The Significance of Tomatine in the Host Response of Susceptible and Resistant Tomato Isolines Infected with Two Races of *Fusarium oxysporum* f. sp. *lycopersici*

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ABSTRACT

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Tomatine concentrations in the xylem fluid of Improved Pearson (IP) and Pearson VF-11 (VF) near-isolines of tomato were compared following wounding or wounding and inoculating with *Fusarium oxysporum* f. sp. *lycopersici* race 1 or race 2. VF is resistant to race 1 but susceptible to race 2; IP is susceptible to both races. Tomatine levels were similar in both isolines prior to treatment. Tomatine remained at the pretreatment level (3×10^{-4} M) in the IP plants regardless of treatment, but increased to fungitoxic levels (10^{-3} M) 2 days after wounding or inoculation in both the resistant VF/race 1 and susceptible VF/race 2 combinations. Tomatine in vitro was inhibitory to the vegetative growth of both races. Tomatine stimulated spore production of both races. The data suggest that tomatine is not a primary determinant of resistance to *F. oxysporum* f. sp. *lycopersici*. The possible role of tomatine in a sequential resistance process involving physical localization responses is discussed.

Additional key words: fungitoxicant, host resistance mechanisms.

The possible involvement of tomatine in the resistance of tomato to *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyd. & Hans. has been examined by several investigators. However, evidence has been conflicting and has failed to establish a direct involvement of tomatine in resistance. Concentrations of tomatine before and after inoculation of resistant and susceptible cultivars have been considered as one means of evaluating the role of tomatine in resistance (8,10,11,14,19–21). The toxicity of crude extracts (9) and of purified tomatine (1) toward race 1 of the pathogen has also been considered. Few studies have used near-isolines (8,13), and only one (20) has included *F. oxysporum* f. sp. *lycopersici* race 2, a more virulent race of the pathogen. Our preliminary investigations indicated that crude xylem extracts were not inhibitory to the germination of conidia of race 1 or race 2. Two objectives of this study were to compare tomatine concentrations in the xylem fluid of near-isolines of tomato inoculated with race 1 or race 2 and compare in vitro the effect of tomatine on race 1 and race 2 with respect to vegetative growth, germ tube extension, and spore germination.

The importance of spore production to the rapid distribution of vascular pathogens within the xylem vessels of the host is well documented (2,6). A host-produced substance that could effectively inhibit sporulation within the vessels could limit the ability of the pathogen to colonize the host and, thus, contribute to resistance. The third objective of this study was, therefore, to determine the effect of tomatine on microconidial production of each race.

MATERIALS AND METHODS

Preparation of spore suspensions. Isolates of *F. oxysporum* f. sp. *lycopersici* were grown on potato-dextrose agar (PDA) and incubated at 27 C in the dark. Microconidia were washed from the cultures with distilled water and filtered through lens paper. The spore concentration was adjusted with the aid of a hemacytometer.

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Inoculation of host. The near-isogenic cultivars of tomato (*Lycopersicon esculentum* (Mill.) Improved Pearson (IP) and Pearson VF-11 (VF)) which are, respectively, susceptible and resistant to race 1, served as hosts. Both cultivars are susceptible to race 2. Tomato plants were grown under greenhouse conditions in 10-cm-diameter pots containing a soil:peat:perlite mix (1:1:1,v/v). Natural daylight was extended with cool-white fluorescent lights to provide a 15-hr photoperiod. Plants grown to the seven- to eight-leaf stage were inoculated according to the method of Conway and MacHardy (4). Wounded, uninoculated plants were treated similarly, but distilled water was used in place of the spore suspension. Nonwounded, uninoculated plants served as controls.

Quantitative determination of tomatine in xylem fluid. Stems were severed between the first and second nodes at 2, 7, 12, or 17 days after wounding or wounding and inoculating. Xylem fluid exuded by root pressure was collected from decapitated plants over a period of 48 hr (17). Each treatment consisted of 25 plants and was repeated three times. Exudate was passed through a Millipore filter (0.45-μm pore size) and stored at −17 C.

