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TOI\1ATINE: ROLE IN RESISTANCE OF TOMATO TO FUSARIUM OXYSPORUM F, SP. LYCOPERSICI RACE 1 AND RACE 2

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THESIS

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TOMATINE: ROLE IN RESISTANCE OF TOMATO TO FUSARIUM OXYSPORUM F. SP. LYCOPERSICI RACE 1 AND RACE 2

BY

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MASTER OF SCIENCE
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March 19, 1938

Date

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ABSTRACT

TOMATINE: ROLE IN RESISTANCE
OF TOMATO TO *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI*
RACE 1 AND RACE 2

by

CHERYL A. SMITH

Previous workers have suggested that the host-produced fungitoxicant, alpha-tomatine, may play a key role in the resistance of tomato plants to the vascular wilt pathogen, *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Hans. & Snyd. race 1. The *in vivo* and *in vitro* studies reported herein were undertaken to examine that hypothesis and to investigate the effect of tomatine on *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) race 2.

Determinations of the concentration of tomatine in the xylem fluid of Improved Pearson (IP) and Pearson VF-11 (VF) near-isolines of tomato were made using chromatographic and spectrophotometric techniques. Plants were sampled at selected time intervals following a severed-taproot inoculation with *Fol* race 1 or race 2. VF is resistant to race 1 but susceptible to the more virulent race 2. IP is susceptible to both races. Another group of plants was

wounded, but not inoculated. Nonwounded-noninoculated plants served as controls.

There was no significant difference in the tomatine content of VF and IP plants prior to inoculation. The tomatine concentration of IP plants remained at pre-inoculation levels (10^{-4} M) regardless of treatment. The concentration of tomatine in the VF isoline increased to fungitoxic levels (10^{-3} M) 2 days after inoculation in both the resistant VF-race 1 and the susceptible VF-race 2 combinations. Tomatine also increased to 10^{-3} M in VF plants 2 days after wounding.

It was postulated that if tomatine plays a role in the resistance of VF to race 1, then race 2 may be less sensitive than race 1 to high levels of tomatine present in VF following infection. The effect of tomatine on mycelial dry weight, colony diameter, spore germination and germ tube length of race 1 and race 2 was compared. Tomatine was inhibitory to both races, but no important differences in sensitivity were observed. It was further postulated that tomatine may contribute to resistance by inhibiting sporulation of Fol, but spore production of both races was stimulated, not inhibited.

It was concluded that tomatine does not play a primary role in resistance, but it may be important in a sequential resistance process. In such a process, vascular occlusion would occur first to limit the upward spread of Fol. Tomatine accumulation below occluded portions of

vessels would then inhibit fungal vegetative growth, thus, limiting the lateral spread of the pathogen to adjacent vessels.

Two additional studies were conducted to examine the effect of Fol race 1 and race 2 microconidia on tomatine. In the first study, it was hypothesized that Fol may detoxify tomatine. If a given population of germinating spores did detoxify tomatine, the percent germination of subsequent populations of spores placed in the tomatine solution would increase. In the second study, it was hypothesized that increasing the spore load in a given concentration of tomatine would reduce the effectiveness of the fungitoxicant, resulting in an increase in spore germination. In both bioassays, percent germination decreased in the buffer control solutions as well as in tomatine. Although conclusions could not be made regarding the original hypotheses, the data provided evidence that the germinating Fol conidia produced a self-inhibitory substance.

SECTION I

THE SIGNIFICANCE OF TOMATINE IN THE HOST RESPONSE OF SUSCEPTIBLE AND RESISTANT TOMATO ISOLINES INFECTED WITH *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI* RACE 1 OR RACE 2

Introduction

The use of tomato cultivars bred for resistance to *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyd. & Hans. has long been the most practical means of controlling this vascular wilt pathogen. However, efforts to discern the nature of the host resistance mechanism have led to conflicting conclusions. Phenolic compounds (Matta et al., 1969) and vascular occlusion (Beckman et al., 1972) have been proposed as determinants of resistance. The possible involvement of host-produced fungitoxicants in resistance to *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) race 1 has also been examined by several investigators. Tomatine is one such fungitoxicant implicated in Fusarium wilt resistance.

Irving et al. (1945) reported that tomato plant extracts contained an inhibitory agent exhibiting marked fungistatic action against *Fol* race 1. The partially purified antibiotic was designated "tomatin". Although some investigators failed to detect any inhibitor in tissue

extracts (Heinze and Andrus, 1945) or xylem fluid (Snyder et al., 1946), further experimentation confirmed the presence of tomatin in tomato tissue (Irving, 1947; Irving et al., 1946; Little and Grubaugh, 1946).

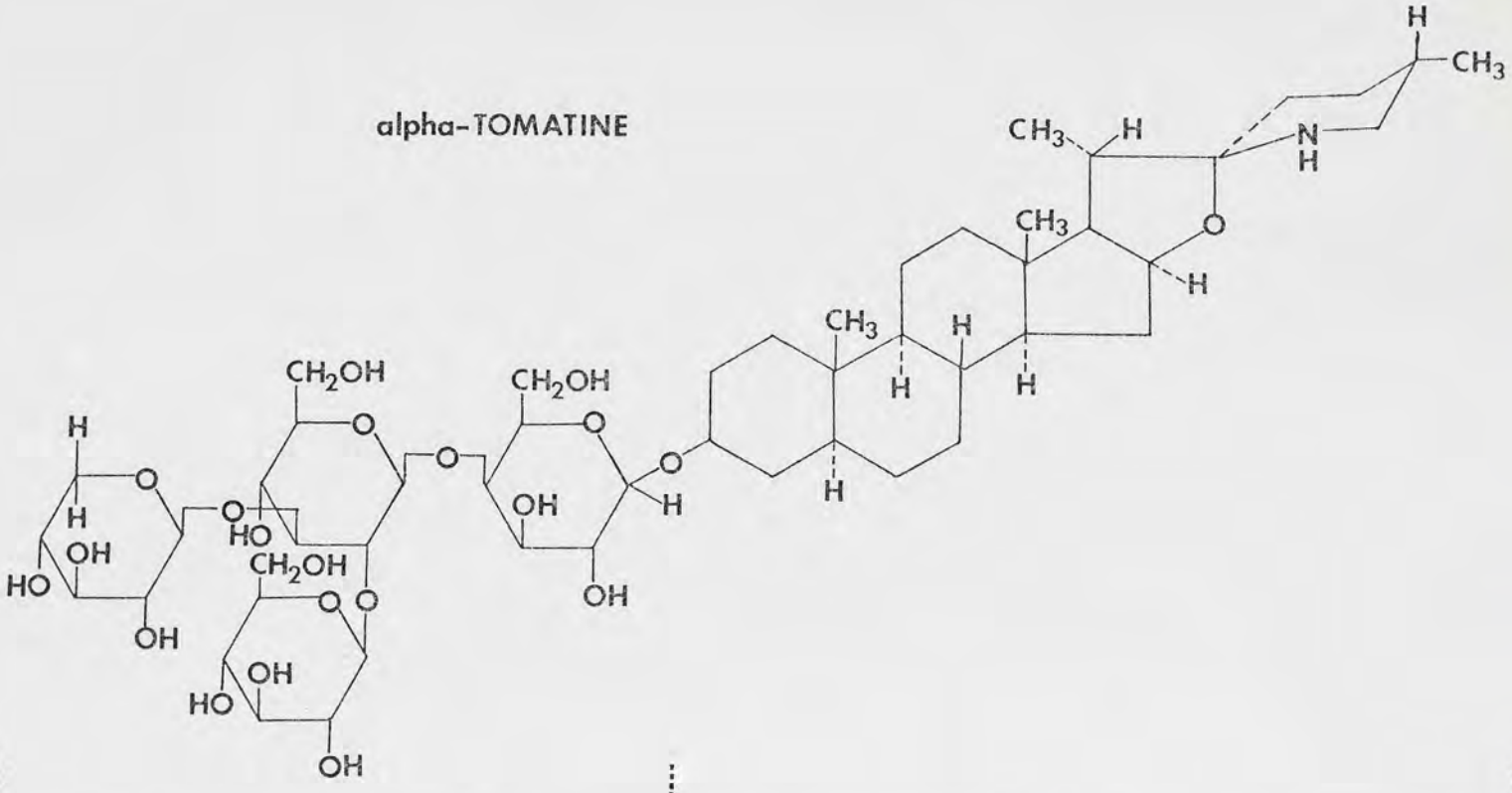
Fontaine et al. (1947) suggested that tomatin might be either wholly or partially responsible for resistance. Kern (1952), however, disagreed with that theory. He measured tomatin levels in roots, stems and vascular fluids of tomato plants, and found that the concentrations in these critical sites were insufficient to inhibit spore germination or mycelial growth. Kern concluded that tomatin could not be responsible for resistance to Fusarium wilt.

Efforts were made to isolate and purify the antibiotic present in the crude tomatin extracts. From tomatin concentrates, Fontaine et al. (1948) were able to isolate crystalline alpha-tomatine. The purified antifungal agent was characterized chemically as a glycosidal alkaloid (Fig. 1). Further chemical and physical data established the steroidal nature of the alkaloid (Fontaine et al., 1951; Uhle and Moore, 1954).

The extent of involvement of tomatine in the plant's defense mechanism was generally ignored until Arneson and Durbin (1968) tested the sensitivity of 30 fungal species to commercially purified tomatine. The test organisms included fungi which were pathogenic, non-pathogenic or saprophytic on tomato plants. The study demonstrated the

Fig. 1. The structural formula for alpha-tomatine. The molecule consists of a tetrasaccharide moiety (two molecules of glucose and one each of xylose and galactose) and an aglycone moiety (tomatidine). Beta$_2$-tomatine is a form of tomatine lacking one glucose unit.

alpha-TOMATINE



inhibitory effect of tomatine on a broad range of fungi. Generally, tomato pathogens, including Fol race 1, were found to be less sensitive to tomatine than were non-pathogens. Arneson and Durbin suggested that the association between tomatine and resistance should be re-evaluated.

Continued efforts to resolve questions regarding the role of tomatine in Fusarium wilt resistance have failed to provide clear-cut answers. Mace and Veech (1971) cited evidence that spore germination or mycelial growth is retarded in the stems of resistant tomato plants inoculated with Fol race 1, suggesting a chemical basis for resistance. Langcake et al. (1972) published a detailed study of an inhibitor produced in response to infection. Based on chromatographic evidence, the investigators identified the compound as tomatine. They reported a substantial increase in the tomatine concentration of tomato stems and roots immediately following infection. However, this increase did not substantiate the involvement of tomatine in resistance because similar increases occurred in both resistant and susceptible cultivars.

McCance and Drysdale (1975) estimated the levels of tomatine in the vascular tissue of race 1-inoculated and noninoculated tomato plants. An increase in tomatine levels occurred in both the resistant and susceptible cultivars following inoculation, confirming the data obtained previously by Langcake et al. (1972). McCance and

Drysdale (1975) disputed Kern's earlier claim that the concentration of tomatine *in vivo* was far too low to inhibit *Fol*. They found that tomatine levels in the taproots of resistant and susceptible cultivars were considerably higher following inoculation than would be required to completely inhibit spore germination or hyphal extension *in vitro*.

Hammerschlag and Mace (1975) provided further evidence that host-produced fungitoxicants could account for resistance. The antifungal activity of root extracts from wounded-noninoculated or wounded-inoculated tomato plants resistant to *Fol* race 1 was nearly twice as great as that of extracts from susceptible plants similarly treated. The inhibitor was identified as tomatine on the basis of chromatographic and bioassay data.

Stromberg and Corden (1974, 1976, 1977) have recently reported that acetone extracts of xylem vessels in resistant and susceptible hosts are fungitoxic to *Fol* race 1 and race 2. Within the infected susceptible cultivar, the race 1 fungal population began to decrease 3 days after inoculation, while the toxicity of the xylem extract decreased. However, in the resistant cultivar the fungal population remained low and the xylem extracts became highly fungitoxic.

