Induction of δ-Cadinene Synthase and Sesquiterpenoid Phytoalexins in Cotton by *Verticillium dahliae*

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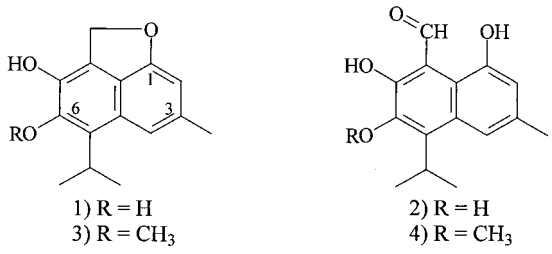
Phytoalexin biosynthesis occurred earlier in the resistant cotton cultivar Seabrook Sea Island 12B2 (SBSI) (*Gossypium barbadense*) than in the susceptible cotton cultivar Rowden (*G. hirsutum*) after inoculation with a defoliating isolate of the pathogen *Verticillium dahliae*. This was demonstrated by significantly higher levels of phytoalexins in SBSI 12 h after inoculation. Furthermore, by 48 h after inoculation of SBSI, the phytoalexins hemigossypol and desoxyhemigossypol achieved levels (23.9 and 10.5 μg/g of fresh tissue, respectively) sufficient to completely inhibit conidial germination. Rowden required 96 h to attain comparable levels. Similarly, the activity of δ-cadinene synthase, a key enzyme required for the biosynthesis of the terpenoid phytoalexins, increased more rapidly in the resistant cotton cultivar than in the susceptible one. The changes in phytoalexin concentrations and enzyme activity are consistent with the hypothesis that phytoalexins are an essential component in protecting the plant from infection by *V. dahliae*.

Keywords: *Gossypium hirsutum; Gossypium barbadense; Malvaceae; Verticillium dahliae; phytoalexins; desoxyhemigossypol; δ-cadinene synthase; plant−pathogen interaction; sesquiterpenes*

INTRODUCTION

Infection by the wilt pathogen *Verticillium dahliae* unleashes a cascade of events in both susceptible and resistant cotton plants. These include the production of the sesquiterpenoid phytoalexins desoxyhemigossypol (dHG, **1**, Scheme 1) and hemigossypol (HG, **2**) (Bell et al., 1975) in the susceptible cultivar Rowden (*Gossypium hirsutum*). The *V. dahliae* resistant cultivar Seabrook Sea Island 12B2 (SBSI) (*Gossypium barbadense*) produces these same phytoalexins, together with significant quantities of their methyl ether derivatives desoxyhemigossypol-6-methyl ether (dMHG, **3**) and hemigossypol-6-methyl ether (MHG, **4**) (Stipanovic et al., 1975). The resistance of SBSI, however, is not derived from its ability to synthesize these methyl ether derivatives because they are less toxic to *V. dahliae* than the parent unmethylated ethers (Mace et al., 1985). In fact, we found that cotton lines bred for increased levels of methylated phytoalexins were more susceptible to *V. dahliae* than their progenitors (Bell et al., 1994). dHG was found to be the most toxic of the phytoalexins, killing all mycelia at a concentration of 15 μg/mL, whereas HG, dMHG, and MHG required 35, 25, and 45 μg/mL, respectively. Further implicating its importance, dHG has a solubility in pH 6.3 water (the pH of infected cotton stem xylem) of 50.2 μg/mL compared to 4.2, 2.9, and 2.0 μg/mL for HG, dMHG, and MHG, respectively (Mace et al., 1985). The solubility of dHG would facilitate its translocation from the site of formation in scattered paratracheal parenchyma cells appressed to infected xylem vessels (Mace et al., 1976) to the fungal propagules in the xylem vessels. All of the phytoalexins are derived from (+)-δ-cadinene (Davis and Essenberg, 1995), and δ-cadinene originates from farnesyl diphosphate (FPP). The enzyme responsible for cyclization of FPP to produce δ-cadinene has been identified as δ-cadinene synthase (δ-CS) (Benedict et al., 1995; Chen et al., 1995, 1996; Davis et al., 1996).

Scheme 1



1) R = H
3) R = CH_3

2) R = H
4) R = CH_3

Early investigations suggested the resistance of SBSI hinges on its ability to respond more quickly to infection. Bell (1969) found that 2−4 days after inoculation, SBSI contained higher concentrations of phytoalexins than Rowden. Histochemical studies were used to detect terpenoid phytoalexins in stele tissue 18 h after inoculation in SBSI, but in Rowden they were not detected until 24 h after inoculation (Mace, 1978).

To substantiate and quantitate earlier investigations, Rowden and SBSI plants were inoculated with defoliating strain V-76 of *V. dahliae*. The concentrations of phytoalexins in inoculated plants were compared to those in a control group that had been inoculated with sterile water. Tissues from these plants were divided into two groups. One group was analyzed for phytoalexin concentrations, and the second was used to assay the level of δ-CS.

