopersicon esculentum* Mill.) to wilt disease caused by *Fusarium oxysporum* Schlecht. f. sp. *lycopersici* (Sacc.) Snyd. and Hans. is an outstanding example of effective plant disease control by resistant cultivars, but the nature of this resistance is not fully understood. Early attempts by Heinze and Andrus (5), Synder et al. (12), and Irving et al. (6) to account for resistance by the presence of fungitoxic materials in roots, stems, or xylem exudates of resistant cultivars were largely unsuccessful. This led Walker (14) in his 1971 review to suggest that the toxigenic differences between xylem extracts from susceptible and resistant cultivars generally are not significant. Recent studies, however, suggest that tomatine (4, 7) and rishitin (10) may inhibit the pathogen in roots and stems of tomato plants.

Beckman (1) postulated that resistance depends primarily on the physical localization of the pathogen in xylem elements through vascular occlusions by tyloses and gels. This hypothesis, however, seems inconsistent with results obtained earlier by Scheffer and Walker (11) in which stem cuttings of resistant and susceptible tomato cultivars were allowed to take up conidia until the xylem vessels contained *Fusarium* spores to the apex of the cuttings. Under these conditions, physical localization of the pathogen within the xylem elements is for the most part by-passed; yet when these cuttings were rooted those of the susceptible cultivar developed severe disease symptoms while those of the resistant cultivar failed to develop symptoms and the pathogen died-out, particularly in the upper portions of the cuttings. These results suggest that fungitoxic materials within the

resistant host may limit the development of the pathogen. This, and Bugbee's (2) demonstration of a simple method of obtaining extracts exclusively from xylem elements, prompted our decision to re-evaluate the influence of xylem toxicants on growth and development of *F. oxysporum* f. sp. *lycopersici* (13).

MATERIALS AND METHODS

Susceptible Bonny Best and single-gene-resistant Jefferson tomato plants were grown in washed silica sand and watered twice daily with Hoagland's nutrient solution. The plants were maintained in a controlled-environment room at 30 C with about 21,520 lux light intensity 16 hr per day.

Forty-day-old plants, generally with six leaves, were inoculated with *Fusarium oxysporum* f. sp. *lycopersici* race 1 through the severed taproot where it was about 5 mm in diameter. The cuttings were placed singly in flasks that contained 50 ml of a suspension with 1×10^5 spores/ml of sterile distilled water and allowed to take up the spore suspension under full illumination at 30 C for 4 hr. Intact plants or cuttings allowed to take up sterile distilled water served as controls. After inoculation or incubation in sterile distilled water, the plants were repotted in washed silica sand and watered twice daily with Hoagland's nutrient solution.

The *Fusarium* population in stems of inoculated plants was estimated using a 2.5-cm section from the internode between nodes that bore the first two true leaves. The stems were surface-sterilized with a 1.3% sodium hypochlorite solution, and rinsed with sterile distilled water. Stem sections were cut, weighed, sliced into 35-40 thin-sections, and mascerated in 10 ml of sterile distilled

water in a Sorvall Omni-Mixer. The stem slurry was diluted and plated in potato-dextrose agar (PDA) adjusted to pH 4.0 with lactic acid and the *Fusarium* content per gram fresh weight of stem was estimated from the number of colonies obtained.

Periodically following inoculation, xylem extracts for fungitoxicity measurements were obtained from two 2.5-cm stem sections excised from the internode between the nodes that bore the first and second true leaves. Short pieces of rubber tubing were fixed to each end of the stem sections and the xylem was extracted under reduced pressure by passing 2 ml of anhydrous acetone through the stem sections in a manner similar to the technique described by Bugbee (2). A fluorescein dye in acetone was passed through a separate set of stem sections to confirm the exclusive extraction of the xylem. Microscopic examination revealed the dye only in the walls of the vessel elements.

