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A POSSIBLE RELATIONSHIP BETWEEN PHYTOALEXIN PRODUCTION IN THE COTTON LEAF AND A PHYTOTOXIC RESPONSE

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

Abstract—A 10-day time-course study on the production of 2,7-dihydroxycadalene, 2-hydroxy-7-methoxycadalene, lacinilene C and lacinilene C 7-methyl ether in cotton leaves induced by cell-free mycelial extracts of *Aspergillus flavus* showed that the cadalenes and the lacinilenes accumulate in a cyclic fashion. The initial increase at 2 days is followed by a greater increase at 6 days after treatment. The location of these compounds was found predominately either in a 6 mm wounded, treated area or in a 3 mm area immediately surrounding the 6 mm treated area of the leaf. Lacinilene C and lacinilene C 7-methyl ether were both phytotoxic in a *Lemna minor* bioassay. Endogenous constituents produced by plant cell damage could have triggered the production of the cadalenes and lacinilenes observed.

INTRODUCTION

Recently it was reported [1] that the two sesquiterpenoid naphthols 2,7-dihydroxycadalene and 2-hydroxy-7-methoxycadalene, together with their oxidation products lacinilene C and lacinilene C 7-methyl ether, respectively, are simultaneously induced in the cotton leaf by a cell-free mycelial extract or a culture fluid extract of *Aspergillus flavus*. Others [2, 3] have reported that these compounds function as phytoalexins in the cotton plant and thus serve as a defensive mechanism against invading microbes. Hargreaves and Bailey [4] demonstrated that the phytoalexin phaseollin from the French bean (*Phaseolus vulgaris*) and other isoflavonoid phytoalexins are probably synthesized in tissues around necrotic cells and are absorbed and accumulate in the dead tissue containing intracelluar hyphae. Their experiments further suggested that cell death is the trigger for subsequent phytoalexin biosynthesis and accumulation, and that constitutive material released by damaged cells initiates phytoalexin biosynthesis. The phytotoxic behaviour of phytoalexins in other microbial–host interactions has also been reported [5–7].

The purpose of this current investigation was (a) to quantify the induced production of the cadalanes and lacinilenes in cotton leaves over a 10 day period, (b) to identify the location of these compounds in relation to the challenged area on the host's tissue, and (c) to investigate the phytotoxicity of the induced compounds.

RESULTS AND DISCUSSION

In the current investigation, the fifth or sixth true leaves of 2-month-old Acala SJ-2 cotton leaves were wounded and treated with the same cell-free hot water soluble extract of *A. flavus* as reported in ref. [1] and the leaves were analysed for cadalanes and lacinilenes each day after treatment for 10 days. Table 1 shows an initial accumulation of these compounds after 2 days with higher accumulations on day 6 after wounded leaves were treated with the heat-soluble mycelia extract of *A. flavus*. A gradual decline of the compounds is apparent after the two peak periods. Control wounded leaves also show increases of the induced compounds on day 6 with a gradual decline towards the end of the test period.

After the second day of treatment with a cell-free mycelia extract of *A. flavus*, the greatest concentration of the compounds was localized in a 3 mm area encircling the 6 mm wounded/fungal-treated area (Table 2). The 6 mm diameter area was brownish in colour and the adjacent 3 mm area had a light yellow-green appearance. Five days after treatment the four compounds were found predominately in the 6 mm diameter wounded/treated area nd this area plus the adjacent encircling 3 mm area were both brownish in colour.

A sensitive bioassay used for determining the allelopathic properties of suspected allelochemicals has recently been reported [8]. This bioassay utilizes the aquatic macrophyte *Lemna minor* L. (lesser duckweed) grown in nutrient medium with or without allelochemicals in wells of 24-well tissue cluster dishes. I found good replication of the materials tested in regard to quantitative measurements of several parameters using this bioassay system. The results obtained with extracts prepared from 15 mm leaf discs surrounding and including wounded areas which were treated with cell-free extracts of *A. flavus* or wounded controls treated with distilled water upon the growth of *L. minor* are shown in Table 3. They indicate an inhibitory effect on the growth of *L. minor* both by number of fronds produced and dry weight expressed as a percentage of control on plants tested with the treated disc extracts. Browning of the roots was also observed in *L. minor* plants exposed to the treated disc extracts.

