Fungal Sensitivity to and Enzymatic Degradation of the Phytoanticipin α -Tomatine

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ABSTRACT

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α -Tomatine, synthesized by *Lycopersicon* and some *Solanum* species, is toxic to a broad range of fungi, presumably because it binds to 3β -hydroxy sterols in fungal membranes. Several fungal pathogens of tomato have previously been shown to be tolerant of this glycoalkaloid and to possess enzymes thought to be involved in its detoxification. In the current study, 23 fungal strains were examined for their ability to degrade α -tomatine and for their sensitivity to this compound and two breakdown products, β_2 -tomatine and tomatidine. Both saprophytes and all five non-pathogens of tomato tested were sensitive, while all but two tomato patho-

gens (*Stemphylium solani* and *Verticillium dahliae*) were tolerant of α -tomatine (50% effective dose > 300 μ M). Except for an isolate of *Botrytis cinerea* isolated from grape, no degradation products were detected when saprophytes and nonpathogens were grown in the presence of α -tomatine. All tomato pathogens except *Phytophthora infestans* and *Pythium aphanidermatum* degraded α -tomatine. There was a strong correlation between tolerance to α -tomatine, the ability to degrade this compound, and pathogenicity on tomato. However, while β_2 -tomatine and tomatidine were less toxic to most tomato pathogens, these breakdown products were inhibitory to some of the saprophytes and nonpathogens of tomato, suggesting that tomato pathogens may have multiple tolerance mechanisms to α -tomatine.

Additional keywords: glucosidase, nondegradative tolerance, saponin.

Many of the class of compounds known as saponins have antimicrobial and insecticidal properties and are considered to be protective agents against potential pathogens and insects (2,17). Saponins are steroidal or triterpenoid compounds that are generally glycosylated at one end of the molecule, possess soap-like properties in water, and have hemolytic and cholesterol-binding properties (27). One member of this group, α -tomatine (Fig. 1), is a steroidal glycoalkaloid found in most organs of the tomato plant at concentrations observed to be inhibitory to microbes in vitro, except in ripe fruit where the compound is normally not detectable (15).

Since concentrations of this compound can accumulate at up to 5% of the fresh weight of tomato tissue (19), it would appear to be a potent barrier to microbial infection. However, the importance of α -tomatine and other phytoanticipins (preformed inhibitory compounds [39]) as a general barrier to microbial infection has been circumstantial, and conflicting data exist for the direct involvement of α -tomatine in plant resistance. One clear aspect of past research is the observation that fungal pathogens of tomato are generally more tolerant to this phytoanticipin than nonpathogens of tomato and saprophytes (2), suggesting that tomato pathogens have evolved specific means to circumvent this chemical barrier.

Previous research has shown that α -tomatine owes its toxic properties to its ability to interact with 3β -hydroxy sterols (34). Tolerance to α -tomatine has been proposed to arise from either membrane characteristics of the fungus or the ability of the fungus to enzymatically detoxify this compound (2). Mutants of *Nectria haematococca* mating population VI whose membranes were reduced in sterol content were more tolerant to α -tomatine (8). Moreover, Pythiaceus fungi such as *Pythium* and *Phytophthora* species do

not produce 3β -hydroxy sterols and are relatively tolerant of α -tomatine (36). However, when sterols are exogenously supplied to these organisms, they readily incorporate the sterols into the membrane and become sensitive to α -tomatine (36). Thus, the membrane characteristics of fungi can directly affect their sensitivity to this saponin.

Enzymatic removal of one or more sugars from the tetrasaccharide moiety of α -tomatine has also been shown to restrict its binding to 3β -hydroxy sterols, thus reducing the toxicity of the compound (3). Hydrolysis of the β -1,2-D glucosyl bond, resulting in the release of D-glucose with the formation of β_2 -tomatine (Fig. 1A), has been reported for *Verticillium albo-atrum* (26) and *Septoria lycopersici* (1). Hydrolysis of all four glycosidic linkages, releasing the four monosaccharides and the aglycone tomatidine, has been observed for *Alternaria solani* (33), while *Botrytis cinerea* and *Fusarium oxysporum* f. sp. *lycopersici* produce an enzyme that cleaves the β -1-D galactosidic linkage, releasing the tetrasaccharide moiety as a single unit (16,24,40). Enzymatic alteration of the alkaloid aglycone tomatidine has been documented, but no pathogens have been reported to possess this ability (4,32).

Fig. 1. Structure of α -tomatine (*O*- β -D-glucopyranosyl-(1,2 glu)-*O*- β -D-xylopyranosyl-(1,3 glu)-*O*- β -D-glucopyranosyl-(1,4 gal)- β -D-galactopyranosyl-tomatidine). β_1 -Tomatine lacks the sugar [B]; β_2 -tomatine lacks the sugar [A]; γ -tomatine does not have either sugar [A] or [B]; δ -tomatine does not have sugars [A], [B], or [C]; and tomatidine lacks all four sugars.

