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INDUCED DEFENCE RESPONSES IN COTTON LEAF DISKS BY ELICITORS FROM *VERTICILLIUM DAHLIAE*

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Key Word Index—*Gossypium hirsutum*; Malvaceae; cotton; chitinase; elicitor; β-1,3-glucanase; PR-proteins; *Verticillium dahliae*.

Abstract—Cotton (*Gossypium hirsutum*) leaf tissue responded with symptoms of chlorosis and necrosis to treatment with heat-released soluble cell-wall fragments from *Verticillium dahliae*. The induction of defence responses in leaf disks by the elicitor led to increased synthesis of anti-fungal lasinilene and cadalene stress metabolites, as well as the rapid deposition of phenolic polymers in the lignin fraction. The coordinated induction and accumulation of chitinases and 1,3-β-glucanases were observed following elicitation; selective low pH extracts of pathogenesis related-proteins revealed a bi-phasic defence hydrolase response that reached a peak within 12 hr, followed by a gradual increase up to 120 hr. Electrophoretic analysis and enzyme staining revealed differences in constitutive and inducible proteins. Three acidic isozymes of chitinase with isoelectric points in the range 3.7–4.2, but only one β-1,3-glucanase isozyme with an isoelectric point of 4.5 were found in the intercellular fluid. The results obtained indicate that the elicitation of cotton leaf disks by *V. dahliae* derived signal molecules exhibits some properties of a multicomponent dynamic system where different protective mechanisms have complementary roles in the overall expression of the defence response. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Verticillium wilt, caused by the ground borne fungus *Verticillium dahliae* Kleb., is a widespread disease that occurs in most cotton producing areas [1]. Little is known about the genetics of the *Gossypium hirsutum–V. dahliae* interaction. Resistance or tolerance against *V. dahliae* was derived from *G. barbadense*, while it is thought that virulence in *V. dahliae* is controlled by a number of genes [2].

Through research on the biochemistry of plant defences, it has been shown that fragments from fungal cell walls can act as powerful signalling agents to activate plant defenses [3]. Plants elaborate a number of inducible defence responses following microbial attack [4]. These responses include *inter alia* the synthesis of antimicrobial phytoalexins which appear to feature prominently in plant disease resistance in many plant:pathogen interactions. In the case of the cotton, the degree of resistance to *Verticillium* wilt has been reported to be related to the amounts of sesquiterpenoids produced by infected tissue [5]. Cell wall reinforcement can also occur in response to pathogen attack through the synthesis of structural hydroxyproline-rich glycoproteins and the deposition of lignin [6]. Pathogenesis-related (PR) proteins, induced in pathological or related situations [7], account for the major quantitative changes in soluble host encoded proteins in infected plants compared to healthy plants. It has been shown that several PR-proteins have cell wall degrading chitinase {poly[1,4-(*N*-acetyl-β-D-glucosaminide)] glycanohydrolase, EC 3.2.1.14}, and 1,3-β-glucanase (1,3-β-D-glucan 3-glucanohydrolase, EC 3.2.1.6) activities [8].

The relationships between different defence responses, their localization and relative contribution towards the total defence response, need to be known before a direct causal relationship between biochemical defense mechanisms and disease resistance can be established. Up to now little or no information was available on the inducible defence responses in cotton. The accumulation of PR-proteins in roots and leaves of cotton seedlings infected with *V. dahliae* as well as vesicular-arbuscal mycorrhizal fungi was recently reported [9]. In this study we have investigated the induction of phytoalexins, lignin and the glycan hydrolases, chitinase and 1,3-β-glucanase, in cotton leaf disks as part of a defence response in reaction to elicitors derived from *V. dahliae*.

Table 1. Identification of lacinilene and cadalene stress metabolites extracted from cotton leaf disks 48 hr after elicitation by elicitors from *V. dahliae*

Compound	R_f values		
	A	B	C
Lacinilene C	0.26	0.22	0.27
2,7-Dihydroxycadalene	0.47	0.47	—
Lacinilene C-methyl ether	0.56	0.59	0.47
2-Hydroxy-7-methoxycadalene	0.63	0.65	—

A, chloroform:acetone:formic acid (80:19:1); B, acetonitrile:chloroform (4:1); C, hexane:ethylacetate:methanol (60:40:1). Compounds were separated on silica gel 60 plates and detected by autofluorescence [12].