The acetone extracts of the xylem tissue from 10 plants were concentrated in a rotary vacuum evaporator at 50 C. The extract residues were brought up to 4 ml in a mixture of acetone: water (8:2, v/v) and were stored at 0 C. To determine their fungitoxicity, aliquots of the extracts ranging from 50 to 1,000 μliters were placed in deepwell slides and the extracts were allowed to go to dryness. A suspension of *Fusarium* microconidia (250 μliters containing about 2,500 spores) was placed in each

well, and the slides were incubated for 46 hr in darkness at 25 C after which spore germination, colony growth and sporulation were recorded. Fungitoxicity was rated according to the scale: 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.

All experiments were suitably replicated and repeated at least three times.

RESULTS

The *Fusarium* population in the stems of the resistant and susceptible cultivars decreased significantly during the 1st day following inoculation, then remained low through the 3rd day (Fig. 1). Between the 3rd and 4th days, the *Fusarium* population in the susceptible cultivar began to increase and eventually peaked on the 8th day at almost 10-fold the initial population. During this rapid proliferation of the fungal population, wilting and yellowing of the leaves appeared (5th day) and the disease symptoms were relatively severe by the 8th day. In the resistant cultivar, the *Fusarium* population remained low and the plants failed to develop disease symptoms throughout the experiment.

Fungitoxicity of the xylem extracts (500 μliters) from inoculated and intact plants of both cultivars was monitored over a 7-day period. Immediately following the inoculation period (time 0), the extracts were all moderately toxic. The extract from the inoculated susceptible cultivar initially allowed germination of the microconidia and development of small colonies, but by the 4th day fungitoxicity had diminished sufficiently to
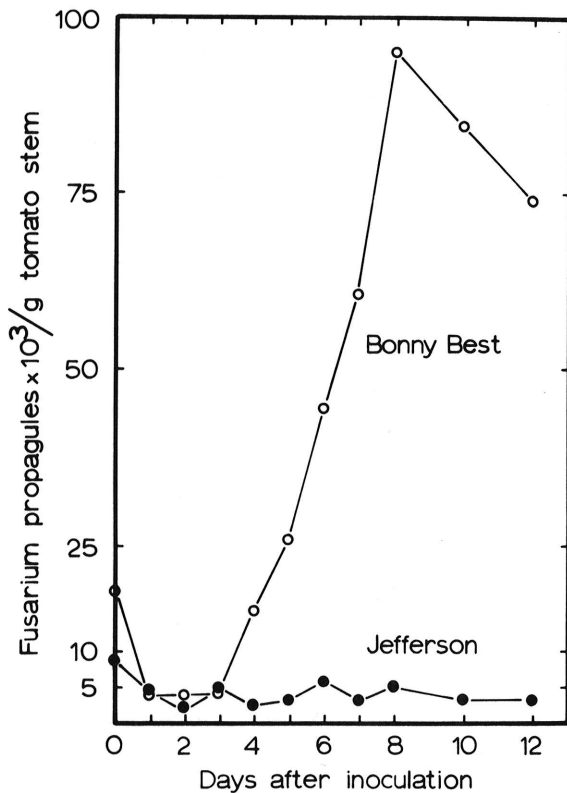

Fig. 1. The population of *Fusarium oxysporum* f. sp. *lycopersici* in stems of Bonny Best (Fusarium wilt-susceptible) and Jefferson (Fusarium wilt-resistant) tomato plants at various times following inoculation with microconidia of the pathogen.
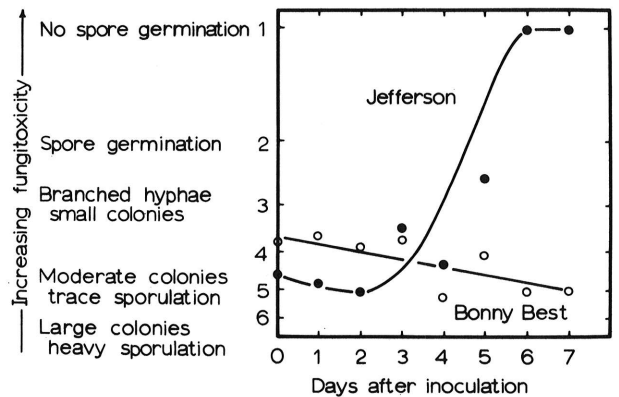