Tomatine was extracted by a modification of the method of Roddick and Butcher (16). Xylem fluid (5 ml) was evaporated to dryness under reduced pressure at 45 C and then dissolved in 10 ml of 50% methanol acidified with two drops of 50% H_2SO_4. Samples (500 μl) were applied as a band to thin-layer chromatography plates (precoated silica gel, 20 × 20 cm), and plates were developed in isopropanol-formic acid-water (73:3:24), v/v). Tomatine located (R_f 0.66) using an iodine chamber (15) or by spraying with modified Dragendorff reagent (5, page 506). In both treatments tomatine gave a bright orange color reaction. The tomatine zone was scraped from untreated chromatograms, eluted into 6 ml of 50% methanol acidified with one drop of 50% H_2SO_4, and the eluate was evaporated to dryness. The residue was dissolved in 10 ml of concentrated H_2SO_4 and incubated at 40 C for 24 hr. The tomatine concentration was determined from the percent transmittance of the H_2SO_4-tomatine chromagen at 325 nm (16). A standard curve was developed by duplicating the above procedure using commercial alpha-tomatine (ICN Life Sciences Group, Cleveland, OH 44128).

Preparation of tomatine solutions for in vitro studies. Commercial tomatine was dissolved in 10 mM HCl and adjusted to pH 4.6 with a phosphate-citrate buffer. A pH of 4.6 was shown to

be optimum for conidial germination of *Fusarium* (18). Control solutions contained the HCl and buffer (pH 4.6), but lacked tomatine. Solutions were filter sterilized (Millipore, 0.2-μm pore size).

Spore germination bioassay. Measured drops of race 1 or race 2 spore suspensions (2×10^5 conidia per milliliter of distilled water) were combined with an equal volume of the test solution on a microscope slide coated with 2.5% cellulose nitrate. The cellulose nitrate coating kept the droplets from dispersing on the slide. Treatments were replicated six times. After 24 hr of incubation at 27 C, spores were stained and fixed with cotton blue in lactophenol. Percent germination was determined for 100 spores in three random fields per replicate. Spores were considered germinated if the germ tube was at least the length of the spore.

TABLE 1. Estimated concentration of tomatine in tomato xylem fluid exudate 2–4, 7–9, 12–14, and 17–19 days after wounding and/or inoculation with *Fusarium oxysporum* f. sp. *lycopersici* race 1 or race 2

Treatment[x]	Host/pathogen combination[y]	Tomatine concentration ($\times 10^{-4}$ M) after days: 2–4	7–9	12–14	17–19
VF					
Nonwounded	...	1 a[z]	...	...	...
Wounded, uninoculated	...	12 bcd	6 a	1 a	1 a
Wounded, inoculated, R1	R	19 d	16 b	1 a	3 a
Wounded, inoculated, R2	S	14 cd	15 b	8 a	7 a
IP					
Nonwounded	...	3 a	...	...	...
Wounded, uninoculated	...	2 a	2 a	1 a	4 a
Wounded, inoculated, R1	S	9 abc	4 a	1 a	1 a
Wounded, inoculated, R2	S	5 abc	1 a	4 a	6 a

[x] VF and IP are near-isolines of tomato cultivars Pearson VF-11 (resistant to race 1 [R1], susceptible to race 2 [R2]), and Improved Pearson (susceptible to both races), respectively.

[y] R = resistant host/pathogen combination; S = susceptible host/pathogen combination.

[z] Each value represents the mean of three observations. Means within a column not followed by the same letter are significantly different ($P = 0.05$) according to Duncan's multiple range test.

Effect of tomatine on colony growth and spore production. Tomatine concentrations of 10^{-3}, 2×10^{-4}, 4×10^{-5}, and 8×10^{-6} M in rehydrated Difco PDA were tested. Control media lacked tomatine. Media in sterile 60 × 16-mm plastic petri dishes were inoculated with 4-mm-diameter agar plugs taken from the margin of 4-day-old race 1 or race 2 colonies. Cultures were incubated at 27 C in the dark. Radial growth (mean of two perpendicular diameters) and spore production (number of microconidia produced per milligram dry weight) were determined every 48 hr for 2 wk for each of three replicates. To determine spore production, spores were washed from each plate with distilled water, the spore suspension was brought up to a constant volume (20 ml), and spores were counted with the aid of a hemacytometer. A separate set of plates was used to obtain dry weights. Colonies were freed from the media by melting and filtration. Colonies were washed to remove traces of medium. The mycelium was then oven-dried at 110 C for 24 hr and weighed. The three dry-weight values were randomly matched with the three total spore counts of the same treatment and the number of spores per milligram dry weight was calculated. Spores from 4- and 8-day-old colonies on 10^{-3} M and 2×10^{-4} M tomatine and the control media were tested for viability using a spore germination bioassay.