RESULTS AND DISCUSSION

Resistance in many plant–pathogen interactions is accompanied by the rapid deployment of a multicomponent defence system. The induction of these defence responses in plant tissues is presumed to be mediated by an initial recognition process between plant and pathogen, involving the detection of signal molecules or elicitors in plants [3, 4].

Elicitor preparation and treatment of leaves

Pooled mycelia of *V. dahliae* (100 g) yielded 8.8 g purified cell walls and 1.26 g heat solubilized, non-dialysable cell wall fraction. The *V. dahliae* elicitor fraction consisted of 70% carbohydrate. The elicitor fraction was qualitatively tested for the ability to induce symptoms in eight-week-old cotton leaves. The leaf tissue reacted hypersensitively with symptoms of interveinal chlorosis and necrosis [10]. The lowest effective elicitor concentration for the induction of defence responses was estimated as 30 μg ml^{-1} from pathogenesis related protein profiles from intercellular fluid harvested at 48 hr (not shown).

The capability of the cell-wall derived elicitor from *V. dahliae* to induce the synthesis of phytoalexins was evaluated after 48 hr of elicitation by extracting the tissue with ethanol and analysing the extracts by TLC. Cadalenes and their oxidation products the lacinilenes were reported to accumulate in leaves of cotton when infiltrated with *Xanthomonas campestris* suspensions [11]. Increased synthesis of yellow–green fluorescing sesquiterpenoids was visible and the following four phytoalexins were identified (Table 1) from published R_f values and colour reactions: lacinilene C, lacinilene C 7-methyl ether, 2,7-dihydroxycadalene, and 2-hydroxy-7-methoxycadalene [12]. The level of induction at 48 hr, compared to 24 hr, was estimated as approximately two- to three-fold. Although phytoalexins are not always effective protectants, they may retard fungal growth until other responses become effective [13].

Lignin deposition

Cotton leaf disks responded to elicitor treatment (Fig. 1) with a rapid initial increase of alkali-insoluble thioglycolic acid-extractable complexes up to 8 hr, followed by a decline and then a gradual increase up to 72 hr. Accumulation of lignin-like material in plant tissue has been associated with attempted fungal infection. It has been hypothesized that the deposition of lignin and the covalent crosslinking with carbohydrate and protein during lignin polymerization result in reinforcement of cell walls and structural rigidity, which interfere with the enzymic hydrolysis and mechanical penetration of plant tissue by fungal pathogens and may also impair the movement of water and diffusible molecules between plant and fungus [14]. It is unclear whether lignification is a primary or secondary determinant of compatibility [6].

Analysis of PR-proteins in whole leaf extracts and intercellular fluid

The presence of PR-proteins has been correlated with increased disease resistance to a number of pathogens [7]. PR-proteins have common and characteristic properties which aid in their detection and isolation. PR-proteins are soluble at low pH and can be extracted under conditions that leave the majority of plant proteins insoluble [15]. The induction of PR-proteins in cotton leaf disks by cell wall elicitors from the pathogen *V. dahliae* was similarly analysed. The electrophoretic profiles of low pH extracts at time periods up to 120 hr were analysed and revealed several inducible proteins against a high background of constitutive proteins (not shown). PR-proteins are relatively resistant to proteases and are well adapted to survive in the intercellular spaces of the leaf [7]. The preparation of intercellular washing fluid provides a fast and effective way for analysis of the apoplastic domain of the plant. The IF extraction procedure allowed the recovery of ± 500 μl of a yellowish fluid per gram leaf tissue. Under optimal conditions, the extract could contain up to 200–300 μg of protein ml^{-1}.

PR-proteins that accumulate in the extracellular compartment, are normally acidic proteins induced by pathogen stress but not by wounding [7, 16]. Consequently, the proteins present in the IF of cotton were analysed by anodic-PAGE under non-denaturing conditions (Fig. 2(A)). The time dependent increase in the concentration as well as the number of proteins can be observed in the R_f range of 0.40–0.45 and 0.7–0.8, which corresponds to the data reported by Liu *et al.* [9]. SDS–PAGE analysis (Fig. 2(B)), revealed M_r values in the range between 17 000–24 000 and 28 000–33 000 for the induced proteins which corresponds to the M_r range of 28 000–33 000 reported for β-1,3-glucanases and chitinases [17].