Understanding that other chemical components besides the compounds under investigation could be contributing to the total disc bioassay prompted a further assay with the purified phytoalexins present in the leaf disc extracts. Preparative TLC separated the lacinilenes

Table 1. Quantitation by TLC fluorodensitometry of the induced components in extracts from leaf discs taken from cotton leaves treated with a cell-free mycelial extract of *A. flavus*

Component	Amount in (μg/g fr. wt)									
	1d	2d	3d	4d	5d	6d	7d	8d	9d	10d
2,7-dihydroxycadalene	tr	13.4	3.1	1.0	tr	28.1	15.0	12.4	12.2	6.3
	(0)*	(0)	(tr)†	(tr)	(6.1)	(3.2)	(9.0)	(5.1)	(5.0)	(3.5)
Lacinilene C	tr	38.4	32.0	12.1	8.6	51.3	30.5	17.3	11.1	8.2
	(0)	(0)	(tr)	(tr)	(8.2)	(18.1)	(20.1)	(10.3)	(10.2)	(6.6)
2-Hydroxy-7-methoxycadalene	tr	25.5	12.1	8.3	3.3	36.4	28.3	20.2	20.0	18.1
	(0)	(0)	(tr)	(tr)	(tr)	(11.3)	(9.3)	(11.1)	(9.2)	(3.2)
Lacinilene C 7-methyl ether	0.5	23.1	3.2	2.6	5.2	38.1	26.2	23.8	21.5	17.3
	(0)	(tr)	(tr)	(tr)	(2.2)	(9.6)	(5.4)	(3.4)	(2.9)	(1.6)

*Wounded leaves treated with distilled water only.
†tr = trace < 1 μg.

Table 2. Percentage distribution after 2 and 5 days of induced components in 6 mm diameter wounded/fungal treated areas and in areas encircling the 6 mm areas in the cotton leaf

Components	Outer diameter (mm)					
	6	9	11	14	16	21
2,7-Dihydroxycadalene	29.1*	58.3	12.6	0	0	0
	86.2†	13.8	0	0	0	0
Lacinilene C	18.9*	77.0	4.1	0	0	0
	92.6†	7.4	0	0	0	0
2-Hydroxy-7-methoxycadalene	34.5*	58.0	4.6	2.1	0.9	0
	81.5†	18.5	0	0	0	0
Lacinilene C 7-methyl ether	42.7*	53.1	4.2	0	0	0
	94.1†	5.9	0	0	0	0

*Two days after treatment.
†Five days after treatment.

Table 3. Effects of 15 mm treated cotton leaf disc extracts on *L. minor* after 1 week of incubation

No. of leaf disc extracts tested*	No. of fronds after 1 week of incubation		Dry wt of *L. minor* as percentage of control
	Treated	Control	
1	22†	28	80
2	7†	26	42
3	11†	16	50
4	10†	28	50
5	9†	27	35
6	14†	32	41
7	11†	28	50
8	10†	30	39
9	10†	18	39

*Treated extracts were prepared from leaves which were wounded and treated with cell-free extracts of *A. flavus*. Control extracts were prepared from wounded leaves treated with distilled water.
†Roots have a brownish discoloration.

from a known number of leaf disc extracts. The lacinilenes' precursors were too unstable to use in this assay system. From the TLC eluted components, aliquots were prepared to represent that amount of component present in one to five leaf discs (Table 4). Both lacinilenes inhibited the growth of *L. minor*; more inhibition occurred in the presence of lacinilene C. Root discoloration occurred from both compounds and some frond bleaching occurred with lacinilene C.

The phytotoxic properties of the two lacinilenes may explain some of the results described in Tables 1 and 2. As in the experiments of Hargraves and Bailey [4], it is possible that cell death in the cotton leaf releases constitutive material (an endogenous elicitor) which initiates phytoalexin biosynthesis and the accumulation of the compounds shown in Table 1. This reasoning could also be used to explain the inducement of these compounds in wounded control leaves on days 5–6 after wounding. The synergistic effect brought on by both a fungal extract and an endogeneous elicitor may explain the peak accumulations of the compounds in treated leaf extracts on days 2 and 6 (Table 1). Synergistic elicitor effects have been observed between the β-glucan elicitor of *Phytophora*

Table 4. Separated components* of cotton leaf discs and controls assayed after 1 week of incubation with *L. minor*

Component	No. of leaf discs extracts†	No. of fronds after 1 week of incubation		Dry wt of *L. minor* as percentage of control
		Treated	Control	
Lacinilene C	1	24‡	32	77
	2	11‡	28	41
	3	8‡§	27	27
	4	11‡§	32	28
	5	9‡§	30	27
Lacinilene C 7-methyl ether	1	28	30	84
	2	22‡	28	81
	3	20‡	32	63
	4	20‡	30	62
	5	18‡	27	64

*Separated by preparative TLC.