Effect of tomatine on spore germination and germ tube extension. Seven tomatine concentrations (10^{-3}, 8×10^{-4}, 4×10^{-4}, 2×10^{-4}, 4×10^{-5}, 8×10^{-6}, and 16×10^{-6} M) were tested. Solutions were prepared using a phosphate-citrate buffer as described above. Control solutions lacked tomatine. All treatments were replicated six times. Percent germination was determined as described above. Germ tube lengths for 25 spores per replicate were measured using a microscope fitted with an eyepiece micrometer.

RESULTS

Quantitative determination of tomatine in xylem fluid. Variation of tomatine concentrations was observed within treatments. Statistical analysis revealed no significant difference in the

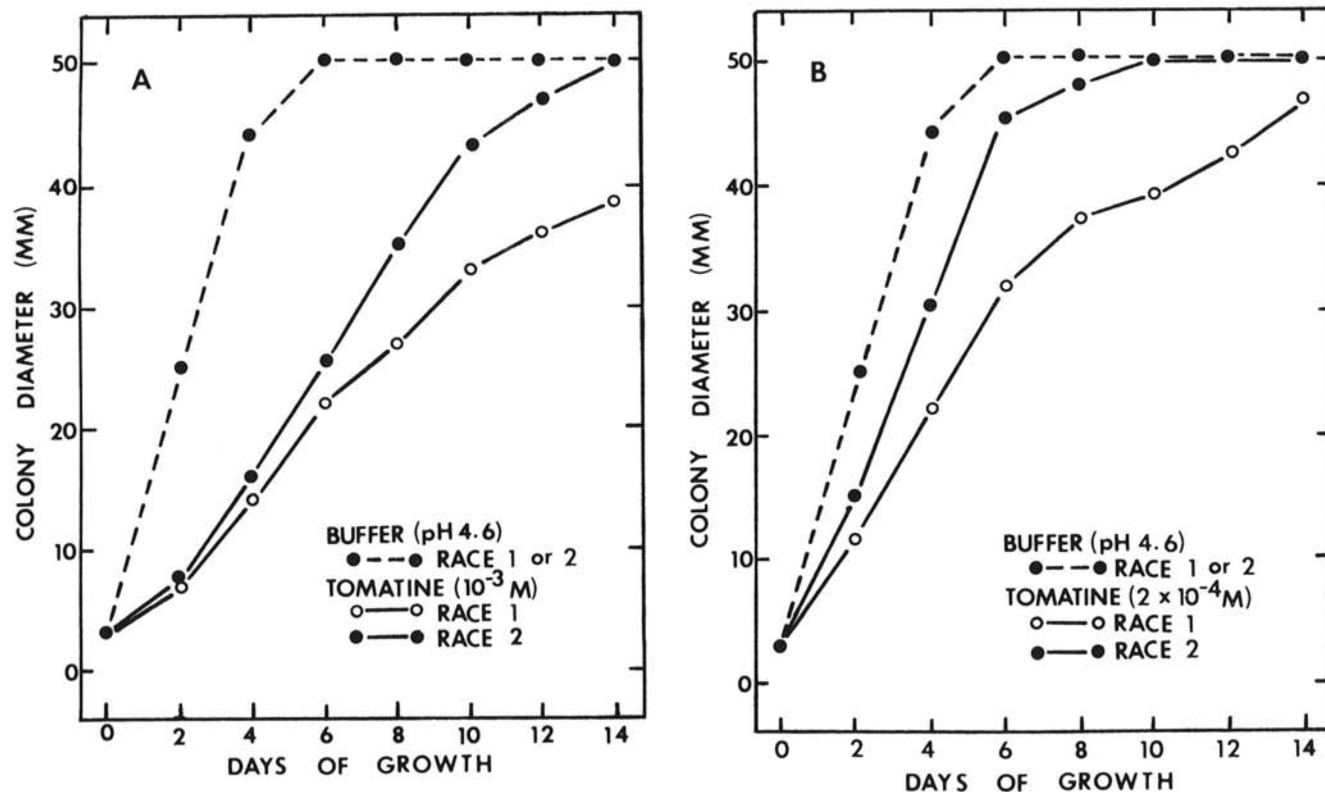


Fig. 1. Colony diameters of *Fusarium oxysporum* f. sp. *lycopersici* race 1 and race 2 cultures grown on buffered potato-dextrose agar containing **A,** 10^{-3} M or **B,** 2×10^{-4} M tomatine, Race 1 and race 2 control cultures were grown on buffered media lacking tomatine.

