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THE ACCUMULATION OF FIVE FLUORESCENT COMPOUNDS IN THE COTTON LEAF INDUCED BY CELL-FREE EXTRACTS OF *ASPERGILLUS FLAVUS*

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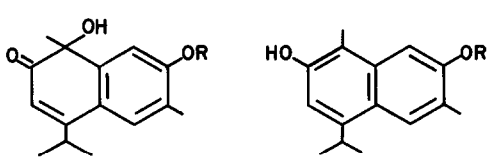
Key Word Index—*Gossypium hirsutum*, Malvaceae, cotton, elicitors, phytoalexins, lacinilene C, lacinilene C 7-methyl ether, 2,7-dihydroxycadalene, 2-hydroxy-7-methoxycadalene, scopoletin, *Aspergillus flavus*

Abstract—Five autofluorescent compounds, lacinilene C, lacinilene C 7-methyl ether, scopoletin, 2-hydroxy-7-methoxycadalene and 2,7-dihydroxycadalene, were induced in cotton leaves in response to treatment with cell-free mycelial extracts or culture fluid extracts of *Aspergillus flavus* It is estimated by thin-layer chromatography fluorodensitometric quantitation, that a 10-fold increase in scopoletin and a 25–30-fold increase in lacinilene C 7-methyl ether resulted when leaves were treated with a cell-free mycelial extract of *A. flavus*

INTRODUCTION

Recently, Essenberg *et al* [1] demonstrated that 2,7-dihydroxycadalene (**2**) and its oxidation product, lacinilene C (**1**), accumulate in leaves and cotyledons of blight-resistant lines of cotton (*Gossypium hirsutum* L) when the tissues were infiltrated with a bacterial suspension of *Xanthomonas campestris* pv *malvacearum* In addition she demonstrated that bacterostasis was produced by these two compounds and that these compounds function as phytoalexins in the cotton plant Stipanovic *et al* [2] reported that the precursors of lacinilene C and lacinilene C 7-methyl ether (**3**) were the sesquiterpenoid naphthols, 2,7-dihydroxycadalene and 2-hydroxy-7-methoxycadalene (**4**) respectively Halloin and Greenblatt [3] found accumulation of the lacinilenes and the cadalenes in cotton boll tissues after he had punctured-inoculated the bolls with *Diplodia gossypina*, the bolls exhibited a bright, yellowish fluorescence under long wave UV light and also resistance of infection by *Diplodia gossypina*

The purpose of the current investigation was to identify the several fluorescence compounds that were elicited in cotton leaves when the leaves were wounded and challenged with cell-free mycelial extracts and culture fluid extracts of *Aspergillus flavus*



(1) R = H
(3) R = Me

(2) R = H
(4) R = Me

Lacinilene C (1), 2,7-Dihydroxycadalene (2), Lacinilene C 7-methyl ether (3), and 2-Hydroxy-7-methoxycadalene (4)

RESULTS AND DISCUSSION

Investigations of the infection of cottonseed by *A flavus* demonstrated the hemagglutination activity (HA) of hot-water soluble mycelial extracts and of culture fluid extracts of 7- to 30-day cultures of *A flavus* [4] An optimum specific HA titer by a mycelial extract was observed in 10-day cultures and in 12-day culture fluid extracts The 10-day mycelial extracts contained 44 6% protein and 37 5% carbohydrate, the 12-day culture fluid extract contained 67 7% protein and 30 3% carbohydrate