†Aliquots prepared from 50 leaf discs to represent the amount of component in 1–5 leaf discs.

‡Roots have a brownish discoloration.

§Some bleaching of fronds resulted.

megasperma and the endogenous elicitor of soybean tissue [9]. Similar synergism has been observed between β-glucan and fatty acid elicitors in potato [10–12].

EXPERIMENTAL

The maintenance of *A. flavus* (SRRC 1000) cultures and the isolation of the fungal elicitor have already been described in a previous paper [1].

Plants and treatment of leaves. Acala SJ-2 cotton plants were grown under greenhouse conditions and were 2 months old at the time of leaf treatment. All wounds were produced with a 6 mm diameter fine carborundum sandpaper disc which was attached to a wooden dowel handle. Two wounds per leaf were produced on the undersides of the fifth or sixth true leaf of each plant. Treated leaves received 10 μl amounts of the cell-free extract in 20 μl H_2O to the abraised wounded area. Control leaves received 20 μl H_2O to the abraised wounded area. Each test consisisted of 20 leaves with 40 areas treated with the fungal extract and 20 control leaves with 40 areas treated only with 20 μl H_2O. In the time-course expt, a 15 mm diameter cork borer was used to excise the leaf discs surrounding the wounded areas on the leaves at the time the leaves were harvested from the plant. In expts designed to determine the location of the phytoalexins surrounding the 6 mm wounded area, cork borers of various diameters were used to excise rings encircling the wounded area.

L. minor *assay with leaf disc extracts. L. minor*, 15 mm discs, were excised after 2 days' treatment around each wounded area, and the discs were placed in test tubes in a manner in which nine separate test tubes contained 1–9 discs. The test tubes were vacuum-infiltrated with 2 ml 50% EtOH, then shaken for 12 hr on a reciprocating shaker. The extracts were filtered from the leaf tissue with Reeve Angel No. 802 filter paper and dried under N_2 purging, vacuum and heat (45°) to a vol. of 0.2 ml. The individual extracts representing 1–9 leaf discs were placed separately in 9 wells of a 24-well tissue culture cluster plate. Two ml of sterile Hoagland's soln [13] plus a single *L. minor* plant (consisting of a mother frond together with its daughter frond) were added to each well of the tissue cluster plate. Control wells contained 2 ml sterile Hoagland's soln plus 0.2 ml of a concentrate remaining from 2 ml of a dried 50% EtOH soln. The tissue culture plate was maintained at 25° ± 2° and received 12 hr of illumination per day from a bank of eight 15-W fluorescent Plant Gro lights. Dry weight was determined after 24 hr at 110°. The results indicate the means of six replicated expts.

To study the effects of lacinilene C and its 7-methyl ether on the growth of *L. minor*, 50 treated and 50 control 15 mm diameter leaf discs were harvested after 2 days' treatment and were vacuum-infiltrated with 20 ml 50% EtOH, shaken for 12 hr, and filtered as previously described above. The total extract was concentrated by N_2, vacuum and heat to dryness. This dried extract was dissolved in 3 ml MeOH and was streaked on three separate prep. silica gel TLC plates and developed in $CHCl_3$–MeCN (4:1). Under 365 nm illumination the fluorsecent areas corresponding to R_f values of lacinilene C (0.22) and lacinilene 7-methyl ether (0.55) were marked, then scraped separately from the TLC plates. EtOAc was added to the scraped silica gel mixture and the eluant was filtered through a fine fritted glass filter. The eluant was dried under N_2, 50% EtOH was added, and from this EtOH mixture aliquots were prepared to represent the amounts of lacinilene C and lacinilene C 7-methyl ether present from 1–5 leaf discs. The tissue culture cluster plates consisted of wells containing sterile Hoagland's soln, *L. minor* plants and the lacinilenes in separate wells representing the amounts of lacinilenes extractable from 1–5 leaf discs. Controls were prepared from wounded leaf discs treated only with H_2O. The results give the means of three replicated expts.

All prep. and TLC fluorodensitometry quantitation procedures were the same as reported previously [1].

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