Phytochemistry, Vol 27, No 11, pp 3429–3431, 1988
Printed in Great Britain

0031-9422/88 $3.00+0.00
Pergamon Press plc

PRODUCTION OF CARPEL WALL PHYTOALEXINS IN THE DEVELOPING COTTON BOLL

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(*Received in revised form* 25 *April* 1988)

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

Abstract—Developing cotton bolls of Deltapine 61, grown under controlled conditions in environmental chambers, were artificially wounded on the carpel surface and treated with an elicitor, composed of cell-free mycelial extracts of *Aspergillus flavus*, at weekly intervals for eight weeks postanthesis Two days after treatment, the bolls were harvested from cotton plants and discs containing the treated surfaces were excised and extracted to determine induction of the sesquiterpenoid naphthol phytoalexins, 2,7-dihydroxycadalene and 2-hydroxy-7-methoxy cadalene, their oxidation products lacinilene C and lacinilene C 7-methyl ether, and the coumarin phytoalexin-scopoletin. Maximum concentrations of the cadalenes and lacinilenes were present in bolls treated at seven weeks postanthesis; scopoletin concentrations were highest in bolls treated three weeks postanthesis To determine the effects of the induced phytoalexins on the growth of *A. flavus*, a second set of wounded elicitor-treated discs were collected at weekly intervals for eight weeks postanthesis, inoculated with *A flavus* and incubated on liquid plant nutrient. Fungal growth displayed a range from 80% of control for treated discs from bolls harvested one week postanthesis to 10% of control for discs from bolls harvested seven to eight weeks postanthesis The induced phytoalexins on elicitor-treated carpel walls appear to be responsible for the reduction of fungal growth observed.

INTRODUCTION

Aflatoxin is a carcinogenic fungal metabolite of *Aspergillus flavus* Link ex. Fries which is prevalent in cotton grown in the southwestern U.S.A. Several investigators have observed that developing cottonseed is more susceptible to *A. flavus* infection during specific stages of the cotton boll maturation and most agree that the mould primarily enters into the boll following insect damage to the carpel surface [1–4]. Lee *et al* [4] found lower aflatoxin levels in cottonseed from bolls inoculated with *A flavus* 19, 26, or 40 days postanthesis than from bolls inoculated 33 days after flowering. Sun *et al.* [1] inoculated several cotton cultivars with *A. flavus* at different stages of boll maturation and found greater amounts of aflatoxin in seed from bolls inoculated at 30 days postanthesis than in bolls inoculated at 20 days or 40 days postantesis. The inducement of the two sesquiterpenoid naphthol phytoalexins 2,7,-dihydroxycadalene and 2-hydroxy-7-methoxy cadalene, their oxidation products lacinilene C and lacinilene C 7-methyl ether and the coumarin scopoletin, has recently been described in cotton leaves inoculated with *A. flavus* [5, 6]. Halloin *et al.* [7] demonstrated the inducement of the lacinilene and cadalene phytoalexins on the outer carpel walls of the cotton bolls in response to a challenge by the fungus *Diplodia gossypina* In a thin-layer chromatographic bioassay we have found the lacinilenes, the cadalenes and scopoletin to be fungitoxic to *A. flavus* (unpublished data).

The purpose of the current investigation was to isolate and quantitate the induced phytoalexins at one week intervals from flowering for a period of eight weeks and to consider if there is a correlation between carpel surface phytoalexin production and resistance to *A. flavus* infection at specific stages of cotton boll development.

RESULTS AND DISCUSSION

In the current investigation 6 mm circular abraded wounds (to simulate insect wounds) were produced on one to eight weeks postanthesis developing bolls of Deltapine 61 cotton plants grown under controlled conditions in controlled environmental conditions. Wounded areas were treated either with a 14-day cell-free *A. flavus* preparation (to elicit phytoalexin production) or with sterile distilled water (to serve as controls). The cell-free *A. flavus* elicitor contained 46.7% protein and 29% carbohydrate. Each week for a period of eight weeks, bolls were inoculated and on the second day after inoculation, the treated bolls were harvested from the plants. Twelve mm discs containing the treated areas were excised from the bolls, extracted and quantified for lacinilenes, cadalenes and scopoletin An induction of the five phytoalexins in each of the eight weeks testing period when disc extracts of the elicitor inoculated bolls were compared to the water inoculated control bolls is shown in Table 1 The concentrations of the lacinilenes and cadalenes increase over the eight weeks testing period and peak either at the seventh or eight week postanthesis boll age. Scopoletin concentrations were just the opposite: higher concentrations were found in extracts of younger bolls, peaking in concentration at three weeks postanthesis.