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Table 1. Mean Concentrations (Micrograms per Gram of Fresh Stele) of Phytoalexins in the First Internode of Rowden (*G. hirsutum*) and SBSI (*G. barbadense*) Cotton Plants at Various Time Intervals after Inoculation with *V. dahliae* (Isolate V-76)

	dHG		HG		dMHG		MHG		total phytoalexins	
time (h)	Rowden	SBSI	Rowden	SBSI	Rowden	SBSI	Rowden	SBSI	Rowden	SBSI
12	0.00[a]	0.42*[b]	0.00	0.00	0.00	0.40*	0.00	0.00	0.00[b]	0.82*
18	0.04	0.73*	0.00	0.25*	0.00	0.52*	0.00	0.01	0.04	1.51*
24	0.56	1.85*	0.10	1.54*	0.03	2.15*	0.00	0.98	0.69	6.52*
36	1.33	6.10*	2.30	13.35*	0.16	7.39*	0.00	6.17*	3.79	33.01*
48	3.27	10.46*	5.94	23.92*	0.31	10.86*	0.02	8.04*	9.53	53.28*
72	4.50	15.53*	19.92	44.95	1.30	20.15*	0.13	10.09*	25.85	90.72*
96	11.06	28.82*	29. 42	77.92*	3.87	65.66*	0.56	27.39*	44.91	199.79*
120	21.08	25.22	64.25	75.39	3.67	54.28*	0.68	23.85*	89.68	178.74*
168	23.29	55.48*	100.97	183.85*	6.46	126.04*	2.48	74.57*	133.21	439.94*
216	18.72	74.33*	114.45	210.77*	16.09	151.24*	8.05	96.77*	157.31	533.11*

[a] Trace quantities found (>0.002 μg/g). [b] The asterisk (*) indicates the entry is significantly greater than the corresponding concentration for Rowden at the 95% confidence level at the time interval indicated (Student's *t* test: two sample, two tailed); outliers were determined according to the method of Rorabacher (1991).

MATERIALS AND METHODS

Chemicals. [1-^{3}H(N)]Farnesyl pyrophosphate (FPP) triammonium salt was purchased from New England Nuclear (Boston, MA). Silica gel TLC plates, Si250-19C, were obtained from Baker Chemical (Phillipsburg, NJ). Coomassie Plus protein assay was purchased from Pierce (Rockford, IL). All other chemicals were from Sigma (St. Louis, MO). Desoxyhemigossypol was prepared as previously described (Stipanovic et al., 1992).

Plant Material. The cotton cultivars Rowden (*G. hirsutum*) and Seabrook Sea Island 12B2 (*G. barbadense*) were pregerminated in paper rolls, transferred to 16-oz plastic cups, grown in the greenhouse, and transplanted to 1-gal pots. After expansion of six true leaves, the plants were moved to growth chambers with a 14-h light cycle at a constant temperature of 27 °C. After achieving 8−10 true leaves, plants were inoculated with *V. dahliae* (defoliating isolate V-76) by injecting 25 μL of either a conidial suspension [(2−5) × 10^7 conidia/mL] or sterile water (controls) with a 22-gauge needle at each of three points located 1 cm below the cotyledonary node and equidistant around the stem. The first internode of each of eight plants of each cultivar was harvested at 0, 12, 18, 24, 35, 48, 72, 96, 120, 168, and 216 h postinoculation (p.i.). The bark was removed from the stem, and the remaining stele was then cross-sectioned into 2−3 mm thick slices. Alternate slices from each plant were combined into two samples. One sample was utilized for analysis of the phytoalexins; the second sample was frozen in liquid nitrogen, combined with slices from the other seven plants from the same species harvested at the same time, and used for enzyme analysis.

Phytoalexin Analysis. The stem slices from each plant were placed in a weighed, capped test tube, the tube was reweighed, and the tissue weight was calculated. An extracting solvent consisting of acetone and 1% aqueous ascorbic acid (9:1, v/v) was added to each tube at the rate of 3 μL of solvent/mg of tissue. The samples were held in a cold room (~2 °C) for ~24 h; tubes were manually shaken twice during this time. Next, the samples were centrifuged and the solution was transferred via pipet to HPLC vials. These vials were stored at −70 °C until analysis.