Few tomatine studies have utilized resistant and susceptible isogenic lines (Hammerschlag and Mace, 1975; Mace and Veech, 1971). A comparative study using

near-isolines would greatly increase the probability that differences between susceptible and resistant host responses to infection could be attributed to the resistance mechanism. The tomato isolines chosen for this study were Improved Pearson (IP) and Pearson VF-11 (VF). VF carries the dominant I gene for resistance to Fol race 1, whereas IP lacks this gene. Race 2, another pathogenic race of Fol, represents a one-step transition from race 1 (Gerdemann and Finley, 1951). Both VF and IP are susceptible to race 2.

The two isolines, in combination with the two pathogenic races, result in one resistant host response (VF-race 1) and three susceptible responses (VF-race 2, IP-race 1 and IP-race 2). Observations can be made on a susceptible and resistant host reaction in two separate cultivars, both inoculated with the same pathogenic race, as well as a susceptible and resistant reaction in a single cultivar inoculated with two different races. A comparative study of this type would provide a valuable tool in furthering our understanding of wilt-resistant and wilt-susceptible responses.

To date, most researchers have used whole stem or root extracts to determine tomatine levels. However, because Fol confines itself to the host vessels, the concentration of tomatire in the xylem fluid, rather than whole tissue, must be considered in evaluating the role of tomatine in resistance. One of the three objectives of

this investigation was to determine the levels of tomatine in the xylem fluid of near-isogenic tomato lines treated as follows: i) nonwounded-noninoculated, ii) wounded-noninoculated, iii) Fol race 1 inoculated and iv) Fol race 2 inoculated.

The toxicity of crude plant extracts (Fisher, 1935; Gottlieb, 1943; Irving et al., 1945; Langcake et al., 1972) and purified tomatine (Arneson and Durbin, 1968; Fontaine et al., 1948) has been examined as one means of evaluating the role of tomatine in resistance. Determinations of the ability of tomatine to inhibit spore germination and mycelial growth of Fol race 1 have been the focus of these fungitoxicity assays. The assumption has been that if tomatine is a determinant of resistance, the mode of operation within the plant is the inhibition of vegetative growth or spore germination. However, the rapid rate of host colonization by vascular pathogens depends not on mycelial growth but on the ability of the fungus to sporulate within the xylem vessels (Beckman et al., 1961). Spores are transported in the transpiration stream until they become trapped on the end walls of a vessel. Conidia germinate, penetrate the obstruction and sporulate in the newly invaded vessel. The new generations of spores are transported in the transpiration stream until they reach the next barrier, and the process is repeated. A host-produced substance which could effectively inhibit sporulation within the xylem vessels could seriously limit

the ability of the pathogen to colonize the host and, thus, contribute to resistance. Host colonization by vegetative growth alone would be slow, allowing more time for the host to respond with other defense mechanisms. To date, no attempt has been made to investigate how tomatine affects sporulation. The second objective of this study was to determine the effect of tomatine on microconidial production of Fol race 1 and race 2.

Fol race 2 is able to overcome the resistance mechanism of tomato plants carrying the single dominant I gene for resistance to race 1. It was hypothesized that, if tomatine determines resistance by inhibiting vegetative growth of the pathogen, then the more virulent race 2 should be less sensitive to tomatine than race 1. Previously, growth inhibition studies have been concerned with race 1, but not with race 2. The third objective of this investigation was to compare the effect of tomatine on race 1 and race 2 with respect to colony growth, germ tube length and spore germination.

Materials and Methods

Cultural Procedures

Host. Near-isogenic lines of tomato (Lycopersicon esculentum Mill.) served as host plants. The isolines IP and VF are, respectively, susceptible and resistant to Fol race 1. Both cultivars are susceptible to the more virulent Fol race 2. Tomato seeds were sown in Jiffy Mix and transplanted at the two-leaf stage to 10-cm-diameter pots containing a 1:1:1 soil:peat:Perlite mixture. Plants were maintained under greenhouse conditions, and daylight was supplemented with cool-white Sylvania fluorescent lighting to provide a 15-hr photoperiod. Soluble 16-32-16 fertilizer was applied weekly.

Pathogen. Isolates of Fusarium oxysporum f. sp. lycopersici races 1 and 2 were obtained from Dr. G. M. Armstrong (Georgia Agricultural Experiment Station, Experiment, Georgia 30212). Race 1 and race 2 cultures were grown on potato dextrose agar (PDA) and incubated at 28 C in the dark. Microconidia for inoculum were washed from the cultures with distilled water and then filtered through lens paper. The spore concentration was adjusted with the aid of a hemacytometer.

Inoculation of host. Plants grown to the seven- to eight-leaf stage were used for inoculation. The plants were uprooted and the roots washed free of soil. Taproots

were severed in the region where they were approximately 2 mm in diameter, 7-10 cm below the cotyledons. The root systems were submerged in a spore suspension of 10^6 microconidia/ml distilled water for 30 min. Plants were then repotted. Another group of plants was wounded by severing the taproot and submerging the root system in distilled water in place of the inoculum. Nonwounded-noninoculated plants served as controls.

Quantitative Determination of Tomatine in Xylem Fluid

Obtaining xylem fluid. Xylem fluid was obtained using a modification of the root pressure technique described by Schnathorst (1970). Twenty-five plants of each treatment were decapitated 2, 7, 12 or 17 days after inoculation. The lower stem of each plant was thoroughly cleansed with distilled water and then disinfested with 70% ethanol. An oblique cut was made between the first and second nodes using a razor blade rinsed in 70% ethanol. Alcohol-sterilized tygon tubing was fitted over the stump and an autoclaved 9" Pasteur pipet was inserted into the tubing. A bend in the tip of the pipet served as a trap for air-borne contaminants. Xylem fluid which exuded into the collecting tube by root pressure was withdrawn by inserting a 22 G needle with a 5-ml syringe into the tygon tubing (Fig. 2). Collections were made for 2 days following decapitation. The exudate was filtered through a Millipore filter (0.45 um pore size) and frozen at -17 C.

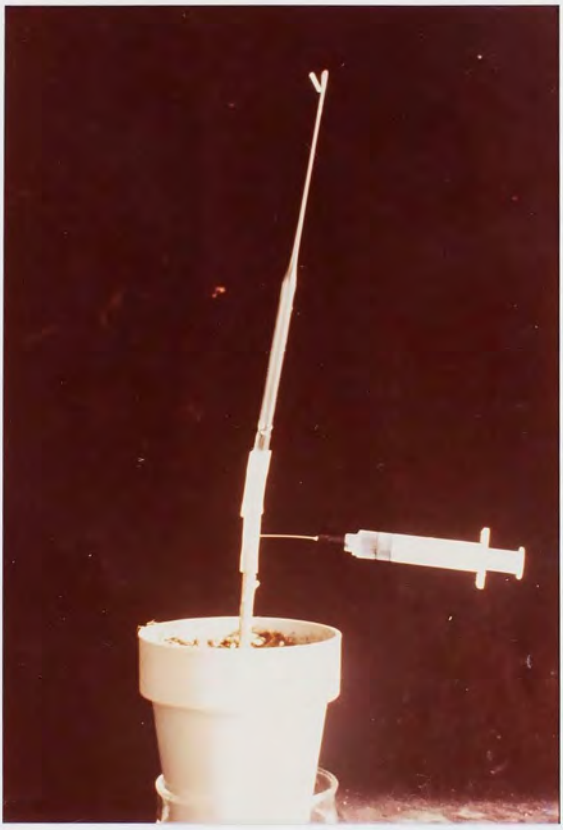


Fig. 2. Method for collecting and extracting xylem fluid exudate from decapitated tomato plants.

Thin layer chromatography. Tomatine was separated from the xylem fluid exudate using thin layer chromatography (TLC). Xylem fluid (5 ml) was evaporated to dryness under reduced pressure at 45 C. Flask contents were taken up in 10 ml of 50% methanol acidified with two drops of 50% H_2SO_4. Aliquots (500 ul) were applied as a 14-cm band on 20 X 20 cm pre-coated silica gel TLC plates. Chromatograms were developed in a solvent system consisting of iso-propanol:formic acid:distilled water (IFW, 73:3:24). After the solvent front had advanced 10 cm to a pre-scored line, plates were removed from the tanks and air dried at room temperature. Tomatine was located at Rf 0.66 either by spraying a plate with modified Dragendorff reagent (Cromwell, 1955, p. 506) or by placing a plate in an iodine chamber. A bright orange color reaction indicated the presence of tomatine. The tomatine zone was scraped from unsprayed chromatograms and eluted in 6 ml of 50% methanol acidified with one drop of 50% H_2SO_4. The silica gel particles were concentrated by centrifuging at 1000 rpm for 7 min. The supernatant was then collected and evaporated to dryness. Concentrated H_2SO_4 (10 ml) was added to the residue and the mixture incubated at 40 C for 24 hours. The percent transmittance of the resulting chromagen was measured at 325 nm on a Beckman DB-G grating spectrophotometer. The molar concentration of tomatine present was determined by reference to a standard curve (Fig. 3) developed using known concentrations of commercial

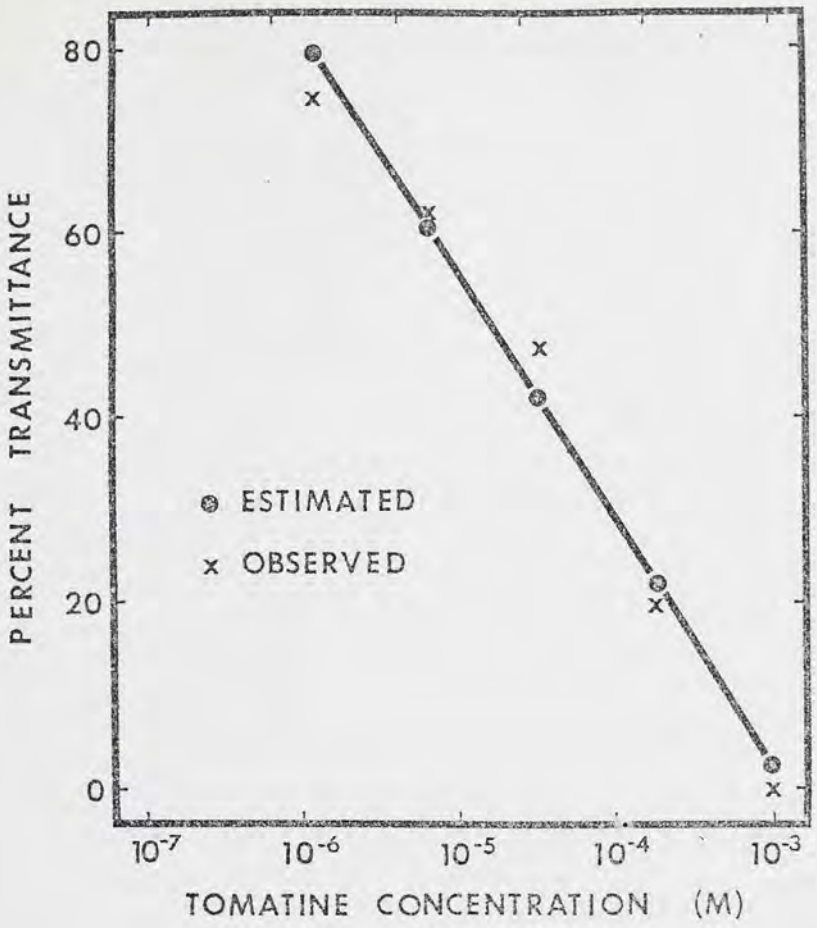


Fig. 3 Standard curve of various concentrations of commercial alpha-tomatine plotted against the percent transmittance of the tomatine-H_2SO_4 chromagen measured at 325 nm. Estimated values were derived from a regression analysis of the observed values (correlation coefficient= 0.988).

alpha-tomatine (ICN Life Sciences Group, Cleveland, Ohio 44128). Total mg tomatine produced per plant was calculated on the basis of the average amount of xylem fluid exuded per plant for each treatment. The data were analyzed statistically using Duncan's multiple-range test (P=0.05).