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Although definitive evidence is lacking regarding the importance of α -tomatine detoxification in pathogenicity by tomato pathogens, the importance of the detoxification of the oat saponin avenacin in pathogenicity has recently been demonstrated. *Gaeumannomyces graminis* var. *avenae*, a root pathogen, produces an enzyme that hydrolyzes the β -1,2- and β -1,4-linked D-glucose molecules from the triterpenoid avenacin, resulting in a less toxic compound (7,37). The gene encoding the avenacinase enzyme was cloned, and avenacinase-deficient mutants of *G. graminis* var. *avenae* were constructed using transformation-mediated gene disruption methods (5). These mutants were no longer pathogenic on oats, which produce avenacin in roots, but retained pathogenicity on wheat, a host that does not produce this saponin. These results provide direct evidence for the involvement of avenacin in plant disease resistance and demonstrate that avenacinase is a pathogenicity trait for *G. graminis* var. *avenae*.

As a preliminary step to determine the importance of α -tomatine tolerance and detoxification for pathogenicity on tomato, 12 tomato pathogens, 7 pathogens of other nontomato hosts, and 2 saprophytic fungi were tested for their in vitro sensitivity to α -tomatine and its derivatives and for their ability to degrade α -tomatine. In addition, the inducibility of the α -tomatine-degrading enzymes was evaluated in one tomato pathogen (*A. solani*) and ergosterol mutants of *Neurospora crassa* were tested for sensitivity to α -tomatine and its derivatives to determine if an organism altered in its sterol composition would also be altered in its sensitivity to these compounds.

MATERIALS AND METHODS

Strains and media. Fungal isolates used in this study are listed in Table 1. Isolates that are indicated as tomato pathogens in Table 2 were originally isolated from tomato. All isolates were grown at 24°C except for *P. infestans*, which was grown at 18°C. *Aspergillus nidulans* was grown on *Aspergillus* medium (44). *N. crassa* was grown on Vogel's medium (41). *Magnaporthe grisea* was grown on oatmeal agar (38). *S. solani* was grown on cornmeal agar (Difco Laboratories, Detroit). *P. infestans* was grown on rye B medium (6). All other isolates were grown on V8 agar medium

(200 ml of V8 juice, 2 g of CaCO₃, and 20 g of agar per liter). All strains except *Pythium aphanidermatum* and *P. infestans* were stored as glycerol stocks at -80°C.

Bioassay for sensitivity of fungi to α -tomatine, β_2 -tomatine, and tomatidine. α -Tomatine and tomatidine were purchased from Sigma Chemical Co. (St. Louis). β_2 -Tomatine was produced by incubating 50 ml of 2.5 mM α -tomatine with 25 mM sodium acetate (pH 5.0) in the presence of purified β_2 -tomatinase (31) for 4 h at 37°C. The reaction was checked for completion by thin-layer chromatography (TLC). The solution was then passed through a 0.2- μ m filter (Nalgene, Rochester, NY) and heated to 60°C for 1 h to inactivate the enzyme. Stocks of α -tomatine, β_2 -tomatine, and tomatidine were kept as 2.5 mM solutions in 25 mM sodium acetate, pH 5.0.

Fungi were tested for their sensitivity to α -tomatine, β_2 -tomatine, and tomatidine by measuring the radius of mycelial colonies grown on medium supplemented with these compounds. All media were adjusted to pH 5.0 with acetic acid prior to autoclaving. Because of the dilution of the media by the addition of the compounds, the media were made at a 1.4 $\times$ concentration. Compounds were added to the media after autoclaving. Bioassay plates contained 2 ml of solid medium in a 10 $\times$ 35-mm petri dish containing 0, 20, 50, 100, 200, 400, 800, and 1,000 μ M of the compound tested except for those used for *N. crassa* wild type and ergosterol-deficient mutants, which contained 0, 20, 60, 90, 120, 180, 240, 500, and 1,000 μ M of the compound tested. An agar plug (6 mm diameter) was taken from the edge of an actively growing colony, the mycelial side was placed on the medium at the edge of the dish, and radial growth was measured every 24 h for 3 to 5 days. Three plates were used for each treatment. The values obtained when the mycelium had advanced approximately 75% across the plate on medium containing no α -tomatine were used to calculate percent inhibition by the test compounds. The concentration of compound that gave 50% inhibition (ED₅₀) of radial growth was extrapolated from these values using the NLIN procedure for nonlinear regression (SAS Institute, Cary, NC). The negative exponential equation $y = b_0[1 - e^{(-b_1 \cdot x)}]$, in which y equals the percent inhibition and x equals the concentration of the compound tested, was used for the nonlinear regression analysis.