Table 1 Quantitation by TLC fluorodensitometry of the induced components in extracts from cotton boll discs treated with cell-free mycelia extracts of *A flavus* at weekly intervals for an eight week postanthesis period

		Amount in (μg/g fr wt of excised discs)							
	Days	1–7	8–14	15–21	22–28	29–35	36 42	43–49	50–56
Component	Weeks	1	2	3	4	5	6	7	8
2,7-Dihydroxycalalene		4 4	18 6	18 2	26 3	34.7	38 6	53 2	3 4
		(2 6)*	(1 5)	(2 6)	(1 5)	(tr)†	(tr)	(–)§	(–)
Lacinilene C		8 3	12 5	10 1	26 3	22 3	25 4	42 8	38 4
		(- -)	(-)	(- -)	(tr)	(3 7)	(3 7)	(5 6)	(4 3)
2-Hydroxy-7-methoxycadalene		—	tr	2 6	7 4	7 7	11 8	24 3	21 5
		(—)	(- -)	(—)	(2 5)	(2 0)	(2 8)	(7 2)	(7 6)
Lacinilene C-7-methyl ether		7 2	10 1	12 5	14 8	14 3	15 2	34 3	26 4
		(—)	(—)	(–)	(-)	(tr)	(2 5)	(3 1)	(3 8)
Scopoletin		41 4	44 6	53 2	26 3	17 2	15 3	8 3	3 4
		(2 6)	(1 5)	(2 6)	(1 5)	(tr)	(tr)	( )	(- -)
Water content (%) of excised carpel discs		87 0‡	87 1	87 8	87 5	83 9	74 8	58 9	49 2

* μg/g fresh wt in wounded bolls treated with sterile distilled H_2O only
† Trace < 1 μg.
‡ Determined by heating at 110° until constant wt
§ None detected
Note (1) non-wounded controls contained < 1 μg component/g fr wt (2) Boll opening occurred 41–49 days

In a separate experiment designed to assay *A. flavus* growth on treated developing boll surfaces, bolls one to eight weeks postanthesis were wounded and treated as described above. Two days after treatment, treated bolls were harvested from the plant, the bolls were surfaced sterilized and discs containing the treated areas were excised. Excised discs were placed on sterile plant nutrient solution in individual wells of 24-well tissue cluster plates and were inoculated with an *A flavus* spore suspension After 10 days incubation the extent of fungal growth was visually compared to the growth of fungus on the control discs (Table 2) Fungal inhibition was apparent at all developing stages of the treated maturing bolls, the greatest inhibition occurring in the seven and eight week postanthesis maturation boll stages; the same stages when concentrations of the lacinilenes and the cadalenes are the highest Aflatoxin determinations were also performed on 10-day incubated discs Aflatoxin values expressed as percentage of control were similar to those values of fungal growth expressed in Table 2

It is interesting to note the highest concentrations of the lacinilene and cadalene phytoalexins occurred during the period of boll opening (41–49 days postanthesis). Lee [8] inoculated sutures with *A flavus* spores at the initiation of boll opening and harvested the bolls after two or four weeks and found no aflatoxin contamination in the seeds of the treated bolls It is possible that these induced phytoalexins could be translocated to deeper tissues within the boll either passively or actively The lacinilene and cadalene phytoalexins are slightly water-soluble [9] and a penetrating carpel wall wound could allow dew or rain to transfer the phytoalexins into deeper tissues of the boll to effect a more widespread defence Of course other mechanisms of protection are possible and it remains to be seen the exact role of these phytoalexins in the protection of cotton bolls from *A flavus* infection Further research is needed to determine the concentrations of these compounds in tissues other than cotton boll carpel walls