Effect of Tomatine on Colony Growth and Spore Production

Solutions of tomatine were prepared to give molar concentrations of 10^{-3}, 2×10^{-4}, 4×10^{-5} and 8×10^{-6} when added to the test media. Pure alpha-tomatine was dissolved in 10 mM HCl and the pH adjusted to 4.6 with a phosphate-citrate buffer. Solutions were sterilized by filtration through a Millipore filter (0.2 um pore size) and added to autoclaved, rehydrated Difco PDA. Buffered PDA lacking tomatine served as a control. Aliquots (8 ml) of media were poured into sterile polyurethane petri dishes (60 X 15 mm).

Test and control media were seeded with a single 4-mm-diameter plug taken from the margin of 4-day-old colonies of Fol race 1 and race 2. All cultures were incubated at 27 C in the dark. Radial growth, dry weight and spore production (microconidia/mg dry wt) of each treatment were determined every 48 hours for two weeks. The data were analyzed statistically using analysis of variance (P=0.01).

Colony growth. Radial growth was determined by

making two perpendicular measurements of the diameter of each of three colonies per treatment. Colonies were freed from the media for dry weight determinations. First, the agar containing the colonies was removed from the plates and autoclaved. Distilled water was used to dilute the agar and, thus, prevent re-solidifying. The diluted medium was then filtered through Whatman #1 filter paper and the remaining fungal material was rinsed with hot distilled water. Colonies were oven-dried at 110 C for 24 hours and then weighed. Each determination was replicated three times.

Spore production. A separate set of three culture plates per treatment was used to measure spore production. Microconidia were washed from each plate with distilled water and then filtered through lens paper. The resulting spore suspension was brought up to a constant volume (20 ml). The total number of spores produced per plate was determined with the aid of a hemacytometer (spores/ml X 20 ml). The three dry weight measurements were randomly matched with the three total spore counts of the same treatment, and spore production was determined on the basis of microconidia/mg dry wt.

Spore viability. Conidia from four- and eight-day-old colonies grown on the control media, 10^{-3} M and 2×10^{-4} M tomatine were tested for viability using a spore germination bioassay. The spore suspensions used in determining microconidial production were adjusted to a

concentration of 2 X 10^5 spores/ml. One drop of the suspension was added to one drop of phosphate-citrate buffer (pH 4.6) on a microscope slide coated with 2.5% cellulose nitrate. Slides were incubated for 24 hours at 26 C. Following incubation, the spores were stained and fixed with 1% cotton blue in lactophenol. Percent germination was determined by counting the number of spores out of 100 which had germinated. Three random fields per replicate were examined for each treatment.

Effect of Tomatine on Spore Germination and Hyphal Extension

Microconidia were harvested from 10-day-old stock cultures of Fol race 1 and race 2 and filtered through lens paper. The spore concentration was adjusted to 2 X 10^5 conidia/ml distilled water with the aid of a hemacytometer. Solutions of tomatine were prepared to give molar concentrations of 10^{-3}, 8 X 10^{-4}, 4 X 10^{-4}, 2 X 10^{-4}, 4 X 10^{-5}, 8 X 10^{-6} and 16 X 10^{-7} when added to the spore suspension. Pure commercial alpha-tomatine was dissolved in 10 mM HCl and the pH adjusted to 4.6 with a phosphate-citrate buffer. A buffer-HCl solution lacking tomatine served as the control. One drop of the test solution was added to one drop of the spore suspension on a microscope slide coated with 2.5% cellulose nitrate. Each treatment was replicated six times. Slides were placed in sterile moist petri dishes and incubated for 24 hours at 26 C. After incubation, spores were stained and fixed with 1% cotton

blue in lactophenol.

Percent germination was determined by counting the number of spores out of 100 which had germinated. Spores were counted as germinated if the germ tube was at least the length of the spore. Three random fields per replicate were examined for each treatment. Germ tube lengths were measured using a microscope with an eye-piece micrometer. Twenty-five spores per replicate were measured for each treatment. The data were analyzed statistically using analysis of variance ($P=0.01$).

Results

Quantitative Determination of Tomatine in Xylem Fluid

There was no significant difference in the molarity of tomatine in the xylem fluid of IP and VF controls (Table 1). There was, however, a significant difference in the response of the two cultivars to the various treatments. The VF isoline consistently produced more tomatine than IP following inoculation or wounding. The molarity of tomatine present in the resistant VF-race 1 and in the susceptible VF-race 2 host-pathogen combinations two days after inoculation was ten times higher than the level present in the control. This maximum molar concentration was maintained until the seventh day after inoculation. From the twelfth day to the seventeenth day, the concentrations of tomatine in VF-race 1 and VF-race 2 plants were comparable to the controls. Wounding also induced a ten-fold increase in the molarity of tomatine in the VF cultivar. The maximum level of tomatine was reached on the second day after wounding, then decreasing to the level of the control by the seventh day.

Inoculation or wounding failed to induce any increase in the molarity of tomatine present in the IP isoline (Table 1). The tomatine concentration in the xylem fluid of the two susceptible IP-inoculated combina-

TABLE 1. Tomatine content (moles) of xylem fluid exudate from stems of Improved Pearson (IP) and Pearson VF-11 (VF) tomato near-isolines at selected time intervals. Plants were nonwounded-noninoculated (CK), wounded-noninoculated (W), inoculated with Fusarium oxysporum f. sp. lycopersici race 1 (R1) or inoculated with Fusarium oxysporum f. sp. lycopersici race 2 (R2). Host-pathogen combinations resulted in either a susceptible (S) or resistant (R) reaction.

Treatment	Host reaction	Tomatine concentration (moles)*			
		Days after treatment			
		2	7	12	17
VF-R1	R	1.9×10^{-3} d	1.6×10^{-3} b	2.3×10^{-5} a	3.4×10^{-4} a
VF-R2	S	1.4×10^{-3} cd	1.5×10^{-3} b	8.1×10^{-4} a	7.1×10^{-4} a
VF-W	-	1.2×10^{-3} bcd	6.0×10^{-4} a	1.1×10^{-4} a	7.8×10^{-5} a
VF-CK	-	1.4×10^{-4} a	1.4×10^{-4} a	1.4×10^{-4} a	1.4×10^{-4} a
IP-R1	S	9.0×10^{-4} abc	4.2×10^{-4} a	1.0×10^{-4} a	7.8×10^{-5} a
IP-R2	S	4.9×10^{-4} abc	1.3×10^{-4} a	4.1×10^{-4} a	5.6×10^{-4} a
IP-W	-	1.9×10^{-4} a	2.2×10^{-4} a	4.0×10^{-5} a	4.1×10^{-4} a
IP-CK	-	3.4×10^{-4} a	3.4×10^{-4} a	3.4×10^{-4} a	3.4×10^{-4} a

*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.

tions and the IP-wounded treatment were comparable to the control on all four sampling dates.

The average yield of exudate per plant ranged from 2-7 ml. Recognizing that variations in yield could conceivably influence quantitative determinations based on molarity, comparisons of the various treatments were also made on the basis of total mg tomatine produced per plant (mg/plant). These data are presented in Table 2. Generally, an examination of tomatine concentrations in mg/plant revealed the same trends observed when tomatine concentrations were expressed as molarity. In the VF isoline, tomatine increased to a level above the control following inoculation or wounding. However, while tomatine molarity within VF-race 1, VF-race 2 and VF-wounded plants increased above the control by a factor of ten, tomatine in mg/plant increased only three- to five-fold for the same treatments. The concentrations of tomatine in mg/plant were comparable for all IP-inoculated, -wounded and control plants, regardless of sampling time.

Effect of Tomatine on Colony Growth and Spore Production

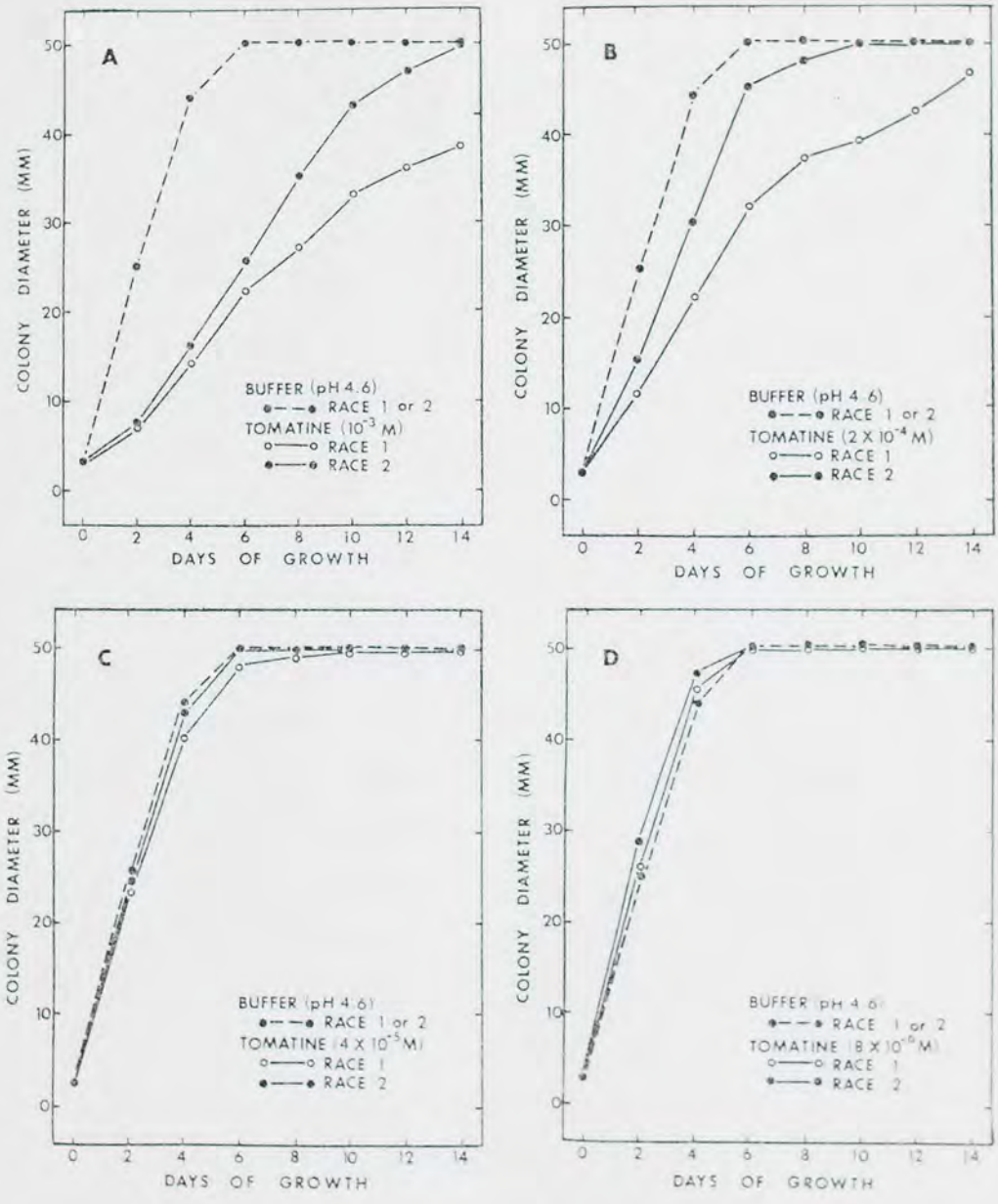
Colony growth. Tomatine significantly inhibited the radial growth of race 1 and race 2, but race 1 was inhibited more than race 2 (Fig. 4-A, B). Differences in the sensitivity of race 1 and race 2 to the inhibitory effect of tomatine became more evident with the progression of time. These differences were observable after 6-8 days

TABLE 2. Tomatine content (mg produced/plant) of xylem fluid exudate from stems of Improved Pearson (IP) and Pearson VF-11 (VF) near-isolines at selected time intervals. Plants were nonwounded-noninoculated (CK), wounded-noninoculated (W), inoculated with Fusarium oxysporum f. sp. lycopersici race 1 (R1) or inoculated with Fusarium oxysporum f. sp. lycopersici race 2 (R2). Host-pathogen combinations resulted in either a susceptible (S) or resistant (R) reaction.