TABLE 1. Fungal strains used in this study

Fungal isolate	Provider
<i>Alternaria alternata</i> (Fr.:Fr.) Keissl. #100ss	D. Gilchrist, Univ. of California, Dept. of Plant Pathol., Davis
<i>A. alternata</i> (Fr.:Fr.) f. sp. <i>lycopersici</i> Grogan et al. T507 ^a	D. Gilchrist
<i>A. solani</i> Sorauer T519 ^a	P. Shoemaker, N. Carolina Mountain Hortic. Crops Res. & Ext., Fletcher
<i>Verticillium dahliae</i> Kleb. T520 ^a	P. Shoemaker
<i>Cryphonectria parasitica</i> (Murrill) Barr. EP155	P. Wang, Univ. of Maryland, Center for Agric. Biotechnology, College Park
<i>Botrytis cinerea</i> Pers.:Fr. T515 ^a	V. Higgins, Univ. of Toronto, Dept. of Plant Pathol., Montreal, Canada
<i>Septoria lycopersici</i> Speg. T512 ^a	V. Higgins
<i>Colletotrichum coccodes</i> (Walr.) Hughes T506 and T508 ^a	V. Higgins
<i>V. albo-atrum</i> Reinke & Berthier T513 ^a	V. Higgins
<i>Fusarium oxysporum</i> Schlechtend.:Fr. f. sp. <i>lycopersici</i> (Sacc.) Snyder & Hans T373 ^a	J. P. Jones, Gulf Coast Res. & Ed. Center, Brandenton, FL
<i>F. moniliforme</i> Sheld. T436 ^a	L. Dunkle, Purdue Univ., Dept. of Botany & Plant Pathol., W. Lafayette, IN
<i>Macrophomina phaeosolana</i> Tassi T437 ^a	G. Odvody, Texas A&M, Agric. Exp. Station, Corpus Christi, TX
<i>Stemphylium botryosum</i> Wallr. #60565	American Type Culture Collection, Rockville, MD
<i>S. solani</i> Weber #11128	American Type Culture Collection
<i>S. solani</i> #18792	American Type Culture Collection
<i>Septoria lycopersici</i> T550 ^a	M. Gleason, Iowa State Univ., Dept. of Plant Pathol., Ames
<i>Nectria haematococca</i> Berk. & Br. MPV1 6-36 ^a	H. VanEtten, Univ. of Arizona, Dept. of Plant Pathol., Tucson
<i>Pythium aphanidermatum</i> (Edson) Fitz.	M. Stanghellini, Univ. of Arizona, Dept. of Plant Pathol., Tucson
<i>Phytophthora infestans</i> (Mont.) deBary T510 ^a	R. Jaime-Garcia, Univ. of Arizona, Dept. of Plant Pathol., Tucson
<i>Magnaporthe grisea</i> (Hebert) Barr. 4392-1-6	M. Orbach, Univ. of Arizona, Dept. of Plant Pathol., Tucson
<i>Neurospora crassa</i> Shear & Dodge 74A FGSC 987	Fungal Genetics Stock Center, Univ. of Kansas Medical Center, Dept. of Micro., Kansas City
<i>N. crassa</i> erg-1 FGSC 2721	Fungal Genetics Stock Center
<i>N. crassa</i> erg-2 FGSC 2723	Fungal Genetics Stock Center
<i>N. crassa</i> erg-3 FGSC 2725	Fungal Genetics Stock Center
<i>N. crassa</i> erg-4 FGSC 3654	Fungal Genetics Stock Center
<i>Aspergillus nidulans</i> (Eidam) G. Wint. UCD1	Fungal Genetics Stock Center

^a VanEtten culture collection number designation.

The *N. crassa* ED₅₀ values obtained for α -tomatine, β_2 -tomatine, and tomatidine were subjected to analysis of variance using the GLM procedure (SAS Institute). ED₅₀ values of the ergosterol mutants were compared to the ED₅₀ value of wild type using the Dunnett test at $\alpha = 0.05$ (13). Each fungus was assayed three times. Reported values are the average of these three assays.

Isolation and identification of α -tomatine degradation products. Fungi were grown for 7 days on 35-mm petri plates containing 2 ml of agar medium supplemented with 20 μ M α -tomatine, a concentration that allowed growth of all fungi. The alkaloids were extracted by placing the mycelium and agar into 15-ml polypropylene tubes with 5 ml of 100 mM HCl and blending with a Brinkman Polytron tissue homogenizer (Brinkman Instruments, Inc., Westbury, NY). After sitting for 1 h at 24°C, the extract was centrifuged for 10 min at 5,000 $\times$ g and the alkaloids were precipitated from the supernatant by the addition of 500 μ l of 30% ammonium hydroxide. After a 30-min incubation on ice, the solution was centrifuged at 5,000 $\times$ g for 30 min, the supernatant carefully removed, and the pellet dissolved in 50 μ l of ethanol. Approximately 25 μ l was spotted onto an aluminum-backed silica gel TLC plate (0.2 mm thick, Kieselgel 60 F₂₅₄; EM Separations, Gibbstown, NJ) and developed in ethyl acetate/methanol/acetic acid/water (30:20:10:1). The TLC plate was dried, dipped in 30% sulfuric acid, and placed at 110°C for 10 min. *R_f* values for α -tomatine, β_2 -tomatine, and tomatidine were 0.23, 0.4, and 0.75, respectively.