Table 2 Estimated fungal growth on treated excised discs inoculated with *A flavus* after 10-day incubation expressed as percentage of control*

Postanthesis		Percent growth on wounded treated discs
Weeks	Days	
1	1–7	80
2	8–14	80
3	15–21	80
4	22–28	60
5	29–35	60
6	36–42	40
7	43–49	10
8	50–56	10

* Determined by visual comparison to growth on wounded non-treated control discs treated with distilled, sterile water

EXPERIMENTAL

Cultures of *A flavus* (SRRC 1000) were maintained and the preparation of the 14-day fungal cell wall elicitor was prepared as reported previously [5]

Plants and treatment of cotton bolls Mature, flowering, cotton plants (Deltapine 61) were maintained under controlled conditions in Environmental Growth Chambers at 60% relative

humidity and with a photoperiod of 20 000 lux for 14 hr at 90°F and for 10 hr at 70°F. Flowers were tagged over an eight week period so that at least 20 bolls were represented for each experimental run Circular abraded wounds (6 mm) were produced by light scraping with a surgeon's scalpel on the carpel surfaces of the developing cotton bolls in areas between suture lines Control bolls were wounded and treated with 20 μl sterile, dist. H_2O and treated bolls were wounded and inoculated with 10 μg dry cell-free *A flavus* elicitor suspended in 20 μl of sterile, dist. H_2O. Two days after inoculation the bolls were harvested and 12 mm circular discs were excised from areas surrounding the wounds, the excised discs were cut only to a depth which included endocarp tissues

Extraction, identification, and quantitation of phytoalexins in developing cotton bolls. All extractions and analytical procedures were executed in subdued illumination For each experimental run, 20 discs were harvested and placed in a 125 ml flask with 40 ml MeOH–Me_2CO (4 1), the flasks were placed on a reciprocating shaker for 12 hr at room temp The extract was decanted and evapd to dryness The residue was resuspendedd in 20 ml EtOAc and filtered through a Millex-SP 0 5 μM filter unit into a tared test tube The filtrate was evapd to dryness at 50° under N_2 and dry wts recorded The residue was dissolved in MeOH to produce a 5% (w/v) solution which was spotted on silica gel TLC plates and developed in MeOH–$CHCl_3$ (1 4) Identification and TLC fluorodensitometric quantitation procedures of the lacinilines, cadalenes and scopoletin were performed as previously reported [5]

A flavus growth and aflatoxin assay with developing boll discs At weekly intervals for an 8 week testing period, 10 bolls were wounded in the same manner as described above and treated with 10 μg cell-free *A flavus* elicitor suspended in 20 μl sterile, dist H_2O, 10 bolls were wounded and treated with sterile, dist. H_2O only, these served as controls After 2 days incubation, the bolls were harvested from the plants and surfaced sterilized with 10% clorox (1 min) and 70% EtOH (0.5 min) and then rinsed several times with sterile dist H_2O Under sterile conditions, 12 mm diameter carpel discs were excised from the area around the wounds. Individual excised discs were placed in individual wells of a 24-well tissue culture cluster plate. Each well contained 0 25 ml sterile Hoagland's plant nutrient solution [10], the empty spaces between the wells were filled with Hoagland's solution to maintain the moisture in the plate. A spore suspension of *A flavus* (0 10 ml) in sterile dist H_2O containing 2.6×10^5 spores/ml was used to inoculate each disc. After 10 days incubation at 27°, the extent of fungal growth on the discs was estimated as a percentage of the controls by visual comparison

Following the recording of fungal growth, aflatoxin determinations were preformed on the 10-day fungal-inoculated treated boll discs and control discs 30 ml of 70% aqueous Me_2CO was added to each weekly set of 10 boll discs and was blended by mortar and pestle The blended mixture was poured into a 125 ml flask and placed on a reciprocating shaker at room temp for 2 hr The crude extract was decanted into a separatory funnel, 30 ml CH_2Cl_2 was added and after mixing, the CH_2Cl_2 layer was separated and was evapd to dryness by vacuum distillation at 40° Aflatoxin determinations were preformed by densimetric quantitation on TLC plates against known standards [11].

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