		Tomatine concentration (mg/plant)*			
		Days after treatment			
Treatment	Host reaction	2	7	12	17
VF-R1	R	0.49 d	0.61 c	0.01 a	0.02 a
VF-R2	S	0.30 bcd	0.52 bc	0.11 a	0.11 a
VF-W	-	0.34 cd	0.24 abc	0.06 a	0.04 a
VF-CK	-	0.07 a	0.07 a	0.07 a	0.07 a
IP-R1	S	0.19 abc	0.22 abc	0.06 a	0.03 a
IP-R2	S	0.07 a	0.06 a	0.19 a	0.21 a
IP-W	-	0.05 a	0.06 a	0.13 a	0.13 a
IP-CK	-	0.11 ab	0.11 ab	0.11 a	0.11 a

*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.

Fig. 4. 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, B) 2×10^{-4} M, C) 4×10^{-5} M or D) 8×10^{-6} M alpha-tomatine. Control cultures were grown on buffered media lacking tomatine.

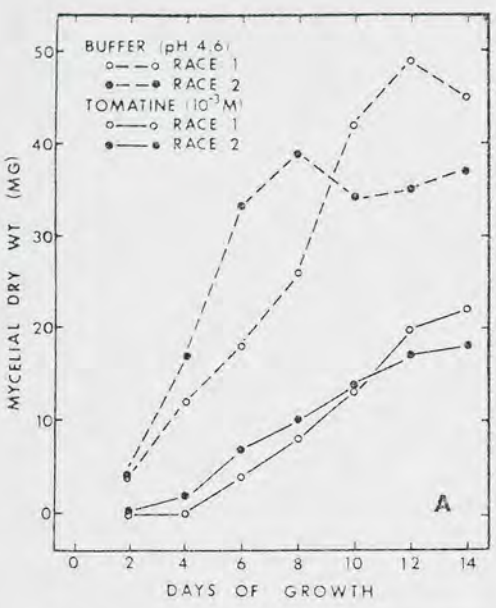

A
COLONY DIAMETER (MM)
DAYS OF GROWTH
BUFFER (pH 4.6)
RACE 1 or 2
TOMATINE (10^{-3} M)
RACE 1
RACE 2
B
COLONY DIAMETER (MM)
DAYS OF GROWTH
BUFFER (pH 4.6)
RACE 1 or 2
TOMATINE (2×10^{-4} M)
RACE 1
RACE 2
C
COLONY DIAMETER (MM)
DAYS OF GROWTH
BUFFER (pH 4.6)
RACE 1 or 2
TOMATINE (4×10^{-5} M)
RACE 1
RACE 2
D
COLONY DIAMETER (MM)
DAYS OF GROWTH
BUFFER (pH 4.6)
RACE 1 or 2
TOMATINE (8×10^{-6} M)
RACE 1
RACE 2

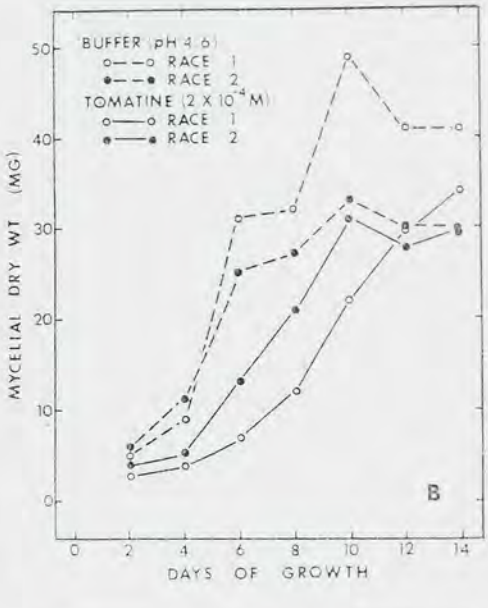
of growth on 10^{-3} M tomatine and after 2-4 days of growth on 2 X 10^{-4} M tomatine. Race 1 and race 2 buffer controls grew to the limits of the petri plates after 6 days. Race 2 took 14 days on 10^{-3} M tomatine and 10 days on 2 X 10^{-4} M tomatine to grow to the limits of the plates. Even after 14 days of growth, colony diameters of race 1 on 10^{-3} M and 2 X 10^{-4} M tomatine were, respectively, 23% and 7% less than the control (Fig. 4-A, B). Race 1 appeared to be slightly inhibited on 4 X 10^{-5} M tomatine after 2 and 4 days of growth, while race 2 was unaffected at that concentration (Fig. 4-C). Neither race was inhibited at the lowest concentration included in this experiment (Fig. 4-D). Colony morphology was also influenced by tomatine. Levels of tomatine which were inhibitory to the radial growth of race 1 or race 2 colonies caused an increase in aerial growth.

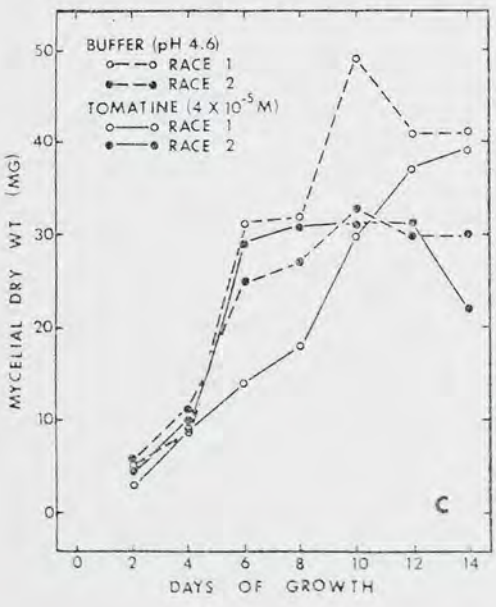
Tomatine also significantly reduced the dry weights of race 1 and race 2 colonies compared to the controls (Fig. 5-A, B, C, D), but there was no significant difference between the two races. Race 1 and race 2 colony growth, as determined by dry weight, was inhibited on 10^{-3} M and 2 X 10^{-4} M tomatine (Fig. 5-A, B). Only race 1 was inhibited on 4 X 10^{-5} M tomatine (Fig. 5-C). Neither race was affected on the lowest concentration (Fig. 5-D).

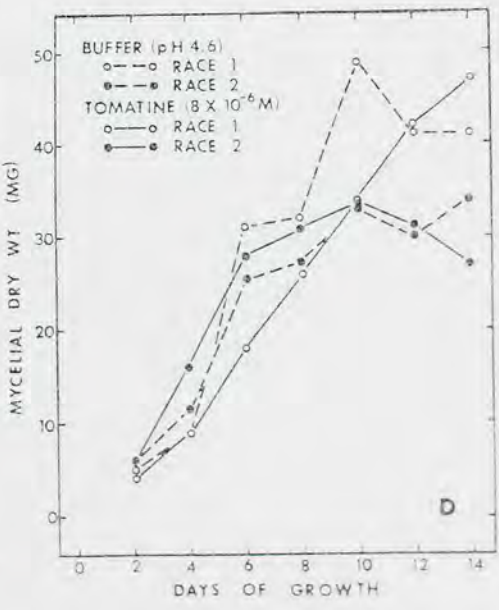
Spore production. Tomatine caused a significant increase in the spore production (microconidia/mg dry wt) of Fol race 1 and race 2, but race 1 was affected more

Fig. 5. Mycelial dry weights 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 alpha-tomatine. Control cultures were grown on buffered media lacking tomatine.