In the present investigation, both the mycelial and culture fluid extracts were used as elicitors, they were independently applied in 10 μg amounts on wounded SJ-2 cotton leaves Forty-eight hours after the elicitors were applied, the leaves were harvested and the wounded areas were removed as leaf discs The discs were vacuum-infiltrated with 50% aqueous ethanol, extracted, and applied to TLC plates as described in the Experimental The plates developed in one of the three solvent systems, were illuminated by UV light (365 nm) This resulted in the appearance of five prominent fluorescent spots on a plate containing leaf extracts that had been treated by either elicitor A TLC plate containing these treated leaf extracts, developed in solvent system II, presented 3 yellow fluorescent spots (R_fs 0 22, 0 46, 0 55), one yellow–green fluorescent spot (R_f 0 65), and a blue fluorescent spot (R_f 0 35) The control wounded leaf contained only small amounts of the blue fluoresent spot (R_f 0 35) and small amounts of the yellow fluorescence spot (R_f 0 55) TLC plates spotted with only the elicitors and developed in each of the three developing solvent systems did not show any migrating fluorescent spots

Authentic standards of lacinilene C, lacinilene C 7-methyl ether and scopoletin were co-chromatographed with leaf extracts treated with a cell-free mycelial extract and extracts of a control wounded leaf The R_f s and the color bands of the treated leaf extracts matched the R_fs and the color bands of the three standards (Table 1)

Table 1 TLC of plant extracts and standards in three solvent systems

Band color (365 nm)	R_f values					
	System I*		System II†		System III‡	
Y	0 26	(0 26)§	0 22	(0 22)§	0 28	(0 28)§
B	0 38	(0 39)‖	0 35	(0 34)‖	0 10	(0 11)‖
Y	0 48		0 46		0 42	
Y	0 58	(0 58)¶	0 55	(0 56)¶	0 45	(0 46)¶
Y–G	0 64		0 65		0 55	

*$CHCl_3$–Me_2CO–HCOOH (80 19 1)
†MeCN–$CHCl_3$ (4 1)
‡Hexane–EtOAc–MeOH (60 40 1)
§Lacinilene C standard
‖Scopoletin standard
¶Lacinilene C 7-methyl ether standard

Two dimensional TLC, using each of the three solvent systems and repeating the initial solvent in the second development, resulted in the fastest moving component (yellow–green band) being converted to lacinilene C 7-methyl ether and the second moving yellow band component being converted to lacinilene C This is in agreement with Stipanovic *et al* [2] who found that the two sesquiterpenoid naphthols, 2-hydroxy-7-methoxycadalene (**4**) and 2,7-dihydroxycadalene (**2**) were the precursors of lacinilene C-7 methyl ether (**3**) and lacinilene C (**1**) and that these naphthols rapidly autoxidize on silica gel to the lacinilenes We have also demonstrated the autoxidation of these naphthols on developed plates after the plates were streaked with these leaf extracts and were heated at 110° for 30 min

Under UV illumination (365 nm), preparative TLC plates streaked with treated plant extracts were scraped from the plates, eluted with methanol, and the dry eluent was analyzed by mass spectrometry The mass spectral data analysis of the major fragment ions of the cadalenes, the lacinilenes, and scopoletin agreed with published data [1, 2, 5]

Although autoxidation of the cadalenes to the lacinilenes occurs, we estimated the quantities of the five accumulative fluorescent compounds from treated leaf extracts by scanning densitometric procedures The results of this quantitation can be seen in Table 2 It is estimated that there is a *ca* 10-fold increase in scopoletin and a 25-30-fold increase in lacinilene C 7-methyl ether when the leaves were treated with the mycelial extract elicitor

Table 2 Quantitation of fluorescent components in plant extracts estimated by TLC fluorodensitometry

Component	μg/fresh tested leaf wt*	μg/g fresh control leaf wt†
Lacinilene C 7-methyl ether	19 6	0 7
2-Hydroxy-7-methoxycadalene	31 0	0
Scopoletin	13 8	1 4
2,7-Dihydroxycadalene	15 8	0
Lacinilene C	40 8	0

*Leaves treated with a cell-free mycelial extract of *A flavus*
†Wounded leaves treated with distilled H_2O only