Fig. 2. Fungitoxicity of xylem extracts from stems of Bonny Best (Fusarium wilt-susceptible) and Jefferson (Fusarium wilt-resistant) tomato plants at various times following inoculation with microconidia of *Fusarium oxysporum* f. sp. *lycopersici*. Fungitoxicity was estimated by inhibition of spore germination and subsequent colony development from about 2,500 microconidia of the pathogen incubated 46 hr in 250 μliters of sterile distilled water containing the residue from 500 μliters of the xylem extracts. Fungitoxicity values are the average of bioassays of extracts from 10 plants from each of six different experiments.

permit more luxuriant colony growth and sporulation (Fig. 2). This drop in toxicity corresponded to the increase in the *Fusarium* population that commenced on the 4th day in stems of the susceptible cultivar (Fig. 1). By the 8th or 9th day after inoculation, fungitoxicity of the extracts from inoculated susceptible plants began to increase when the population of the pathogen began to decline (Fig. 1), but the disease symptoms at this time were already severe.

Fungitoxicity of the extracts from noninoculated intact plants (Fig. 4-A) of both cultivars was similar to that obtained from the inoculated susceptible cultivar. In the wounded, noninoculated control (Fig. 4-B), the response of the susceptible cultivar was similar to that in the corresponding intact, noninoculated and *Fusarium*-inoculated plants, but in the resistant cultivar moderate fungitoxicity developed on the 3rd day then rapidly decreased. The development of high fungitoxicity on the 5th through the 7th days typical of *Fusarium*-inoculated plants did not occur in the wounded, noninoculated controls.

The extract from the inoculated resistant cultivar, although initially somewhat less fungitoxic than that from the susceptible cultivar, increased significantly in toxicity after a period of 3-4 days following inoculation and by the 6th day completely inhibited germination of the microconidia (Fig. 2). A smaller volume of the extract (250 μliters) also completely inhibited spore germination.

The assay suspensions containing nongerminated microconidia were diluted 1:99 in molten PDA (42 C) and immediately poured into petri plates to determine the
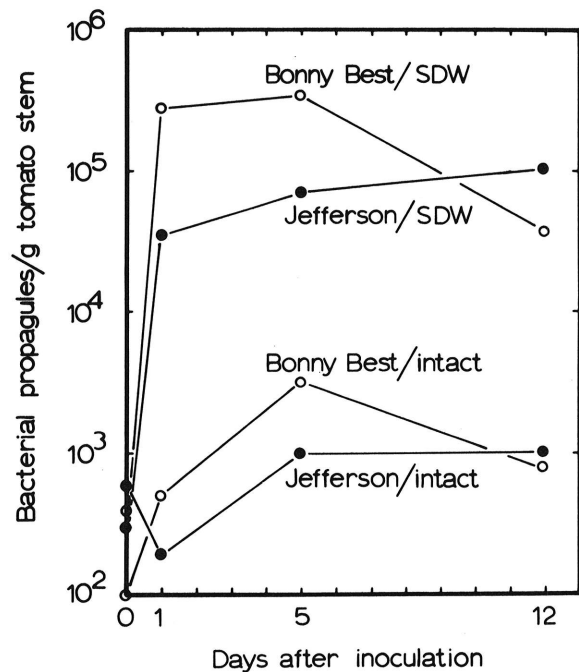
viability of the spores. In all cases, these spores failed to initiate colonies, which indicated that the extract from the resistant cultivar had been fungicidal.