BUFFER (pH 4.6)
RACE 1
RACE 2
TOMATINE (10^{-3} M)
RACE 1
RACE 2
MYCELIAL DRY WT (MG)
DAYS OF GROWTH
A

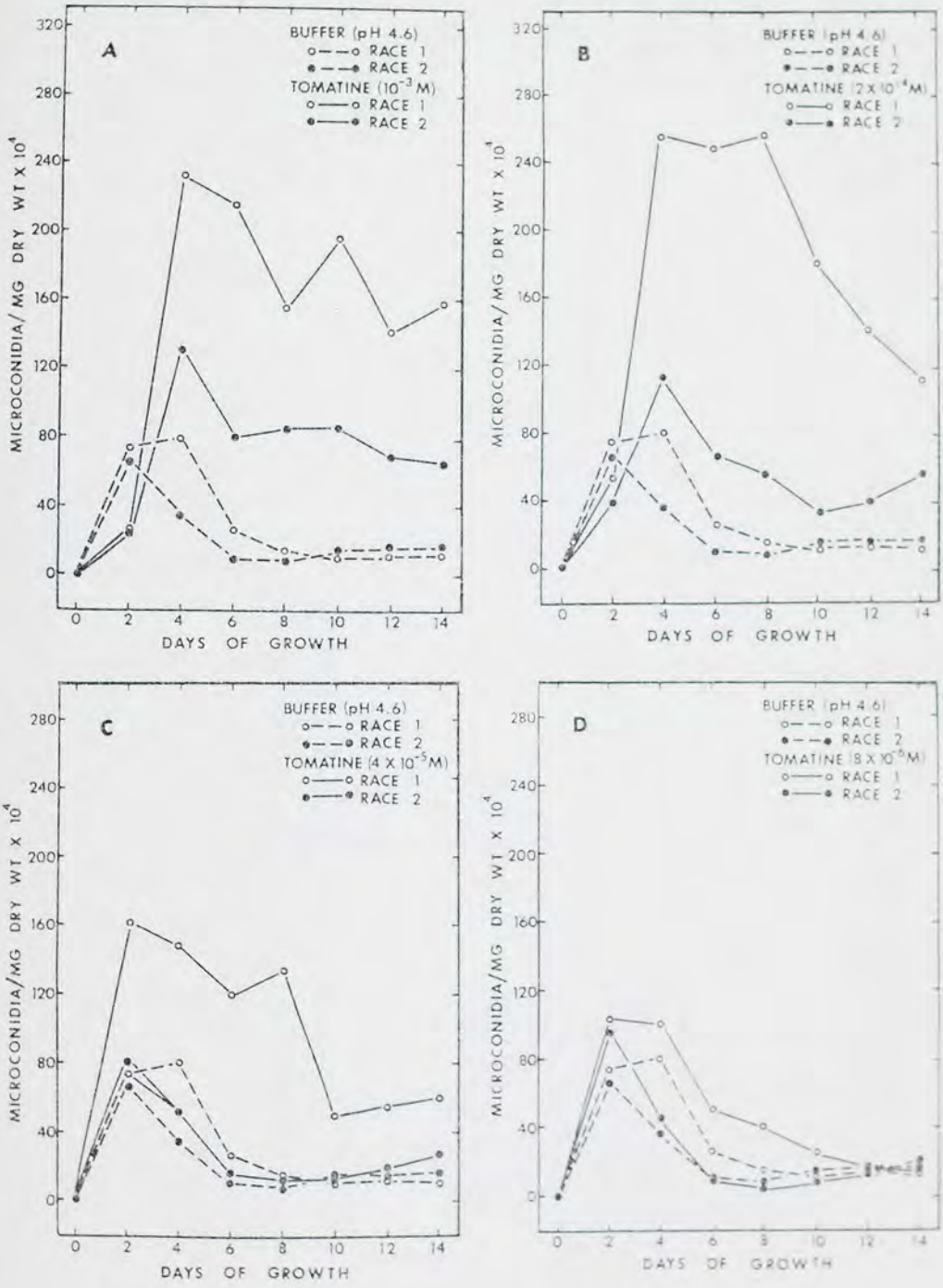

BUFFER (pH 4.6)
RACE 1
RACE 2
TOMATINE (2×10^{-4} M)
RACE 1
RACE 2
MYCELIAL DRY WT (MG)
DAYS OF GROWTH
B


BUFFER (pH 4.6)
RACE 1
RACE 2
TOMATINE (4×10^{-5} M)
RACE 1
RACE 2
MYCELIAL DRY WT (MG)
DAYS OF GROWTH
C


BUFFER (pH 4.6)
RACE 1
RACE 2
TOMATINE (8×10^{-6} M)
RACE 1
RACE 2
MYCELIAL DRY WT (MG)
DAYS OF GROWTH
D

than race 2 (Fig. 6). An initial inhibition of sporulation in colonies grown on 10^{-3} M or 2 X 10^{-4} M tomatine was followed by a dramatic increase in the number of spores produced/mg dry wt, reaching a peak after 4 days of growth. Cultures grown on the buffer control, on 4 X 10^{-5} M tomatine or on 8 X 10^{-6} M tomatine reached their maximum levels of spore production after 2 days of growth (Fig. 6-C, D). At the peak levels of spore production on 10^{-3} M and 2 X 10^{-4} M tomatine, race 1 produced three times more spores/mg dry wt and race 2 produced two times more spores/mg dry wt than their respective controls. Race 1 colonies grown on 4 X 10^{-5} M tomatine reached a level of spore production twice as high as the peak level observed in the control. Race 2 growing on 4 X 10^{-5} or 8 X 10^{-6} M tomatine and race 1 growing on 8 X 10^{-6} M tomatine reached levels of spore production comparable to their respective controls. Once spore production reached a maximum in race 1 and race 2 buffer controls, spore production quickly dropped to a near-minimum and then leveled off. Microconidial production in cultures grown on tomatine generally remained at maximum or near-maximum levels for several days after reaching the peak in spore production. After 6-8 days of growth on 10^{-3} M tomatine, race 1 was producing 23 times more spores/mg dry wt and race 2 was producing 11 times more spores/mg dry wt than their respective controls (Fig. 6-A). During the same time period on 2 X 10^{-4} M tomatine, race 1 was producing 16

Fig. 6. 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} or D) 8×10^{-6} M alpha-tomatine. Control cultures were grown on buffered media lacking tomatine.


A
BUFFER (pH 4.6)
RACE 1
RACE 2
TOMATINE (10^{-3} M)
RACE 1
RACE 2
MICROCONIDIA/MG DRY WT X 10^4
DAYS OF GROWTH
B
BUFFER (pH 4.6)
RACE 1
RACE 2
TOMATINE (2×10^{-4} M)
RACE 1
RACE 2
MICROCONIDIA/MG DRY WT X 10^4
DAYS OF GROWTH
C
BUFFER (pH 4.6)
RACE 1
RACE 2
TOMATINE (4×10^{-5} M)
RACE 1
RACE 2
MICROCONIDIA/MG DRY WT X 10^4
DAYS OF GROWTH
D
BUFFER (pH 4.6)
RACE 1
RACE 2
TOMATINE (8×10^{-6} M)
RACE 1
RACE 2
MICROCONIDIA/MG DRY WT X 10^4
DAYS OF GROWTH

times more spores/mg and race 2 was producing 7 times more spores/mg than the controls (Fig. 6-B). Race 1 spore production on 4×10^{-5} M tomatine was 7 times greater than the buffer control (Fig. 6-C). The difference between race 1 cultures on 8×10^{-6} M tomatine and the control was less drastic (Fig. 6-D). Race 2 spore production on the two lowest concentrations of tomatine (4×10^{-5} and 8×10^{-6} M) was comparable to the controls (Fig. 6-C, D).

Spore viability. The viability of race 1 and race 2 microconidia produced on 10^{-3} M and 2×10^{-4} M tomatine was compared to the viability of conidia produced on the control media. The percent germination of race 1 and race 2 spores was comparable (85-95%), whether the spores were from colonies grown on tomatine or from colonies grown on the buffer control.

Effect of Tomatine on Spore Germination and Hyphal Extension

Spore germination. Tomatine significantly restricted spore germination of both races. There was no difference in the sensitivity of race 1 and race 2, except at 10^{-3} M tomatine (Fig. 7). At that concentration, the more virulent race 2 was surprisingly more sensitive than race 1. While spore germination of race 1 was 60% less than the control, race 2 spore germination was inhibited by 80%. Spore germination was only slightly inhibited (12-24%) at 4×10^{-4} M tomatine. Below 4×10^{-4} M, spore germination in tomatine was comparable to the buffer

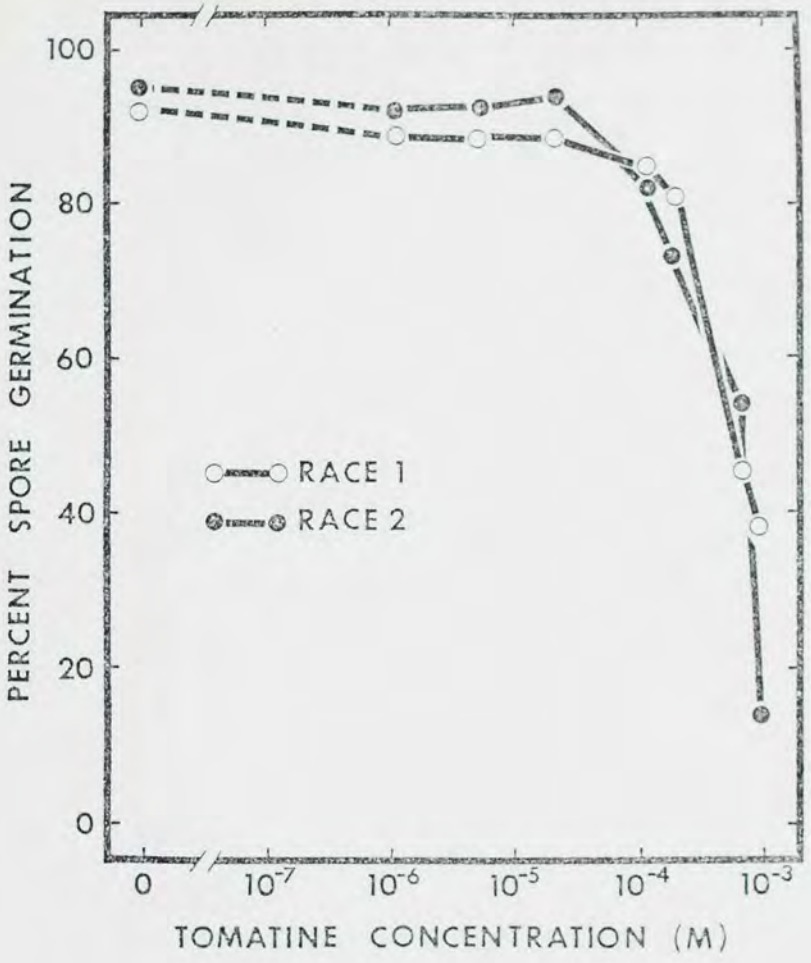


Fig. 7. Effect of various concentrations of buffered (pH 4.6) alpha-tomatine on germination of Fusarium oxysporum f. sp. lycopersici race 1 and race 2 microconidia. Control solutions lacked tomatine. Conidia were incubated for 24 hours at 26 C.

controls. The ED_{50} for the inhibition of spore germination was 8×10^{-4} M.

Hyphal extension. The inhibitory effect of tomatine on germ tube length was comparable for both race 1 and race 2 (Fig. 8). Tomatine concentrations of 10^{-3} M and 8×10^{-4} M were highly inhibitory to Fol, resulting in an 80-90% reduction in germ tube length compared to the controls. The germ tubes of microconidia in 4×10^{-4} M or 2×10^{-4} M tomatine were less than one-half the length of the control germ tubes. The three lowest molar concentrations (4×10^{-5} M, 8×10^{-6} M and 16×10^{-6} M) resulted in less than a 25% inhibition of germ tube length. The ED_{50} for the inhibition of hyphal extension was 6×10^{-5} M tomatine.

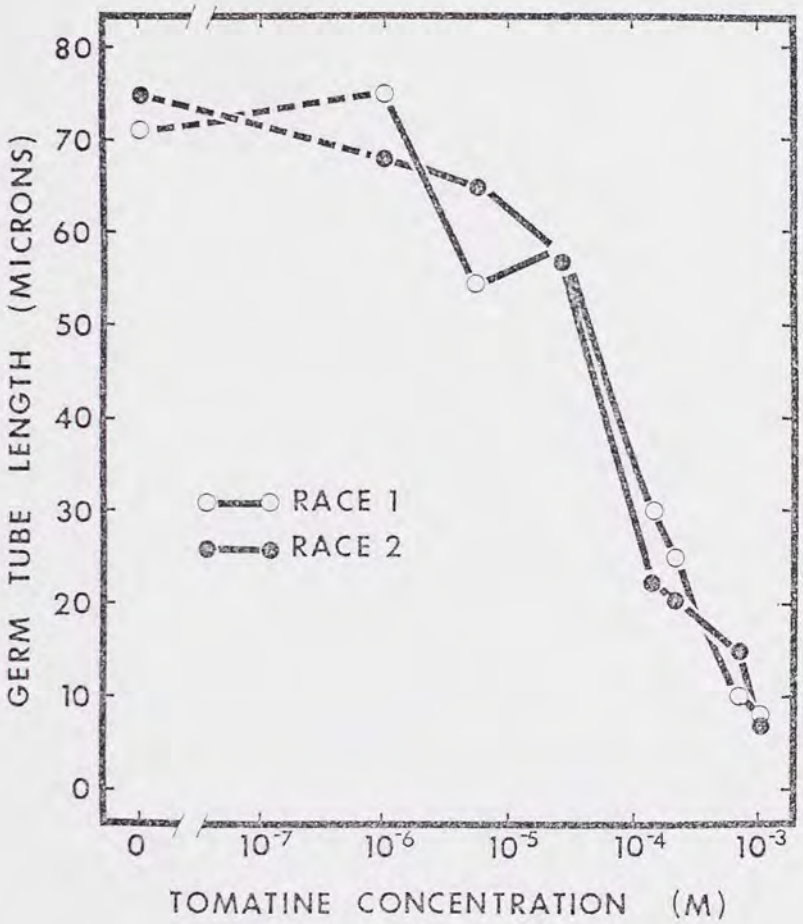


Fig. 8. Effect of various concentrations of buffered (pH 4.6) alpha-tomatine on the germ tube length of Fusarium oxysporum f. sp. lycopersici race 1 and race 2 microconidia. Control solutions lacked tomatine. Conidia were incubated for 24 hours at 26 C.

Discussion

Two types of host resistance mechanisms are known to operate in vascular wilt diseases (Talboys, 1964, 1972). "Extra-vascular determinants" are those host responses which act to limit cortical invasion and, thus, prevent pathogen entry into the vascular system. Lignituber formation and cortical cell suberization can function as defense mechanisms in the pre-vascular stage of pathogenesis (Talboys, 1958). Resistance in pre-vascular tissue has also been attributed to increased synthesis of antifungal compounds in response to infection (Bell, 1969). It is conceivable that tomatine could similarly act as an extra-vascular determinant of resistance to Fol, but this has not yet been investigated.

Root damage may enable many wilt pathogens, including Fol, to by-pass extra-vascular determinants. The present study was designed to examine the nature of the host resistance response once vascular infection has occurred. The pathogen was introduced directly into the xylem vessels via a severed-taproot inoculation technique. As such, the main concern here is with the second type of mechanism, "vascular determinants" of resistance. Vascular determinants are host responses which operate within the vascular tissue to limit systemic distribution of the pathogen. Phenols, vascular occlusion and antifungal compounds have been implicated as vascular determinants of

tomato resistance to Fol race 1.

Matta et al. (1969) demonstrated in resistant tomato plants that phenols increased to a maximum concentration only 3 days after inoculation. Phenols increased gradually in susceptible plants, not reaching a maximum concentration until 18 days after infection. Matta et al. suggested that phenols could either act directly to inhibit fungal growth or aid in the formation of physical defense barriers.