In some experiments, a compound possessing an *R_f* value of 0.57 was observed on the TLC plates. Silica from a preparative TLC plate in which the unknown compound migrated was removed from the plate, eluted with ethanol, evaporated to dryness for mass spectrum (MS) analysis, and resuspended in deuterated pyridine for nuclear magnetic resonance (NMR). α -Tomatine and tomatidine were used as standards for the MS and NMR analyses.

Partial characterization of *A. solani* tomatine-degrading enzymes. *A. solani* mycelium was scraped from the surface of the agar medium of a 7-day culture and placed in 200 ml of clarified V8 liquid medium (V8 juice medium minus the agar was centrifuged at 10,000 $\times$ g, the pellet discarded, and the liquid autoclaved). Cultures were shaken at 200 rpm for 3 days at 24°C. My-

celium was harvested onto Miracloth (Calbiochem-Behring, La Jolla, CA) by vacuum, and it and the culture filtrate were used immediately for enzyme purification.

Proteins in the culture filtrate were precipitated by the addition of ammonium sulfate to 80% saturation and stirring for 1 h at 24°C before centrifuging for 30 min at 12,000 $\times$ g. The pellet was resuspended in 50 ml of 50 mM sodium acetate (pH 5.0) and dialyzed against 4 liters of the same buffer for 24 h at 4°C. The dialyzed proteins were concentrated to 5 ml using an Amicon ultrafiltration cell and a YM-30 filter membrane (Amicon Inc., Beverly, MA). Protein concentration was determined with the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard. Enzyme assays were carried out in 500 μ l of 500 μ M α -tomatine with 50 mM sodium acetate (pH 5.0) using 100 μ g of protein at 24°C for 2 h. The alkaloids were precipitated with 50 μ l of 30% ammonium hydroxide, centrifuged at 3,000 $\times$ g for 15 min at 24°C, and analyzed by TLC as described above.

To determine if the α -tomatine-degrading enzymes of *A. solani* increased after exposure to α -tomatine, 1.0 g of *A. solani* mycelium from a 3-day culture was incubated in 25 ml of 50 mM sodium acetate (pH 5.0) with and without 500 μ M α -tomatine for 0, 2, 4, 6, 8, and 24 h. The mycelium was collected by vacuum filtration, washed once with 50 mM sodium acetate (pH 5.0) with 50 mM NaCl, and homogenized for 1 min in 25 ml of 50 mM sodium acetate (pH 5.0) with 50 mM NaCl and 10 g of glass beads (50 to 70 U.S. sieve; Sigma Chemical Co.) using a Bead Beater (Biospec Products, Bartlesville, OK). The homogenate was centrifuged for 30 min at 15,000 $\times$ g and the supernatant collected and concentrated to 5 ml using an Amicon ultrafiltration cell and a YM-30 filter (Amicon Inc.). Protein (100 μ g) from the concentrate was incubated in 500 μ l of 500 μ g of α -tomatine with 50 mM sodium acetate (pH 5.0) for 2 h at 24°C. The alkaloids in the reaction mixture were analyzed as above.

RESULTS

The sensitivity of fungi to α -tomatine and its degradation products. Saprophytic fungi, most nonpathogens of tomato, and

TABLE 2. Fungal sensitivity to α -tomatine, β_2 -tomatine, and tomatidine and degradation products produced by those fungi