Selection of suitable controls for the experiments described above presents a difficult problem. If intact
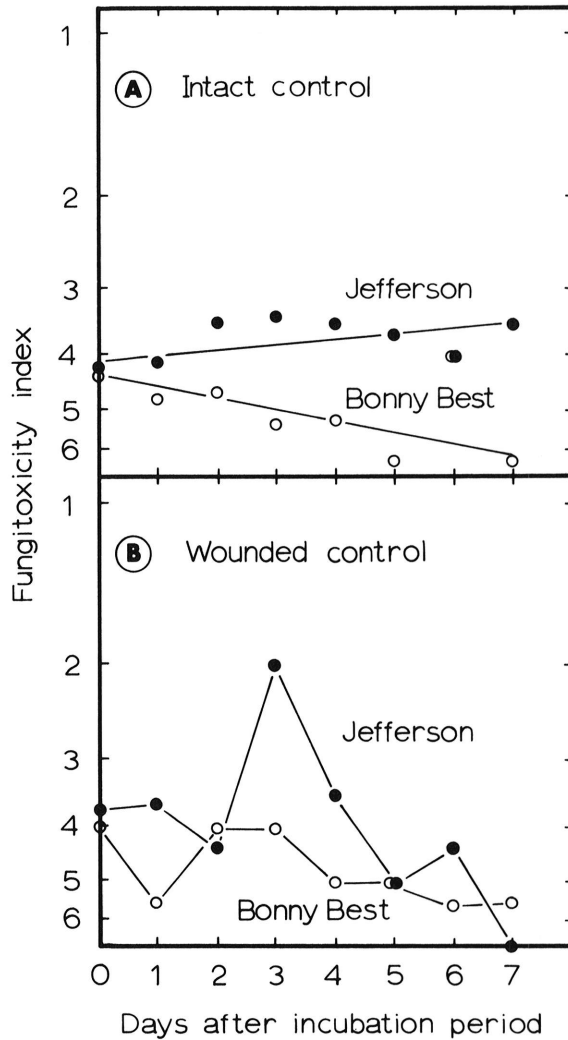

Fig. 4-(A-B). Fungitoxicity of xylem extracts from stems of Bonny Best (Fusarium wilt-susceptible) and Jefferson (Fusarium wilt-resistant) tomato plants. A) Intact noninoculated control plants, and B) wounded control plants incubated 4 hr in sterile distilled water then transplanted to silica sand. Fungitoxicity was estimated at various times after the incubation period by spore germination and subsequent colony development from about 2,500 microconidia of *Fusarium oxysporum* f. sp. *lycopersici* incubated 46 hr in 250 μliters of sterile distilled water that contained the residue from 500 μliters of the xylem extracts. Fungitoxicity values are the averages of bioassays of extracts from 10 plants from each of six different experiments 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. 3. The bacterial population in stems of Bonny Best (Fusarium wilt-susceptible) and Jefferson (Fusarium wilt-resistant) tomato plants at various times following incubation of stem cuttings in sterile distilled water (SDW) or in stems from intact plants.

plants are used there is no control for the wounding during the inoculation procedure, but if plants are treated like the inoculated plants except for incubation in sterile distilled water instead of a *Fusarium* microspore suspension, a significant bacterial population develops in the stems. Stem sections from tomato cuttings (like those used for determination of the *Fusarium* population, but incubated in sterile distilled water) were mascerated and the slurry was diluted, plated in PDA (pH 6.0), and the bacterial colonies were subsequently counted. The bacterial population in the resistant and susceptible stems infused with sterile distilled water rose sharply during the first 24 hr then remained at a relatively high level through the 12th day (Fig. 3). As with the *Fusarium* population in inoculated plants, the bacterial population in the stems of plants resistant to *Fusarium* wilt was significantly lower than that in the susceptible cultivar during the first 5 days after inoculation. Intact plants of both cultivars had a relatively low bacterial population (Fig. 3). The bacterial population in Jefferson water-infused control plants may have been responsible for the moderate fungitoxicity that developed on the 3rd day (Fig. 4-B), but the bacteria did not induce the high fungitoxicity on the 5th through the 7th day that was typical of the *Fusarium* inoculated plants (Fig. 2). Nevertheless, the high bacterial population is a potential confounding factor in selecting controls for experiments of this type.