Vascular occlusion is a general mechanism by which plants, once infected, can restrict systemic invasion (Beckman, 1964, 1966, 1968; Beckman and Halmos, 1962). Tyloses act as a physical barrier which the pathogen is unable to penetrate. Beckman et al. (1972) compared resistant VF-race 1 and susceptible IP-race 1 host-pathogen interactions. Resistant plants were characterized by a rapid host response, resulting in maximum vascular occlusion 2 days after inoculation. Further occlusion was maintained, effectively sealing off infection. Susceptible plants also were initially characterized by a rapid host response 1-2 days after inoculation; however, further tylose development was retarded. Final occlusion did not occur in the susceptible plants until the pathogen had already become systemic.

Sinha and Wood (1967) suggested that tyloses may not be the only factor involved in the resistance of tomato plants to vascular wilt organisms. They presented evidence

that an antifungal compound may also be operating to confirm resistance in Verticillium wilt of tomato.

Early investigators of Fusarium wilt (Irving, 1947; Irving et al., 1945) postulated that certain tomato cultivars were resistant because of the presence of antibiotics inhibitory to the fungus. These substances would be absent in susceptible cultivars. Tomatine was originally thought to be such a compound. Further investigation revealed that tomatine was present in both susceptible and resistant tomato cultivars (Irving, 1947). This was also found to be true in the present study. The concentration of tomatine in the xylem fluid prior to infection (10^{-4} M) was comparable in the VF and IP isolines, resistant and susceptible, respectively, to Fol race 1.

Following the discovery of tomatine in resistant and susceptible plants, researchers turned next to investigate the effect of infection on tomatine levels (Irving, 1947; Langcake et al., 1972; McCance and Drysdale, 1975). It is apparent from the discussion of tyloses and phenolics as determinants of resistance that the critical factor is the rate at which the plant is able to respond to infection. Timing is also critical in any consideration of tomatine as a vascular determinant. Since localization occurs within 2-4 days after infection, any other mechanism which is a primary factor in resistance should be evident within that period. An increase in tomatine concentration immediately following infection in a resistant plant could

act to inhibit the pathogen until vascular occlusion has been completed.

The results presented herein indicate that the concentration of tomatine in the resistant host-pathogen combination did increase to fungitoxic levels during the critical period following vascular infection. The data also indicate a significant difference in the resistant VF-race 1 and susceptible IP-race 1 and IP-race 2 reactions. Maximum tomatine production (approximately 10^{-3} M) within VF infected with race 1 was reached 2 days after inoculation and was maintained until the seventh day. By the twelfth day after inoculation, tomatine decreased to the level of the control (approximately 10^{-4} M). The molarity of tomatine in the susceptible IP-race 1 and IP-race 2 combinations did not increase significantly above the pre-infection level at any time during the course of the experiment. The differences observed here apparently support the view that tomatine plays a primary role in resistance to Fusarium wilt.

These results are supported by Stromberg and Corden (1974, 1977) and Hammerschlag and Mace (1975) who also demonstrated differences in tomatine levels in resistant and susceptible hosts following infection. In contrast, Langcake et al. (1972) and McCance and Drysdale (1975) reported that comparable increases in the concentration of tomatine occurred in both the resistant and susceptible cultivars 2-4 days after inoculation.

If post-infection tomatine levels are critical to the resistance of VF to race 1, then the susceptible VF-race 2 reaction should yield results comparable to those of the susceptible IP-race 1 and IP-race 2 reactions. However, this was not the case here. Rather, tomatine in VF-race 2 plants increased to a level comparable to that of the resistant VF-race 1 combination. This is in disagreement with Stromberg and Corden (1976) who suggest that susceptibility to race 2 is due to failure of fungitoxicants to accumulate in response to infection.

Although rapid increases of tomatine in both susceptible- and resistant-type reactions would suggest that tomatine cannot be a primary factor in the resistance of VF to Fol race 1, there is an alternative explanation. The more virulent Fol race 2 could be less sensitive than race 1 to the inhibitory effects of tomatine. If this were the case, then race 2 could overcome the resistance mechanism in VF merely by being able to tolerate levels of tomatine that would otherwise be fungitoxic. To test this hypothesis, the relative sensitivities of race 1 and race 2 were compared by examining the effect of tomatine on colony growth, germ tube length and spore germination. It has already been established that tomatine is inhibitory to mycelial growth and spore germination of Fol race 1 at concentrations present in tomato plants (McCance and Drysdale, 1975).

The data presented here indicate that, as expected,

tomatine is more inhibitory to the colony growth of race 1 than race 2. This difference in sensitivity between the two races argues for the involvement of tomatine in resistance. However, two lines of evidence argue against this involvement. When race 1 and race 2 colony diameters were compared after 2 days of growth on 10^{-3} M or 2 X 10^{-4} M tomatine, there was no significant difference between the two races. It was only after 4 days of growth that differences in the diameters of race 1 and race 2 became apparent. Although race 2 was considerably more successful than race 1 as the organism continued to colonize the media, both races grew at the same rate initially. It is not this greater tolerance in the later stages of colonization, but a more immediate one which would be important to race 2 in the VF isoline. The fact that tomatine was equally inhibitory to race 1 and race 2 during the initial period of growth was further supported by slide bioassay data. There was no significant difference in the sensitivity of the two races to tomatine when germ tubes were measured after 24 hours of growth. These data are supported by Stromberg and Corden (1976) who also concluded that race 1 and race 2 spores were equally sensitive to a fungitoxicant in acetone stem extracts.

Although the evidence presented above makes one question tomatine's role in resistance, it is true that tomatine inhibits colony growth and germ tube length at concentrations present in tomato tissue. The concentration

present in infected plants (10^{-3} M) was highly inhibitory to growth, while the concentration found in healthy plants (10^{-4} M) was moderately inhibitory to growth.

Tomatine was more effective in inhibiting hyphal extension than in restricting spore germination. Spore germination was not inhibited at concentrations below 4×10^{-4} M tomatine, and even 8×10^{-4} M tomatine was only moderately inhibitory to germination. This is in agreement with a recent study by McCance and Drysdale (1975) who also presented evidence that tomatine is more inhibitory to hyphal extension than to spore germination. In contrast, Fontaine et al. (1947) indicated that tomatine was fungicidal to spores of Fol race 1, but only partially inhibitory to vegetative growth. The results reported here show that race 2 is not less sensitive than race 1 to the effect of tomatine on spore germination. Only at 10^{-3} M tomatine was any difference between the races observed. At that concentration, the more virulent race 2 was actually more sensitive than race 1.

Thus, an examination of all the data from these in vitro studies indicates that race 2 is actually not any more tolerant than race 1 of fungitoxic levels of tomatine. This argues against tomatine as the sole mechanism of resistance.

It was hypothesized here that tomatine may play a key role in resistance by inhibiting spore production. The importance of spore production in the rapid distribution of

vascular pathogens within vessels of infected plants is well documented (Beckman, 1964; Beckman and Halmos, 1962; Beckman et al., 1961; Dimond, 1955). However, data presented here indicate that tomatine is a stimulant, not an inhibitor, of sporulation. Tomatine stimulated spore production at concentrations known to be present in vivo. The molarity of tomatine in xylem fluid of healthy resistant or susceptible plants was approximately 10^{-4} M. Following infection, the level of tomatine increased to 10^{-3} M in both a susceptible (VF-race 2) and a resistant (VF-race 1) host-pathogen combination. The greatest stimulation of race 1 and race 2 spore production was also induced by 2×10^{-4} M and 10^{-3} M tomatine.

If tomatine causes a proliferation of spores in vivo, the role of tomatine in resistance appears even more doubtful. Pathogen build-up through massive spore production is a characteristic feature of susceptible, not resistant, host-pathogen interactions (Elgersma et al., 1072). An in vivo stimulation of fungal propagules would not be important to host colonization in resistant reactions because vascular occlusion through rapid tylose formation can physically localize wilt pathogens. Therefore, the upward movement of conidia within the vessels would be blocked in the resistant cultivar. In susceptible cultivars, however, vascular occlusion is delayed. Tyloses form too late to act as an effective barrier and are, thus, by-passed by the fungus. Stimulation of spore production

in a susceptible plant would then be likely to contribute to further host colonization by the pathogen. In light of the data presented here, the hypothesis that tomatine could play a role in resistance by inhibiting spore production appears unlikely.

Sinha and Wood (1967) working with Verticillium wilt, and Mace and Veech (1971) working with Fusarium wilt, postulated that an inhibitory agent present in the upper stem of resistant tomato plants may be responsible, at least in part, for resistance. It seems unlikely, however, that the presence of inhibitory levels of tomatine in the upper stem would be of primary importance to Fusarium wilt resistance. Elgersma et al. (1972) found that there was almost no advance of Fol race 1 beyond the initial uptake of inoculum in the resistant VF isoline, while extensive secondary distribution beyond the initial uptake was observed in the susceptible IP isoline. Conway (1976) concluded from his work that VF is resistant to race 1 because secondary distribution is limited, but susceptible to race 2 because secondary distribution is extensive. It is clear from these two studies that resistance results only when Fol is confined to the lower portion of the plant. Vascular occlusion was cited as the mechanism responsible for restricting secondary distribution. If tylose development is retarded, as in a susceptible host-pathogen interaction, colonization becomes extensive. Once the pathogen begins to colonize the upper stem following natural

infections, resistance mechanisms are not effective and susceptibility results. The presence of an inhibitor in the upper plant axis would be insufficient to confirm resistance once extensive colonization has taken place. Nevertheless, an antifungal compound located in the upper portion of a resistant plant could act to restrict the growth of those few fungal propagules which manage to escape the localization process.

It may be that tomatine is still contributing to resistance, but not as the primary mechanism of host defense. Tomatine may be effective in the lower stem as part of a sequential resistance process. Vascular occlusion in resistant plants may not only be important in restricting the distribution of the parasite, but may also impede the flow of the transpiration stream, resulting in the further accumulation of tomatine in the infected vessels of the resistant plant. These localized concentrations of tomatine may very well exceed 10^{-3} M and approach the concentration required to completely inhibit mycelial growth or spore germination. First, tylose development would form a barrier restricting the upward spread of the pathogen, and then the build-up of tomatine behind this stationary front would effectively prevent the lateral spread of the fungus from vessel to vessel. Clearly, occlusion is the primary mechanism of resistance in such a system.

SECTION II

THE EFFECT OF *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI* RACE 1 AND RACE 2 ON THE ANTIFUNGAL ACTIVITY OF TOMATINE

Introduction

Fontaine et al. (1947) were the first to suggest that the host-produced fungitoxicant, tomatine, may be responsible for the resistance of certain tomato cultivars to *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyd. & Hans. race 1. Tomatine was found in both resistant and susceptible tomato plants prior to infection. After infection, a high level of tomatine was maintained in resistant plants, while tomatine gradually disappeared from the susceptible cultivar. Tomatine was absent from wilted and dying tomato plants (Irving, 1947). Kern (1952) later confirmed those results.

Arneson and Durbin (1967) suggested that the decrease in tomatine content of infected plants indicated that in susceptible tomato plants *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) race 1 was either able to degrade tomatine, or the fungus induced the host to degrade tomatine. Recently Stromberg and Corden (1974) came to a similar conclusion. They presented evidence that *Fol* race 1 fungal populations increased within an infected susceptible cultivar three days after inoculation, while

the fungitoxicity of acetone xylem extracts decreased. However, in the resistant cultivar, the fungal population remained low, and the xylem extracts became highly fungitoxic. The persistence of a high level of an inhibitory substance in a resistant plant, in contrast to a decrease in this substance within a susceptible plant following infection, led the authors to conclude that susceptibility was due to detoxification of the inhibitor.