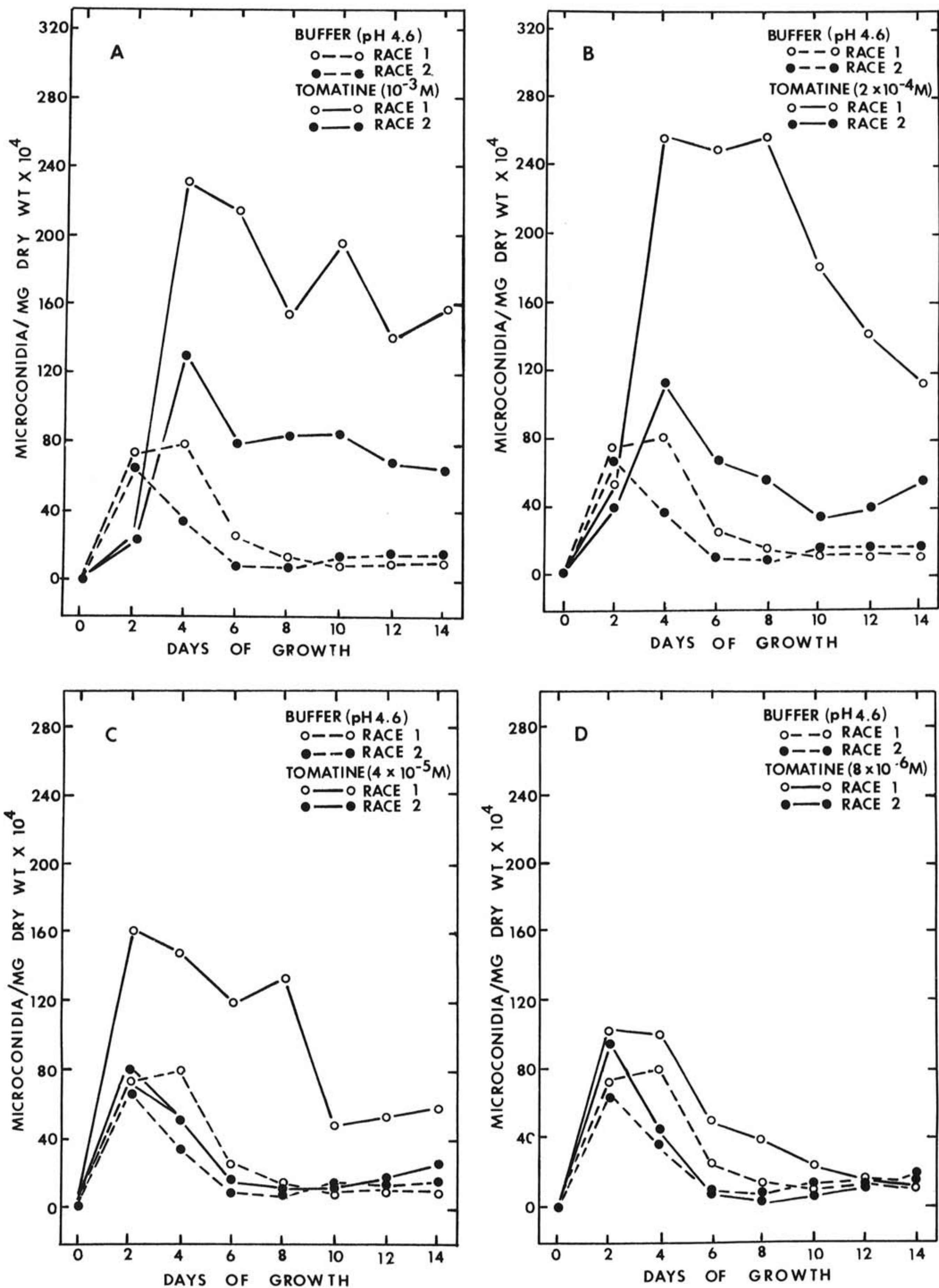


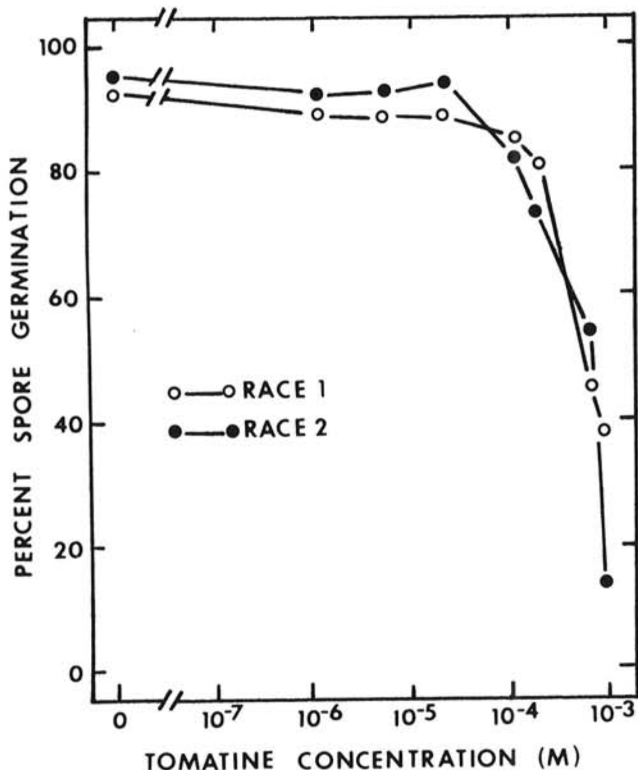
Fig. 2. Microconidial production of *Fusarium oxysporum* f. sp. *lycopersici* race 1 and race 2 cultures grown on buffered potato-dextrose agar containing **A,** 10^{-3} M; **B,** 2×10^{-4} M; **C,** 4×10^{-5} M; or **D,** 8×10^{-6} M tomatine. Race 1 and race 2 control cultures were grown on buffered media lacking tomatine.

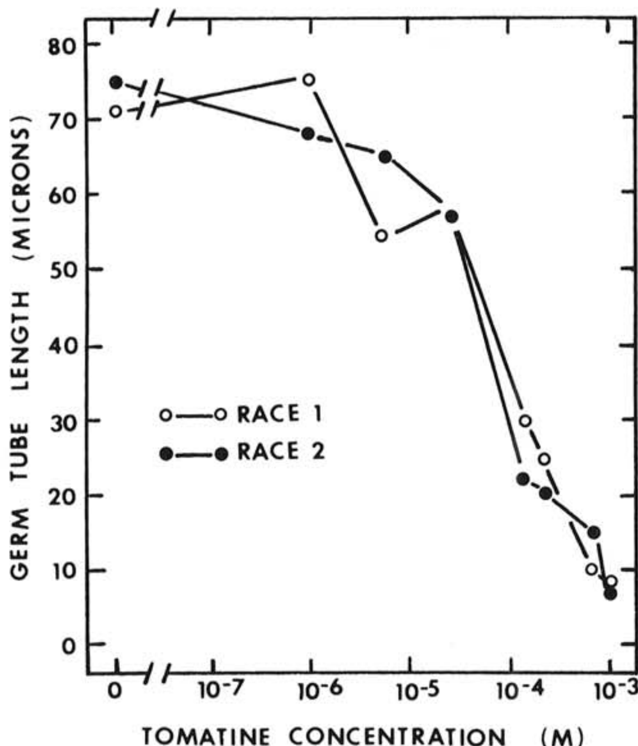
concentration of tomatine in the xylem fluid of IP and VF prior to wounding and inoculating. There was, however, a significant difference in the response of the isolines to the various treatments (Table 1). Wounding or wounding and inoculating failed to induce any change in tomatine concentrations in the xylem fluid of IP. Tomatine increased only in VF during the first 4 days after wounding or wounding and inoculating, but these levels were maintained for 7–9 days after treatment only when VF was inoculated as well as wounded. A comparison of tomatine concentrations on the basis of total milligrams of tomatine produced per plant revealed the same trends as those observed when tomatine concentrations were expressed as molarity.

Effect of tomatine on colony growth and spore production. Tomatine concentrations of 10^{-3} M and 2×10^{-4} M inhibited mycelial growth of both races, but race 1 was inhibited more than race 2 (Fig. 1). These differences were observable after 6–8 days of growth on 10^{-3} M, and after 2–4 days of growth on 2×10^{-4} M tomatine. Growth of race 1 and race 2 on 4×10^{-5} M and 8×10^{-6} M tomatine was similar to the buffer control.

Tomatine stimulated spore production (microconidia per milligram dry weight) of both races, but race 1 was stimulated more than race 2 (Fig. 2). Percent germination of spores of race 1 and race 2 was comparable (85–95%) whether spores were from colonies on tomatine or on the buffer control medium.