DISCUSSION

Our results suggest that fungitoxic materials produced or released in the xylem vessels of Bonny Best and Jefferson tomato cultivars initially inhibit the growth of the pathogen within the host. In the susceptible cultivar, the pathogen eventually grows extensively in the xylem vessels, and development of severe disease symptoms closely follows the proliferation of the pathogen. Crude extracts from the xylem of the susceptible cultivar do not become highly fungitoxic until 8 or 9 days after inoculation when the symptoms are already severe. In the resistant cultivar, within 3-4 days after inoculation the crude extract becomes highly toxic, the fungal population within the stem remains low, and disease symptoms fail to develop.

Past failures to isolate fungitoxic materials from the xylem tissue and sap of tomato cultivars resistant to Fusarium wilt may have resulted from use of improper solvent or extraction methods, the lability of the toxicant, its continual metabolism to nontoxic materials, or to binding of the toxicant within the plant tissues. Maintenance of effective phytoalexin concentrations is generally dependent on their concentration at the initial site of host-pathogen interaction with limited translocation within the plant.

Our inoculation methods were selected to enhance characterization of the host-pathogen interaction sequence. By allowing the spore inoculum to be taken into the cuttings for 4 hr under high transpiration conditions, we obtained a relatively high inoculum concentration throughout the root and stem xylem. Thus, our stem samples contained a synchronous culture of *Fusarium* spores interacting with host cells. Conversely, stem sections from plants growing in infested soil or root-dip

inoculated contain xylem tissues at various stages of interaction with the pathogen. In these plants, *Fusarium* advances up stems primarily by mycelial growth (8), and thus, characterization of the sequence in host-pathogen interaction is confounded by lack of uniformity within relatively large tissue samples. This could account for some failures to isolate significant quantities of fungitoxic materials from infected plants.

Preliminary experiments indicated that the total polyphenolic content of the crude xylem extracts correlates with their fungitoxicity (M. E. Corden, *unpublished*). Matta et. al. (9) suggested that acquired resistance of tomato plants after inoculation with nonpathogenic formae of *F. oxysporum* may depend on release of phenolic substances in response to the nonpathogen, but the identity and fungitoxicity of these polyphenolics were not determined.

Recently, tomatine (4, 7) and rishitin (10) were isolated from *Fusarium*-inoculated tomato plants, their in vitro fungitoxicity to *Fusarium* was demonstrated, and the activity of these compounds in restricting the pathogen in the resistant host was suggested. Correlative studies of changes in the *Fusarium* population within the host and the occurrence of tomatine and rishitin in the xylem vessels may aid in assessing the in vivo effectiveness of these compounds.

The concentration of inhibitory materials in tomato stem vessels is sufficient to suppress growth of the pathogen in the host. For example, the crude extract (250 μliters) from 1.25 cm of resistant plant stems (about 1.0 g) collected 6 days after inoculation completely inhibited germination of 2,500 *Fusarium* spores (Fig. 2). Inoculated resistant plants contained 2,000 to 6,800 propagules/g of stems from plants 1-12 days after inoculation (Fig. 1), and thus, the toxicant-to-fungus ratio in the bioassay approximated the ratios in inoculated plants.

Beckman (1) has dismissed the role of chemical inhibition of *Fusarium* in wilt resistance primarily on limited but equal growth of the pathogen out of the cut ends of inoculated petioles of a resistant and a susceptible cultivar into sterile distilled water. This experimental design, however, does not provide a valid estimate of the growth of *Fusarium* in the host's vascular elements during pathogenesis. The presence of fungitoxic materials in the xylem at effective concentrations, and reductions in the *Fusarium* population following inoculation of resistant plants (11) suggests that physical localization of the pathogen probably is not the sole or even a primary defense mechanism in tomato to Fusarium wilt. Recently, Collins and Nielsen (3) made a similar suggestion for Fusarium wilt of sweet potatoes.

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