Isolate	ED ₅₀ (standard deviation)			Percent inhibition at 1 mM			Enzymatic degradation products
	α -Tomatine	β_2 -Tomatine	Tomatidine	α -Tomatine	β_2 -Tomatine	Tomatidine	
<i>Neurospora crassa</i>	13 μ M (2)	122 μ M (5)	51 μ M (13)	100	100	100	No ^a
<i>Magnaporthe grisea</i>	22 μ M (3)	31 μ M (3)	>1,000 μ M	100	100	17	No
<i>Cryphonectria parasitica</i>	21 μ M (2)	302 μ M (6)	81 μ M (12)	100	100	93	No
<i>Nectria haematococca</i> MPVI	34 μ M (2)	80 μ M (3)	>1,000 μ M	100	100	17	No
<i>Aspergillus nidulans</i>	45 μ M (5)	98 μ M (4)	81 μ M (16)	100	100	92	No
<i>Stemphylium solani</i> 11128 ^b	85 μ M (3)	92 μ M (3)	300 μ M (17)	100	79	98	TD ^c
<i>Fusarium moniliforme</i>	93 μ M (4)	502 μ M (7)	>1,000 μ M	93	77	23	No
<i>Macrophomina phaeosolana</i>	100 μ M (3)	>1,000 μ M	>1,000 μ M	80	47	44	No
<i>Verticillium dahliae</i> ^b	107 μ M (3)	502 μ M (6)	>1,000 μ M	90	74	43	TD
<i>Botrytis cinerea</i>	143 μ M (3)	600 μ M (7)	>1,000 μ M	100	61	16	TD
<i>Stemphylium solani</i> 18792 ^b	302 μ M (7)	301 μ M (6)	304 μ M (12)	100	82	85	TD
<i>Phytophthora infestans</i> ^b	303 μ M (6)	Not tested	>1,000 μ M	91	Not tested	38	No
<i>Colletotrichum coccodes</i> T506 ^b	394 μ M (8)	>1,000 μ M	>1,000 μ M	51	33	14	TD
<i>Stemphylium botryosum</i> ^b	420 μ M (12)	502 μ M (9)	>1,000 μ M	70	56	16	TD
<i>Colletotrichum coccodes</i> T508 ^b	423 μ M (9)	>1,000 μ M	>1,000 μ M	54	30	11	TD
<i>Pythium aphanidermatum</i> ^b	433 μ M (9)	Not tested	451 μ M (15)	100	Not tested	93	No
<i>Verticillium albo-atrum</i> ^b	439 μ M (7)	>1,000 μ M	>1,000 μ M	65	31	0	β_2 ^d
<i>Alternaria alternata</i> f. sp. <i>lycopersici</i> ^b	493 μ M (8)	>1,000 μ M	>1,000 μ M	73	35	08	TD
<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i> ^b	912 μ M (14)	>1,000 μ M	>1,000 μ M	53	35	11	TD
<i>Alternaria solani</i> ^b	>1,000 μ M	903 μ M (14)	>1,000 μ M	47	61	35	TD
<i>Alternaria alternata</i> ^b	>1,000 μ M	>1,000 μ M	>1,000 μ M	45	15	10	TD
<i>Septoria lycopersici</i> T550 ^b	>1,000 μ M	>1,000 μ M	>1,000 μ M	41	21	7	β_2
<i>Septoria lycopersici</i> T512 ^b	>1,000 μ M	>1,000 μ M	>1,000 μ M	10	0	0	β_2

^a No = none detected.

^b Pathogen on tomato.

^c TD = tomatidine.

^d β_2 = β_2 -tomatine.

the tomato pathogens *S. solani* isolate 11128 and *V. dahliae* were highly sensitive to α -tomatine, with little or no growth at concentrations higher than 200 μ M (e.g., *Aspergillus nidulans*) (Fig. 2A). All fungi with ED_{50} values $\leq 107 \mu$ M (Table 2) had this pattern of response to this compound. Most showed large increases in growth inhibition with relatively small increases in α -tomatine concentration before an asymptotic threshold was achieved at 95 to 100% growth inhibition. For most of the tomato pathogens tested there was a moderate inhibition of growth with increasing concentrations of α -tomatine, but a complete inhibition of growth was not obtained at the concentrations tested (e.g., *Colletotrichum coccodes*) (Fig. 2B and Table 2). Percent inhibition at 1 mM α -tomatine was used to compare the relative toxicity of α -tomatine (and its breakdown products) for fungi whose ED_{50} values were >1 mM (Table 2). A third type of response was observed only for *Septoria lycopersici* T512, which revealed little inhibition at all concentrations of α -tomatine tested (Fig. 2C).

The fungal isolates were also tested for sensitivity to β_2 -tomatine and tomatidine, the hydrolysis products of α -tomatine (Fig. 1). Although β_2 -tomatine was generally less toxic than α -tomatine (Table 2), the relative sensitivity to β_2 -tomatine compared with that to α -tomatine varied greatly. For example, *S. solani* and *A. solani* ED_{50} values were within 10% of those observed for α -tomatine, while *V. dahliae* and *Cryphonectria parasitica* were 5 and 15 times as tolerant of β_2 -tomatine than of α -tomatine, respectively. Almost all fungi were more tolerant of the aglycone tomatidine than of α -tomatine, but the degree of tolerance varied, especially among the nonpathogens of tomato as can be seen in

Fig. 2. Growth responses observed for selected representatives of saprophytic fungi, nonpathogens of tomato, and tomato pathogens when grown on medium supplemented with α -tomatine. **A**, *Aspergillus nidulans*; **B**, *Verticillium albo-atrum*; and **C**, *Septoria lycopersici* isolate T512. Lines represent the growth curve, as determined by NLIN program for nonlinear regression (SAS Institute Inc.), that best fit the observed data points.