HPLC analysis of the phytoalexins in the sample extracts was carried out with a computer-controlled Hewlett-Packard 1090 liquid chromatograph equipped with an autosampler, a diode array detector, and a Keystone Scientific, Inc., MOS-Hypersil-1 (5 μm) glass-lined column (25 cm × 4 mm). The column was held at 40 °C, and the eluting solvent was a mixture of 0.07% aqueous H_3PO_4 and 0.07% H_3PO_4 in MeOH maintained at a flow rate of 1.25 mL/min. A solvent gradient was utilized such that the H_2O/MeOH ratio was varied in a linear fashion between the set points of the following timetable: 80 H_2O:20 MeOH at 0 min, 70:30 at 7 min, 20:80 at 12 min, 10:90 at 19 min, 0:100 at 19.2 min, and 0:100 at 23. The eluant was monitored at 235 nm (bandwidth = 20 nm). The injection volume was 50 μL for most samples and 25 μL for samples containing the highest concentrations of the phytoalexins. Peak identities were confirmed by retention time and by comparison of the sample UV spectra to those of authentic compounds. The phytoalexin concentrations in the sample extracts were determined by comparison to a standard curve of each compound. Concentrations in the plant tissue were calculated by assuming complete equilibration between the tissue and extraction solvent.

Isolation of δ-Cadinene Synthase (δ-CS). Approximately 1 g of tissue was ground in liquid nitrogen and homogenized with 8 mL of extraction buffer [50 mM MOPS pH 7.5, 0.35 M sucrose, 10 mM 2-mercaptoethanol, 5% insoluble PVP, and the protease inhibitors (*trans*-epoxysuccinyl-L-leucylamino-(4-guanidino)butane) (E-64), 5 μg/mL; leupeptin, 5 μg/mL, and phenymethanesulfonyl fluoride (PMSF), 170 μg/mL]. The slurry was homogenized for 5 s with a polytron and filtered through cheesecloth. The filtrate was centrifuged first at 3000*g* for 5 min, at 10000*g* for 30 min, and then at 105000*g* for 1.5 h. The 105000*g* pellet was rinsed three times and resuspended in freshly prepared buffer containing 50 mM potassium phosphate, pH 7.5, 30 mM DTT and the protease inhibitors. Glycerol was added to the 105000*g* supernatant to 15% (v/v) concentration. The activity of δ-CS was measured in the soluble fraction. Protein content was estimated by using the Pierce Coomassie Plus protein assay according to the manufacturer's instructions.

δ-Cadinene Synthase Activity. The reaction mixture contained 200 μL of the supernatant fraction described above; 20 μL of 0.1 M reduced glutathione (GSH), pH 7.5; 40 μL of 0.2 M KF; 20 μL of 0.1 M $MgCl_2$; and 0.55 nmol of [1-^{3}H]FPP containing 5 × 10^6 dpm (2.5 μCi). The reaction mixture was incubated for 20 min at 30 °C. The reaction was stopped with the addition of 2 mL of hexane/ethyl acetate (3:1, v/v). The aqueous layer was extracted two times with 2 mL of the solvent mixture. The amount of ^{3}H was determined in the organic phase by liquid scintillation counting. Eighty-five percent of the label corresponds to δ-cadinene (Benedict et al., 1995).

RESULTS

Rowden and SBSI differ greatly in their response to the wilt pathogen *V. dahliae*. Under the experimental conditions described, Rowden is severely defoliated, whereas SBSI shows little or no symptoms (Bell and Presley, 1969). Here we examined the relative rates at which the two cultivars are able to synthesize fungitoxic terpenoids and δ-CS following inoculation.

Phytoalexins. Phytoalexins were undetectable in healthy control plants of both Rowden and SBSI. Following inoculation, dHG, the most toxic phytoalexin, was the first detected. At 12 h p.i., SBSI had accumulated 0.42 μg of dHG/g of fresh tissue, whereas

Rowden required 24 h to exceed this level (Table 1). In the critical early stages of infection, levels of dHG in SBSI were significantly higher than in Rowden, and the levels of dHG remained higher in SBSI throughout the study (216 h). An earlier plate bioassay study showed that dHG and HG killed >50% of V-76 mycelia (LD_{50}) at 15 and 30 μg/mL, respectively (Mace et al., 1990). By 72 h p.i., the concentration of dHG and HG had exceeded these levels in SBSI. In Rowden, it required 96−120 h to achieve these concentrations.

Enzyme Activity. Activity for δ-CS was negligible in the control plants for both cultivars throughout the test period. In the early stages of infection, enzyme activity increased more quickly in inoculated SBSI plants than in Rowden. δ-CS activity in SBSI was maximal at 48 h [55 nmol of δ-cadinene h^{-1} (mg of protein)$^{-1}$]. It took over twice as long (120 h) for Rowden to surpass this level of activity, and in Rowden the activity continued to increase up to 168 h.