In summary we have demonstrated that scopoletin, lacinilene C, lacinilene C 7-methyl ether, 2-hydroxy-7-methoxycadalene, and 2,7-dihydroxycadalene are simultaneously induced in the cotton leaf when the leaf is treated with a cell-free mycelial extract or culture fluid extract of *A flavus* It is interesting that these extracts which were used as elicitors contain hemagglutinins [4], but there is no evidence that these hemagglutinins are responsible for the elicitation demonstrated

EXPERIMENTAL

Fungi and culture conditions A flavus (SRRC 1000) was maintained on potato dextrose agar plates, and fungal spores were inoculated into the defined medium of Adye and Mateles [6] contained in (2 8 l) Fernbach culture flasks The inoculated media was kept at 29°, without shaking for 10–14 days

The mycelia were washed with distilled H_2O, homogenized with 0 1 M phosphate buffer, filtered, defatted with $CHCl_3$–MeOH (1 1), washed with Me_2CO, and air dried [4] Mycelia were resuspended in H_2O, autoclaved for 3 hr at 121°, filtered on filter paper, and the filtrate vol was concd by vacuum distillation, dialysed against H_2O at 2° and lyopholized

The culture fluid extract was obtained by filtering the culture fluids on paper, concentrating the vols to 150 ml by vacuum distillation below 40°, dialysing against distilled H_2O at 2°, then lyopholizing [4]

Thin layer chromatography (TLC) Pre-coated TLC plates of silica gel 60 (without fluorescent indicator, 0 25 mm thickness, EM Laboratories) were used after activation by heat at 110° for 10 min Preparative TLC plates were prepared with silica gel (EM Reagents) to concentrate the fluorescent compounds for mass spectral analysis A Finnigan Model 4000 was used with a direct probe to obtain MS data Solvent systems were System I, $CHCl_3$–Me_2CO–HCOOH (80 19 1), System II, MeCN–$CHCl_3$ (4 1), and System III, hexane–EtOAc–MeOH (60 40 1) After the plates were developed, fluorescent spots were located under UV illumination (365 nm)

TLC quantitation analysis Plates were spotted with treated leaf extracts and standards densitometrically scanned at 365 nm with a Schoeffel Spectrodensitometer (Model SD 3000) to obtain a semi-quantitative analysis of the fluorescent spots Authentic standards of lacinilene C, lacinilene C 7-methyl ether, and scopoletin were utilized and 2,7-dihydroxycadalene was quantified against the lacinilene C standard and 2-hydroxy-7-methoxycadalene was quantified against the lacinilene C 7-methyl ether standard Calibration curves were developed for quantifications Quantitation was obtained only from leaf extracts treated with a 10-day hot water soluble mycelium culture

Plants and treatment of leaves Acala SJ-2 cotton plants were grown under greenhouse conditions and were 2 months old at time of leaf treatment Both treated and control leaves were scratched lightly with the blade of a scalpel to produce a wound of *ca* 5 mm in diameter Two areas per fifth or sixth true leaf per plant were scratched in areas midway between the log axis of the leaf and midway between the outer margin of the leaf to the midvein The mycelial and culture fluid extracts were applied independently in 10 μg amounts in 20 μl distilled H_2O to the scratched areas on the leaves Distilled H_2O was applied to the scratched control leaves Each test consisted of 10 leaves with 20 areas treated with the extracts and 10 control leaves with 20 areas treated only with distilled H_2O Data represent averages of three separate experiments

Two days after treatment leaves were harvested and the

wounded areas were excised with a 15 mm diameter cork borer All preparative and analytical procedures were done in subdued light The leaf discs, 20 discs at a time, were placed in 50% aq EtOH and were vacuum infiltrated, the flasks were subsequently placed on a reciprocating shaker for 12 hr at room temp The discs were removed from the EtOH extract by filtration and the extract was concd to dryness by rotary evaporation under vacuum at 50° The dry residue was dissolved in MeOH to produce a 5% (w/v) soln and the extract was spotted on TLC plates

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