TABLE 3. Sensitivity of *Neurospora crassa* sterol mutants to α -tomatine and its derivatives (ED_{50})

Isolate ^a	α -Tomatine	β_2 -Tomatine	Tomatidine
<i>Neurospora crassa</i> 74A	15 μ M (2) ^b	120 μ M (4)	51 μ M (3)
<i>Neurospora crassa</i> <i>erg</i> -1	31 μ M (3) ^c	125 μ M (4)	50 μ M (3)
<i>Neurospora crassa</i> <i>erg</i> -2	51 μ M (3) ^c	313 μ M (6) ^c	100 μ M (5) ^c
<i>Neurospora crassa</i> <i>erg</i> -3	79 μ M (4) ^c	329 μ M (7) ^c	52 μ M (4)
<i>Neurospora crassa</i> <i>erg</i> -4	75 μ M (3) ^c	172 μ M (6) ^c	97 μ M (4) ^c

^a All isolates were completely inhibited by 1 mM α -tomatine, β_2 -tomatine, and tomatidine except the *N. crassa* *erg*-3 mutant on β_2 -tomatine, which was inhibited 98%.

^b Numbers in parentheses represent standard deviation.

^c Value is statistically different from wild-type ED_{50} value using the Dunnett test at $\alpha = 0.05$.

both the ED_{50} values and their percent inhibition at 1 mM tomatidine (Table 2). Seventeen of the twenty-three fungal isolates tested were highly tolerant ($ED_{50} > 1$ mM) as compared with their sensitivity toward α -tomatine and, in all of these cases, tomatidine was less toxic than α -tomatine. However, *N. crassa*, *C. parasitica*, *Aspergillus nidulans*, and the tomato pathogens *S. solani* and *Pythium aphanidermatum* were either moderately or largely inhibited by the aglycone (Table 2). Tomatidine was more toxic than β_2 -tomatine to *Aspergillus nidulans*, *C. parasitica*, and *N. crassa*.

To test the effect of membrane sterol composition on the sensitivity to α -tomatine and its derivatives, the toxicity of these compounds was tested on four ergosterol (*erg*) mutants of *N. crassa* (Table 3). Mutations in the genes of the ergosterol biosynthetic pathway increased the α -tomatine and β_2 -tomatine tolerance of *N. crassa*, except for the isolate carrying the *erg*-1 mutation whose tolerance to β_2 -tomatine did not statistically differ from wild type (Table 3). The *N. crassa* mutants *erg*-2 and *erg*-4 were twice as tolerant of tomatidine as the wild type and were statistically different from wild type. Growth of all isolates was totally inhibited at 1 mM α -tomatine, β_2 -tomatine, and tomatidine except for *erg*-3, which had a slight amount of growth (2%) at this concentration of β_2 -tomatine.

Fungal degradation of α -tomatine. The degradation products of α -tomatine produced by the fungi tested are listed in Table 2. No degradation products were detected from *Aspergillus nidulans*, *C. parasitica*, *F. moniliforme*, *M. grisea*, *Macrophomina phaeo-*

Fig. 3. Thin-layer chromatography (TLC) plate showing degradation products of α -tomatine produced by *Alternaria solani*. Approximately 10 μ g of the alkaloid standards and extracts was spotted onto each lane of the TLC plate. Top of figure represents the solvent front. Lane 1, α -tomatine standard ($R_f = 0.23$); lane 2, β_2 -tomatine standard ($R_f = 0.4$); lane 3, tomatidine standard ($R_f = 0.75$); lane 4, reaction products after incubating α -tomatine for 2 h with protein from culture filtrate of *A. solani* grown in the absence of α -tomatine; lane 5, reaction products after incubating α -tomatine with mycelial extracts for 2 h from mycelium that was not grown in the presence of α -tomatine; and lanes 6 through 9, reaction products after incubating α -tomatine with mycelial extracts for 2 h from mycelium that was grown in the presence of α -tomatine for 2 h (lane 6), 4 h (lane 7), 8 h (lane 8), and 24 h (lane 9).

lina, *Nectria haematococca*, *N. crassa*, *Pythium aphanidermatum*, and *P. infestans* when these fungi had completely grown through and across the agar surface on medium supplemented with 20 μ M α -tomatine. *Septoria lycopersici* and *V. albo-atrum* degraded α -tomatine to β_2 -tomatine. All other isolates were able to degrade α -tomatine to tomatidine.