Little research has been done on the microbial transformation of tomatine. As a result, only a few fungi have been shown to be capable of detoxifying tomatine. Arneson and Durbin (1966) demonstrated that extracts from healthy tomato leaves inhibited spore germination and mycelial growth of Septoria linicola and S. lactucae, both non-pathogenic on tomato plants. However, extracts of leaves heavily infected with the tomato leaf spot pathogen, S. lycopersici, were not toxic to those fungi. The authors suggested that S. lycopersici detoxified tomatine following infection. This was supported in a later study (Arneson and Durbin, 1967) which showed that S. lycopersici detoxified alpha-tomatine by enzymatically hydrolyzing one glucose unit from the tomatine molecule. The resulting compound was $beta_2$-tomatine (Fig. 1). Although alpha-tomatine occurred in healthy tomato leaves, $beta_2$-tomatine was also present in leaves infected with S. lycopersici.

These data, coupled with the fact that other fungal

parasites of tomato have also been reported to be relatively insensitive to tomatine, led Arneson and Durbin (1967) to hypothesize that these parasites also may detoxify tomatine. It was also suggested that the ability to overcome the toxicity of alpha-tomatine may be necessary for the success of tomato pathogens.

Botrytis cinerea, which causes a latent infection in young, green tomato fruits, is capable of detoxifying alpha-tomatine (Verhoeff and Liem, 1974). In this case, tomatine was hydrolyzed to tomatidine, a derivative of alpha-tomatine (Fig. 1) which is non-toxic to B. cinerea.

One of the two objectives of the present study was to determine if Fol race 1 or race 2 was capable of detoxifying tomatine. It is known that tomatine is inhibitory to spore germination of both Fol races. It was hypothesized that if a given population of spores was able to detoxify tomatine, then the percent germination of subsequent populations of spores placed in the tomatine solution would increase. The percentage of spore germination would then continue to increase as more tomatine was detoxified with each successive population of spores.

With the exception of Stromberg and Corden (1977), few researchers have considered toxicant concentration-to-fungal population ratios in their investigations of the effect of tomatine on Fol. It was hypothesized that spore concentration may be important in determining the effectiveness of a given concentration of tomatine on spore

germination. If this were the case, then as spore levels increased and the tomatine concentration remained constant, less tomatine would be available per spore and the germination percentage would increase. Conversely, as spore levels decreased, more toxicant would be available per spore, and spore germination would decrease. The second objective of this study was to determine the effect of spore load on the percent germination of Fol race 1 subjected to a known concentration of tomatine.

Materials and Methods

Isolates of *Fusarium oxysporum* f. sp. *lycopersici* races 1 and 2 were obtained from Dr. G. M. Armstrong (Georgia Agricultural Experiment Station, Experiment, Georgia 30212). Stock cultures were maintained on silica gel (Perkins, 1962). Microconidia for spore suspensions were harvested from 10-day-old cultures grown on potato dextrose agar. Spores were filtered through lens paper and the concentration adjusted with the aid of a hemacytometer.

Two solutions were tested, one containing 8×10^{-4} M tomatine and one without tomatine. Pure alpha-tomatine (ICN Life Sciences Group, Cleveland, Ohio 44128) was dissolved in 10 mM HCl and the pH brought up to 4.6 with a phosphate-citrate buffer. Buffered solution lacking tomatine served as the control. Tomatine and buffer control solutions were sterilized by filtration through a Millipore filter (0.2 um pore size). The pH of the solutions was tested after spores had been incubated.

Percent germination was determined by counting the number of spores out of 100 which had germinated. Spores were considered germinated if the germ tube was at least the length of the spore. Three random fields per replicate were examined for each treatment. The data were analyzed statistically using Duncan's multiple-range test ($P=0.05$).

Effect of Successive Spore Populations on Germination

Samples (2.5 ml) of tomatine or buffer control solutions were added to 2.5 ml of a Fol race 1 or race 2 spore suspension in sterile 25 ml Erlenmeyer flasks. Spore suspensions were adjusted to give a concentration of 1×10^5 microconidia/ml when added to the two test solutions. Each of the four combinations was replicated six times. Flasks were placed on a shaker table (70 cycles/min) and incubated at room temperature (25-26 C) for 24 hours. After incubation, a drop was removed from each flask, and percent spore germination was determined. Microconidia were removed from the flask contents by filtration through a Millipore filter (0.2 um pore size). Flasks from each combination were divided into two groups. Race 1 spores were added to the first group of conditioned solutions; race 2 spores were added to the second group. Percent germination was determined after 24 hours on the shaker table. Solutions were filtered free of spores and the process was repeated twice. Each time, group one flasks received race 1 spores and group two flasks received race 2 spores.

Effect of Spore Concentration on Germination

Slide bioassay. Fol race 1 spore concentrations of 1×10^4, 1×10^5, 1×10^6 and 3×10^6 microconidia/ml were tested. One drop of the tomatine solution or buffer solution was added to one drop of spore suspension on a

microscope slide coated with 2.5% cellulose nitrate. Each treatment was replicated six times. Slides were placed in sterile, moist petri dishes and incubated at 26 C for 24 hours. Following incubation, spores were stained and fixed with 1% cotton blue in lactophenol and the percent germination determined.

Shaker table bioassay. Fol race 1 spore concentrations of 1×10^5 and 1×10^6 microconidia/ml were tested. Samples (2.5 ml) of tomatine or control solution were added to 2.5 ml of spore suspension in sterile 25 ml Erlenmeyer flasks. Each treatment was replicated three times. Flasks were placed on a shaker table (70 cycles/min) and incubated at room temperature (25-26 C). After 24 hours, a drop was removed from each flask and placed on a microscope slide. Spores were stained and fixed with 1% cotton blue in lactophenol and the percent germination determined.

Results

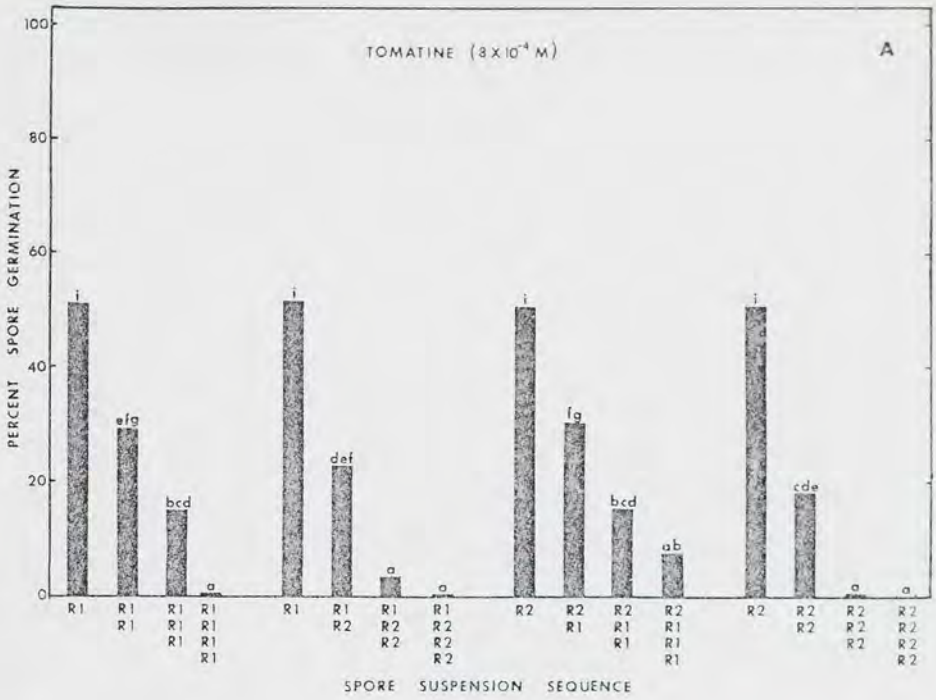
Effect of Successive Spore Populations on Germination

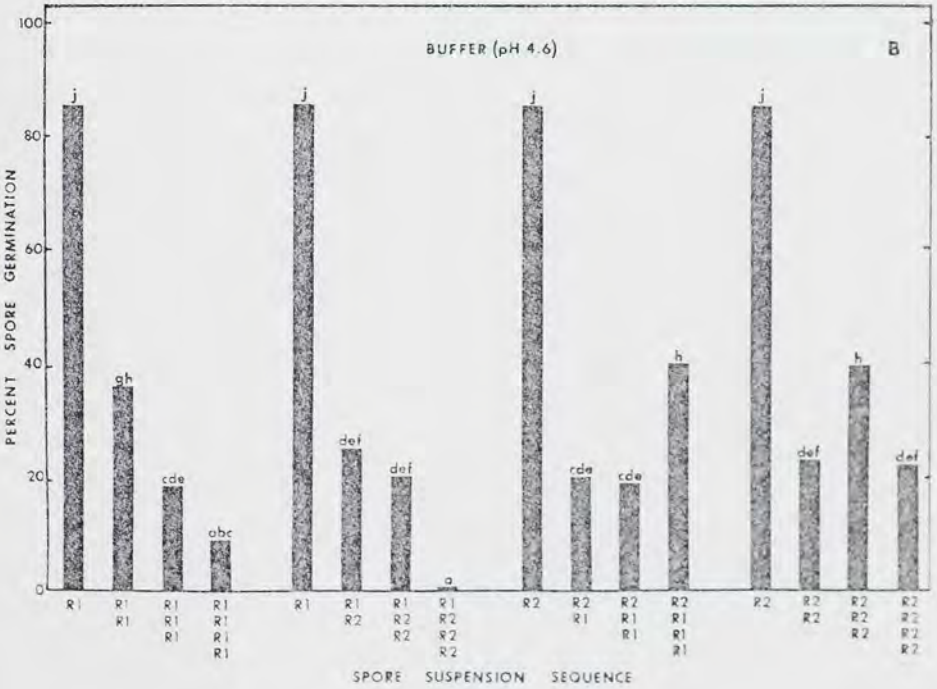
Tomatine significantly inhibited germination of race 1 and race 2 spores in the initial suspension, but there was no significant difference in the response of the two races (Fig. 9-A). Percentages of spore germination of race 1 in the initial buffer control solutions were also comparable to race 2 (Fig. 9-B). Spore germination in conditioned tomatine or buffer control solutions decreased significantly compared to germination in the initial suspension. Germination was reduced as much as 59-78% in conditioned buffer solutions, while a 41-65% reduction in germination was observed for spores in conditioned tomatine solutions. Spore germination continued to decrease with each successive spore suspension. Generally, percent germination of conidia in the conditioned tomatine solutions was not significantly different from germination in the conditioned buffer control solutions, when comparing similar spore suspension sequences. The pH of all solutions remained at 4.6.

Effect of Spore Concentration on Germination

Slide bioassay. The percent germination of spores in the buffer control or tomatine solutions decreased

Fig. 9. The effect of various spore suspension sequences on the germination of microconidia of Fusarium oxysporum f. sp. lycopersici race 1 (R1) or race 2 (R2) in A) an 8 X 10^{-4} M tomatine solution or B) a buffer control solution. Means labeled with the same letter are not significantly different, P=0.05, according to Duncan's multiple-range test.