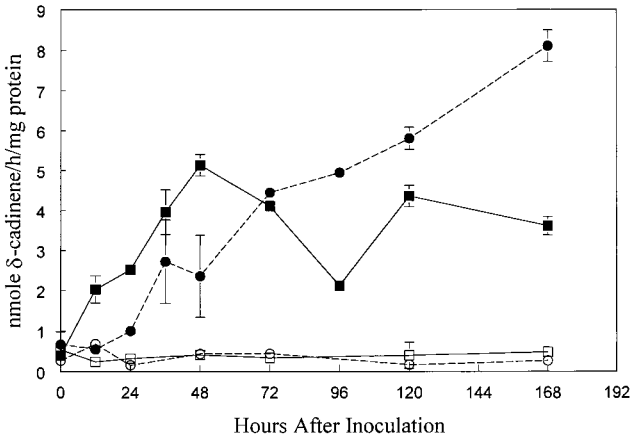

Figure 1. Changes in δ-cadinene synthase activity in cotton cultivars inoculated with *V. dahliae*: Seabrook Sea Island, resistant (■, inoculated; and □, control); Rowden, susceptible (●, inoculated; and ○, control).

DISCUSSION

Induction of Phytoalexins. In an earlier in vitro study, Mace et al. (1985) found that *V. dahliae* conidia germination was totally inhibited by dHG at 10 μg/mL (95% inhibited at 4 μg/mL), by HG at 30 μg/mL (95% inhibited at 10 μg/mL), and by dMHG at 15 μg/mL (95% inhibited at 10 μg/mL). Table 1 shows that at 36 h after inoculation, concentrations of both dHG and HG in SBSI stele tissue exceed that required to inhibit 95% conidia germination, and by 48 h the level of dMHG surpasses this threshold. Rowden required 72 h to attain comparable levels. Higher levels of phytoalexins in SBSI than in Rowden shortly after inoculation support the early study by Bell (1969) that used a crude measure of terpenoids. The early induction of phytoalexin production explains in part how SBSI halts the spread of the pathogen, whereas in Rowden the concentration of phytoalexins increases too slowly and fails to prevent the spread of the pathogen via the xylem stream (Bell, 1969; Bell and Presley, 1969).

Phytoalexin concentrations were determined on fresh tissue taken from the entire first internode, but only part of the xylem vessels in this tissue was infected. Earlier histochemical studies by Mace et al. (1976, 1989) have shown that the phytoalexins are not evenly distributed in stem tissue but rather occur in specialized, often solitary, paravascular parenchyma cells appressed to infected xylem vessels; uninfected vessels are essentially free of phytoalexins. Our method of inoculation infects less than one-third of the xylem vessels. Thus, the concentration of the phytoalexins at the site of infection can be expected to be up to 3 times those indicated in Table 1. By this measure, SBSI has achieved ED_{100} levels (i.e., levels that completely inhibit conidia germination) of dHG, HG, and dMHG in 36 h, whereas Rowden requires twice as long to achieve this concentration of dHG and HG.

Induction of δ-Cadinene Synthase. δ-Cadinene is a direct precursor to the cotton phytoalexins, and the δ-CS activties in SBSI and Rowden differ significantly (Figure 1). δ-CS activity increases rapidly in SBSI during what may be the critical 12 h following inoculation and remains higher up to 48 h. The initial buildup in activity probably is required to provide the substrate (i.e., δ-cadinene) for further modification by other enzymes to produce dHG. After 48 h, δ-CS activity in SBSI decreases but the concentration of total terpenoids continues to increase (Table 1). In contrast, δ-CS in Rowden continued to increase from 48 h up to 168 h and remained high until the end of the test period (i.e., 216 h). At this time the plant was almost completely defoliated. This continued increase in δ-CS activity in Rowden is probably due to the spread of infection from the initial sites of inoculation to surrounding tissue. Thus, in Rowden more tissue is infected than in SBSI, and a continual increase in δ-CS activity at new infection sites is expected. The δ-CS activity from 72 to 216 h p.i. to the end of the test period was highest in Rowden, and more xylem vessels were infected. However, the concentration of total phytoalexins in Rowden was less than that in SBSI throughout this period. This may indicate that the level of other enzymes in the pathway or cell viability is limiting the production of phytoalexins in Rowden.

The changes in phytoalexin concentrations and in enzyme activity found in this study provide additional support to our hypothesis that the phytoalexins are an essential component in the cotton plant's response to infection by pathogens such as *V. dahliae*.

ABBREVIATIONS USED

δ-CS, δ-cadinene synthase; SBSI, cotton cultivar, Seabrook Sea Island 12B2, *Gossypium barbadense*; dHG, desoxyhemigossypol; HG, hemigossypol; dMHG, desoxyhemigossypol-6-methyl ether; MHG, hemigossypol-6-methyl ether; V-76, *Verticillium dahliae* pathogen that defoliates cotton; HPLC, high-performance liquid chromatography; FPP, farnesyl diphosphate.

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