Effect of tomatine on spore germination and germ tube extension. Tomatine significantly reduced spore germination of race 1 and race 2 (Fig. 3). There was no difference in the sensitivity of the races except at 10^{-3} M tomatine, and race 2 was more sensitive than race 1 at that concentration. The inhibitor effect of tomatine on germ tube length was comparable for both races (Fig. 4).





Figs. 3 and 4. Effect of buffered (pH 4.6) tomatine concentrations on **3**, germination and **4**, germ tube length of *Fusarium oxysporum* f. sp. *lycopersici* race 1 and race 2 microconidia. Control solutions lacked tomatine. Conidia were incubated for 24 hr at 26 C.

DISCUSSION

Restriction of fungal spread by vascular occlusion within 2–4 days after infection has been identified as a primary mechanism of resistance to Fusarium wilt of tomato (3,7). If any other mechanism is to be considered a primary mechanism, it must also be operative within the same time period. Tomatine did increase to a fungitoxic level within 2–4 days after inoculation only in the VF isoline. Wounding and infection by nonpathogenic microflora that entered through wounds, appeared to be the main stimuli for the increase in tomatine, because inoculation of the VF plants with race 1 or race 2 following wounding did not result in a further increase in the tomatine level. Inoculation with *Fusarium* in addition to wounding and epiphytic microflora, however, extended the period of high tomatine concentration to 9 days following treatment. By the 12th day after inoculation, tomatine returned to pretreatment levels in the inoculated plants. Thus, tomatine did increase to fungitoxic levels in the VF isoline within the crucial time period following inoculation, but the response occurred within both the resistant (VF/race 1) and susceptible (VF/race 2) combinations. This result disagrees with the report of Stromberg and Corden (20), who suggested that susceptibility to race 1 is due to the failure of fungitoxicants to accumulate in response to infection.

Our root pressure technique may not have permitted sampling from xylem vessels in which blockage had occurred due to tyloses. Some tomatine may have become trapped by these occlusions. The findings of Beckman et al (3) indicate that complete vascular occlusion was delayed in the susceptible host/pathogen combination until 7 days or more after inoculation. On the other hand, complete occlusion occurred in the resistant combination 2–3 days after inoculation. If tomatine was trapped by the vascular occlusions, little effect would be expected in the first sampling (2–4 days after inoculation) and then only in the resistant host/pathogen combination. Occlusions should have little influence on tomatine exudation in the susceptible combinations until the 12–14 day sampling.

Rapid increase of tomatine in both the susceptible and resistant reactions within VF suggests that tomatine does not influence resistance, at least during the early stages of pathogenesis. However, VF is susceptible only to the more virulent race 2. Thus, we hypothesized that race 2 invades VF xylem vessels extensively because race 2 is less sensitive than race 1 to the inhibitory effects of tomatine. If this hypothesis were true, then it could still be argued that tomatine contributes significantly to resistance in the VF/race combination. However, race 2 and race 1 were equally sensitive to tomatine when percent spore germination and germ tube lengths were measured after 24 hr of growth in selected tomatine

concentrations. Stromberg and Corden (20) also concluded that race 1 and race 2 were equally sensitive to a fungitoxicant in acetone extracts of stems. Thus, an examination of the data from these in vitro studies also argues against tomatine activity as a sole or primary mechanism of resistance.

We proposed that tomatine may play a key role in resistance by inhibiting spore production rather than inhibiting spore germination or hyphal growth. The inhibition of spore production might contribute greatly to resistance, because the rapid rate of host colonization by vascular pathogens is related more to sporulation and spore transport than to mycelial growth within vessels (2,6). Unexpectedly, tomatine in vitro stimulated sporulation. If tomatine causes a similar proliferation of spores in vivo, the role of tomatine in resistance appears even more doubtful. Pathogen buildup through the production of large quantities of spores is a characteristic feature of susceptible, not resistant, host/pathogen interactions (7).

Tomatine may contribute to resistance in the lower stem, but not as a primary mechanism of defense. A more likely role suggested by the evidence to date is that tomatine may be effective as part of a sequential two-component resistance process, as proposed by McCance and Drysdale (14) for Fusarium wilt of tomato and Mace (12) for Verticillium wilt of cotton. In this two-component system: (i) vascular occlusion restricts the upward distribution of the pathogen and impedes water movement which results in (ii) localized accumulations of tomatine and/or other fungitoxicants that may exceed the concentrations required to inhibit mycelial growth and spore germination, thus preventing lateral spread of the fungus to adjacent vessels.

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