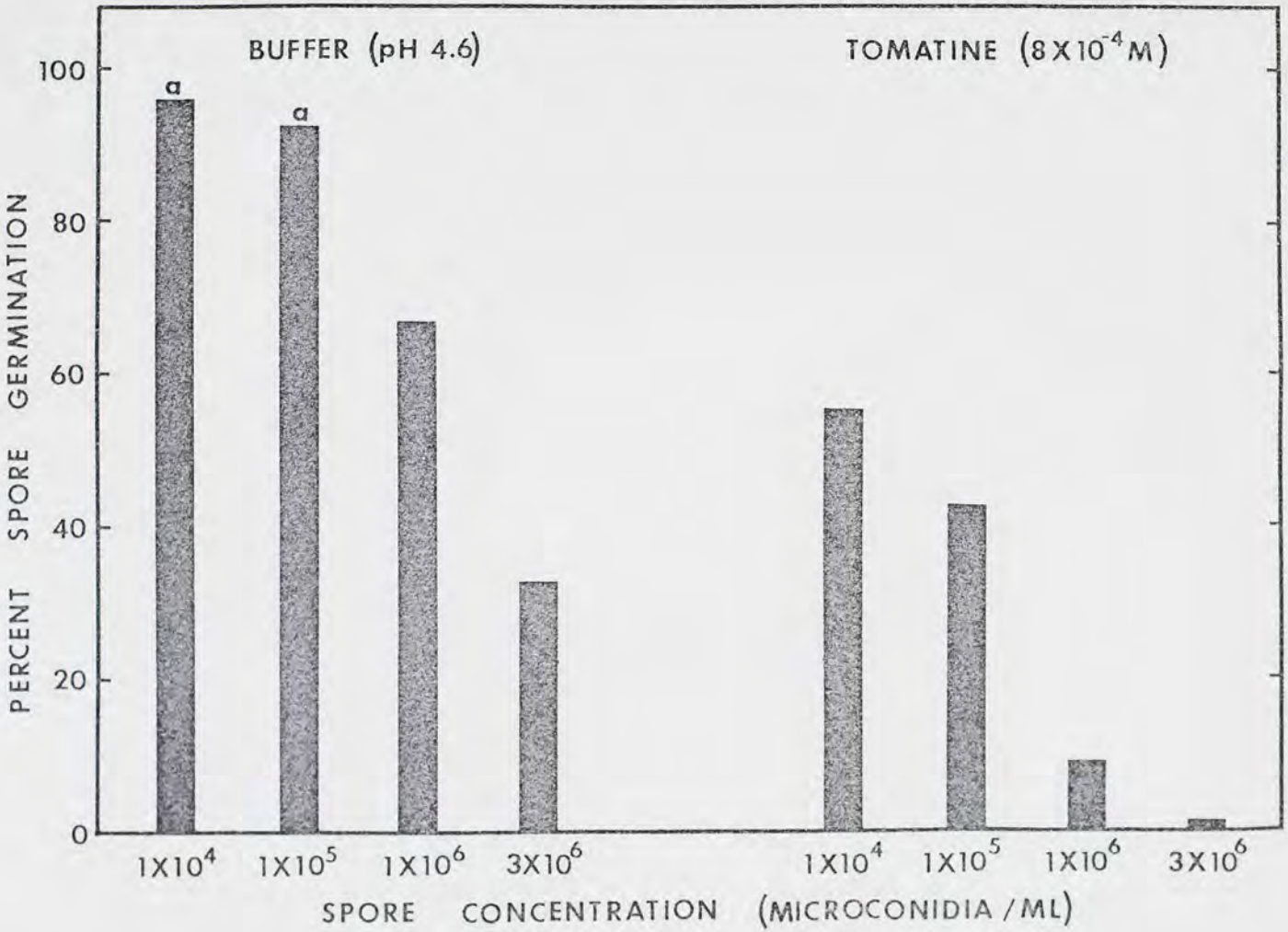

TOMATINE (8 X 10⁻⁴ M)
A
PERCENT SPORE GERMINATION
100
80
60
40
20
0
i
efg
bcd
a
i
def
a
a
i
fg
bcd
ab
i
cde
a
a
R1
R1 R1
R1 R1 R1
R1 R1 R1 R1
R1
R1 R2
R1 R2 R2
R1 R2 R2 R2
R2
R2 R1
R2 R1 R1
R2 R1 R1 R1
R2
R2 R2
R2 R2 R2
R2 R2 R2 R2
SPORE SUSPENSION SEQUENCE


BUFFER (pH 4.6)
B
PERCENT SPORE GERMINATION
100
80
60
40
20
0
j
gh
cde
abc
j
def
def
a
j
cde
cde
h
j
def
h
def
R1
R1 R1
R1 R1 R1
R1 R1 R1 R1
R1
R1 R2
R1 R2 R2
R1 R2 R2 R2
R2
R2 R1
R2 R1 R1
R2 R1 R1 R1
R2
R2 R2
R2 R2 R2
R2 R2 R2 R2
SPORE SUSPENSION SEQUENCE

significantly as spore concentration increased (Fig. 10). Germination of conidia at concentrations of 1 X 10^4 and 1 X 10^5 microconidia/ml was comparable in the control solutions. As the spore concentration was increased ten-fold from 1 X 10^5 to 1 X 10^6 microconidia/ml, germination was reduced by 29%. A three-fold increase in spores from 1 X 10^6 to 3 X 10^6 conidia/ml resulted in a 53% reduction in germination . Germination of conidia in the tomatine solution was significantly less than germination in the buffer solution at the same spore concentration (Fig. 10). Increasing the spore level in tomatine from 1 X 10^4 to 1 X 10^5 microconidia/ml caused a 26% drop in germination. An 84% decrease in germination occurred when the spore concentration increased from 1 X 10^5 to 1 X 10^6 conidia/ml. The largest reduction (92%) in spore germination occurred when 3 X 10^6 conidia/ml was tested in tomatine. The pH of all solutions remained at 4.6.

Shaker table bioassay. Percent germination of spores at a concentration of 1 X 10^5 microconidia/ml was similar whether spores were incubated on the shaker table or on microscope slides (Fig. 10, 11). However, the effect of spore load on germination was more pronounced when the shaker table was used than when slides were used. When the spore concentration in the control solution was increased from 1 X 10^5 to 1 X 10^6 microconidia/ml, germination decreased by 87% (Fig. 11). The same increase in spore concentration in the tomatine solution resulted in a

Fig. 10. The effect of selected spore concentrations on the germination of microconidia of Fusarium oxysporum f. sp. lycopersici race 1 incubated for 24 hours on microscope slides. Means labeled with the letter a are not significantly different, P=0.05, according to Duncan's multiple-range test.


BUFFER (pH 4.6)
TOMATINE (8X10⁻⁴M)
PERCENT SPORE GERMINATION
100
80
60
40
20
0
a
a
1X10⁴
1X10⁵
1X10⁶
3X10⁶
1X10⁴
1X10⁵
1X10⁶
3X10⁶
SPORE CONCENTRATION (MICROCONIDIA / ML)

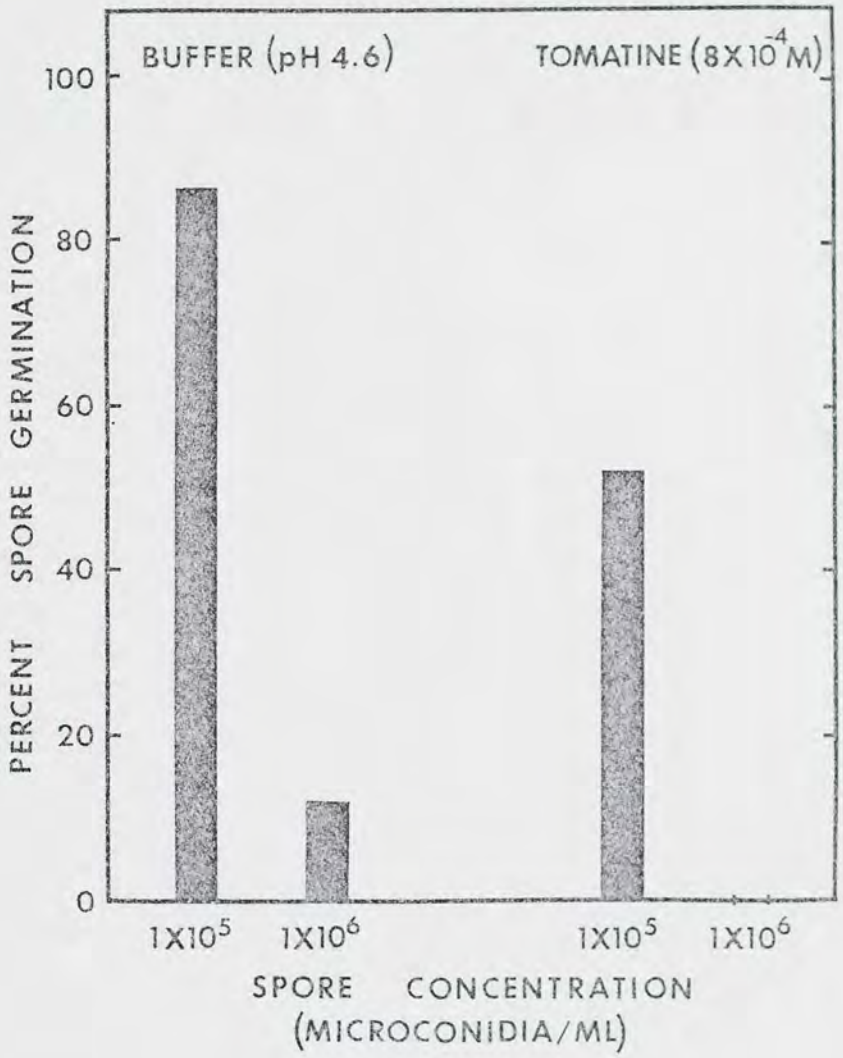


Fig. 11. The effect of selected spore concentrations on the germination of microconidia of Fusarium oxysporum f. sp. lycopersici race 1 incubated for 24 hours in flasks on a shaker table.

complete (100%) inhibition of germination. The pH of all solutions remained at 4.6.

Discussion

The focus of previous studies has been to investigate the effect of tomatine on spores (Langcake et al., 1972; McCance and Drysdale, 1975). It was the intent of this experiment to determine what effect spores may have on tomatine. In the first assay, the possibility that Fol race 1 and/or race 2 could detoxify tomatine was examined. The possibility that increased spore load would lessen the inhibitory effect of tomatine on spore germination was examined in the second set of assays. It was expected, in both cases, that the effectiveness of tomatine would decrease, resulting in an increase in spore germination. Instead, a dramatic decrease in germination was observed, but in the buffer solutions as well as in the tomatine solutions. Thus, the original hypotheses can neither be substantiated nor denied. Nevertheless, the data do provide evidence that Fol race 1 and race 2 spores produced a substance which was self-inhibitory.

Spores generally do not germinate in the culture in which they are produced (Macko et al., 1976). This may be due to dormancy, but more commonly it is due to the presence of inhibitory substances. Spores of many fungi germinate poorly or not at all when they are excessively crowded on a surface or in a dense suspension (Cochrane, 1958; Macko et al., 1976). The crowding effect alone is not sufficient evidence for the presence of a self-inhibitor,

but it does suggest that an inhibitory substance may be present.

Evidence from the two bioassays performed here indicated that physical crowding alone did not account for the inhibition. Considering the spore load assay alone, one could argue that decreasing germination percentages with increasing spore concentration was due to a physical crowding effect. However, the other bioassay technique showed that the effect was really due to the presence of an inhibitory substance. In this assay, spore concentration remained the same, while one population of spores was followed in sequence by another population. The further decrease in spore germination with each subsequent spore suspension indicated that a chemical inhibitor produced by the spores remained in the solution when the population was removed. As the compound built up in solution with each successive population, the inhibitory effect also increased. The total effect was similar to that of increasing the actual spore numbers in the solution.

Reduced spore germination due to the production of self-inhibitory substances has been demonstrated for a number of other fungal species. Conidia of Glomerella cingulata in liquid shake cultures germinated at a concentration of 1×10^5 spores/ml, but less than 1% of the spores germinated when the concentration was increased to 1×10^9 spores/ml (Lingappa and Lingappa, 1965, 1966). An inhibitory fraction was extracted from the conidia. The rusts,

particularly Puccinia graminis tritici (Allen, 1955), provide a classic example of the self-inhibitory phenomenon.

Solutions conditioned by race 1 or race 2 were inhibitory to both race 1 and race 2. The toxic compound was apparently produced by both races and exerted the same inhibitory effect on the two races. Spore germination in conditioned solutions was restricted regardless of whether spores were incubated in tomatine or buffer. In fact, any initial differences in the germination of conidia in the two solutions were later obscured.

The self-inhibiton phenomenon could have some very interesting implications if it occurs in tomato plants infected with Fol. Spores which build up behind the physical barriers produced in response to infection may fail to germinate due to the excretion and accumulation of this inhibitory compound. The self-inhibitory factor, along with tomatine accumulation, could contribute to resistance by restricting lateral fungal spread in infected vessels.

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