Degradation of α -tomatine by *A. solani*. An ammonium sulfate precipitation of the culture filtrate of *A. solani* grown in the absence of α -tomatine, when incubated for 2 h with α -tomatine (Fig. 3, lane 4) and β_2 -tomatine (data not shown), caused the release of tomatidine, indicating that enzymes required for the hydrolysis of α -tomatine to tomatidine are constitutive and secreted from *A. solani*. To determine if tomatinase activity increased by pretreatment with α -tomatine, a final concentration of 500 μ M α -tomatine was added to a 3-day liquid culture, and after 0, 2, 4, 8, and 24 h, crude protein extracts of the mycelium were assayed for α -tomatine-degrading activity by incubating the extracts for a reaction period of 2 h with α -tomatine. With protein extracted from mycelium not previously exposed to α -tomatine, β_2 -tomatine was the only degradation product detected after the 2-h incubation period (Fig. 3, lane 5), unlike extracts from culture filtrates that were observed to degrade α -tomatine to tomatidine. Crude protein extracts from *A. solani* mycelium collected after 2 to 8 h after exposure to α -tomatine converted α -tomatine to an unknown derivative (Fig. 3, lanes 6 to 8). Tomatidine was the only alkaloid detected by TLC (Fig. 3, lane 9) with the protein extract obtained from mycelium 24 h after exposure to α -tomatine.

Identification of δ -tomatine. The 500-MHz proton NMR spectrum of the unknown compound (Fig. 3, lanes 6 to 8) in pyridine when compared with the spectrum of α -tomatine (42) strongly suggested that this compound was δ -tomatine (Fig. 1). The aglycone peaks included methyls at delta 0.65 (*s*, H-19), 0.80 (*d*, J = 6 Hz, H-27), 0.85 (*s*, H-18), and 1.07 (*d*, J = 7 Hz, H-21); protons

alpha to N at 2.82 (*m*, H-26beta) and 2.93 (*t*, J = 11 Hz, H-26alpha); and protons alpha to O at 3.82 (*m*, H-3) and 4.17 (*m*, H-16). There was only one anomeric proton at delta 4.95 (*d*, J = 7 Hz, 1'), with other sugar protons at 3.97 (*m*, 5'), 4.15 (*m*, 3'), 4.25 (*m*, 6'), 4.40 (*m*, 2'), 4.50 (*m*, 6'), and 4.61 (*br s*, 4'). There was a single major parent ion peak at 577 when MS was performed on this compound that corresponded to the predicted molecular weight of δ -tomatine.

DISCUSSION

Some of the tomato pathogens used in the current study have been shown previously to be tolerant of α -tomatine (2). Although our assay conditions were different, we also observed that most tomato pathogens were tolerant of this compound, while saprophytes and pathogens of hosts other than tomato were sensitive to α -tomatine (Table 2 and Fig. 4). Although some tomato pathogens were found to be fairly sensitive to α -tomatine (*S. solani* and *V. dahliae*), their modes of pathogenicity may offer an explanation for these observations. *S. solani* is a leaf pathogen, and leaves may contain α -tomatine at levels of up to 1 mM. However, like another leaf pathogen, *Cladosporium fulvum*, that is also quite sensitive to α -tomatine (12), *S. solani* grows within the intercellular space and middle lamella of the leaf (11). Here, the advancing hyphae may not come into contact with α -tomatine, as this compound is stored in cell vacuoles (29). A similar situation may hold true for *V. albo-atrum* and *V. dahliae* because, as wilt pathogens, they are limited mainly to the xylem tissue. These modes of pathogenicity differ from *Septoria lycopersici*, which directly penetrates the leaf (35), and *A. solani*, which can directly penetrate the leaf and produce a toxin that may result in the release of α -tomatine (30).

All of the tomato pathogens tested were able to degrade α -tomatine except for *Pythium aphanidermatum* and *P. infestans* (Table 2).

Fig. 4. Fungal sensitivity to α -tomatine and their ability to degrade this compound. The ED₅₀ is shown for each isolate. Horizontal bars at each ED₅₀ value represent standard deviation. A solid bar indicates that the isolate is a tomato pathogen, while a transparent bar indicates that the isolate is a saprophyte or non-pathogen of tomato. An asterisk indicates that the isolate has the ability to degrade α -tomatine.

This is the first report that *A. alternata*, *A. alternata* f. sp. *lycopersici*, *Colletotrichum coccodes*, *S. solani*, and *V. dahliae* are able to degrade this compound. Most of the tomato pathogens degraded α -tomatine to tomatidine (Table 2). However, two tomato pathogens, *Septoria lycopersici* and *V. albo-atrum*, form β_2 -tomatine as the final product as previously reported (1,26). One isolate that was considered to be a nonpathogen of tomato, *B. cinerea* from grape, was able to degrade α -tomatine to tomatidine. This isolate, however, has not been tested for its ability to parasitize tomato.

Based on the available data, there does not seem to be a common enzymatic mechanism by which tomato pathogens degrade α -tomatine, since different species remove a single sugar moiety (*Septoria lycopersici* [1], *V. albo-atrum* [26]), two or more sugars sequentially (*A. solani* [33, this study], *Colletotrichum coccodes* [R. W. Sandrock, unpublished data]), or the tetrasaccharide moiety as a single unit from α -tomatine (*B. cinerea* [40], *F. oxysporum* f. sp. *lycopersici* [16]). Degradation of α -tomatine by *A. solani* was previously reported to occur via sequential hydrolysis of the four sugar residues in a crude enzyme preparation from tomato fruit pulp tissues (33). Since ripening tomato fruit also has tomatinase activity (28) and no standards were available for the verification of the breakdown products, we have retested the previous results. For the current study, mycelium of *A. solani* was grown in liquid culture both in the presence and absence of α -tomatine, and it was shown that the culture filtrates of *A. solani* contain tomatinase activity and that the tomatinase activity within the mycelium increased after exposure to α -tomatine (Fig. 3). α -Tomatine was degraded to β_2 -tomatine, the previously unidentified intermediate δ -tomatine, and tomatidine. These results do not indicate the number of enzymes involved in the degradation of α -tomatine. Since some saponinases are able to hydrolyze more than one glycosidic linkage (43), determination of the number of enzymes involved in the degradation of α -tomatine to tomatidine by *A. solani* requires further biochemical characterization.

Previous researchers have suggested that the hydrolysis products of α -tomatine are less toxic because of their inability to complex with membrane-bound 3β -hydroxy sterols (3,23,36). In this study, β_2 -tomatine and the aglycone tomatidine were generally less toxic, but these compounds were inhibitory to some fungi (Table 2). In addition, α -tomatine was inhibitory to *P. infestans* and *Pythium aphanidermatum*, which lack these sterols. Assuming that there are few sterols in the medium that these two fungi can utilize, this suggests that α -tomatine and its derivatives may possess additional properties other than binding to 3β -hydroxy sterols that render them toxic. If this is true, it is possible that tomato pathogens have acquired additional means to tolerate this other mode of toxicity. Of particular interest are *Septoria lycopersici* and *V. albo-atrum*, which are not able to degrade β_2 -tomatine, yet are very tolerant of this compound compared with most of the saprophytes and nonpathogens of tomato that are unable to degrade β_2 -tomatine. Tomato pathogens may have evolved an additional means to tolerate a tomatine-like structure that is not based on enzymatic alteration of the saponin. Such "nondegradative" tolerance towards plant phytoalexins and polyene antibiotics that interact with sterols or membrane lipids has been identified as a possible means to overcome the toxicity of these compounds (8-10,20,21).

Nevertheless, both the structural and quantitative traits of the sterols present in a membrane can affect the saponin-sterol interaction in vitro (22,23,36). The differences in the tolerance of the ergosterol mutants of *N. crassa* to α -tomatine and its metabolites indicate that changes in the type of sterol can effectively increase tolerance (Table 3). Ergosterol, a 3β -hydroxy sterol, is the major sterol component of *N. crassa* and constitutes 80% of the total sterol content (14). *erg* mutants of *N. crassa* lack ergosterol due to mutations in genes involved in the ergosterol biosynthetic pathway and accumulate the 3β -hydroxy sterols fecosterol (*erg*-1),

24(28) dehydroergosterol (*erg*-2), $\Delta^{8,14}$ sterols (*erg*-3), and zymosterol (*erg*-4) (14,18). Although the interaction of these sterol intermediates with α -tomatine in vitro is unknown, results from these in vivo tests suggest that the membranes are altered so that they are less sensitive to these alkaloids, suggesting different degrees of interaction with the different sterols. In addition, however, other research has shown that the presence of glycolipids in cholesterol-containing membranes results in an increase in the membrane-disrupting properties of α -tomatine, not because of the structure and composition of the glycolipid, but because of the concentration of the glycolipids (23). Based on these data, it is possible that alterations in the sterol and lipid compositions of the tomato pathogens may protect them from the residual toxic effects of the degradation products of α -tomatine.

With the discovery that the avenacinase gene is essential for the pathogenicity of *G. graminis* var. *avenae* (5), it will be interesting to assess the importance of α -tomatine degradation by pathogens of tomato. This assessment should more easily be addressed using *F. oxysporum* f. sp. *lycopersici* and *Septoria lycopersici*, since it appears that a single enzyme in each of these species is involved in the degradation of α -tomatine (24,31). Moreover, the gene encoding the β_2 -tomatinase gene from *Septoria lycopersici* has been cloned, and this should facilitate the formation of " β_2 -tomatinase-deficient" mutants via gene disruption (24,25,31). Formation of tomatinase-deficient mutants in these fungi will also provide information as to whether a nondegradative tolerance mechanism exists and, if so, whether this tolerance is to α -tomatine, the degradation products of α -tomatine produced by these fungi, or both.

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