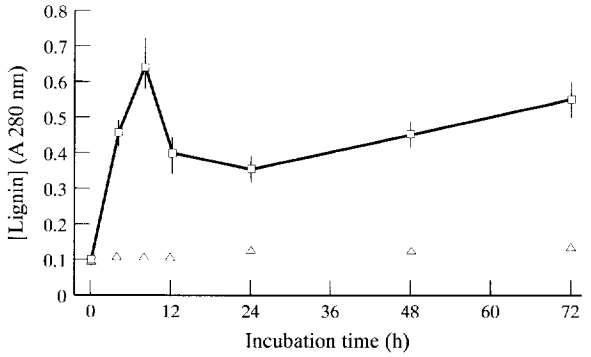

Fig. 1. Time course of lignification, measured as alkali-insoluble thioglycolic acid-extractable complexes, in cotton leaf disks responding to cell wall derived elicitor molecules (30 μg ml^{-1}) from *V. dahliae*. Controls (△) were treated with sterile distilled water; vertical bars indicate standard deviation.

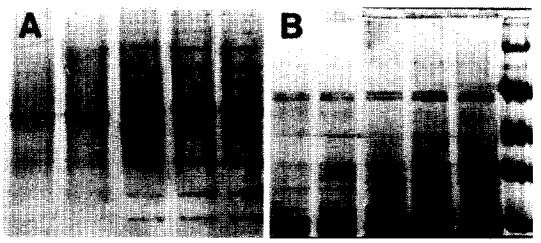

Fig. 2. Electrophoretograms of intercellular fluid extracted from cotton leaf disks treated with elicitors from *V. dahliae*; (A) 10% anodic PAGE of lane 1, 0 hr control; lane 2, 120 hr water treated control; lanes 3–5, extracts at 24, 48, 72 and 120 hr and (B) 15% SDS–PAGE of lane 1, 0 hr control; lane 2, 120 hr water treated control; lanes 3–5, extracts at 24, 48, 72 and 120 hr; lane 6, M_r calibration proteins; 21 000, 30 000, 46 000, 69 000 and 96 000.

1,3-β-*Glucanase and chitinase activities in whole leaf extracts*

Assays of total 1,3-β-glucanase activity revealed a biphasic response consisting of a rapid (6–12 hr after elicitor treatment) transient induction followed by a slow rise up to 120 hr (Fig. 3(A)). A similar pattern can be seen for the time course study of chitinase activity (Fig. 3(B)). For both enzymes, the controls (leaves treated with sterile distilled H_2O) were not much higher than the basal levels at 0 hr. The implication of plant hydrolases in defence against fungal pathogens appears to be significant since 1,3-β-glucanase and chitinase, especially in combination, have been shown capable of hydrolysing fungal cell walls *in vitro* [18]. The germination of conidia from *V. dahliae* was reported to be significantly reduced in solutions containing PR-proteins from cotton [9]. Since both hydrolases, considered as direct antimicrobial defence enzymes, act synergistically in the degradation of fungal cell walls, coordinated induction kinetics as observed in this study would be important for the effective inhibition of fungal growth [18]. The enzymes may also have a function in recognition and signalling events between plant and pathogen, since they are potentially able to release elicitors of defence reactions from the walls of some pathogens or of the plant cells themselves [3].

The reported half-lives of 40–70 hr for chitinase and 1,3-β-glucanase permit the accumulation of high levels of these enzymes [7]. Different isoforms accumulate at different intra- and extracellular locations such as in the apoplast and the vacuole [7, 19, 20]. It has been proposed that the extracellular, acidic isoforms may serve as a first line of defence against invading pathogens, with the intracellular, basic isoforms stored in the vacuole, as a second line of defence [21].

Activity profiles of 1,3-β-*glucanases and chitinases in the intercellular fluid*

The activity profile for 1,3-β-glucanase ($R_f = 0.43$), following 10% anodic PAGE, can be seen in Fig. 4(A). Only a single band for 1,3-β-glucanase was observed after activity staining. This result was confirmed by isoelectric focusing where a single acidic isoform with a pI value 4.5 was detected in a pH 3–10 gradient (results not shown).

Isoelectric focusing in a 3.0–10.0 pH gradient indicated the presence of at least two chitinases with pI values of 3.7 and 4.2 (data not shown). The activity profiles of these chitinases separated by anodic PAGE were very similar to the glucanases except that three isoforms with R_f values of 0.26, 0.38 and 0.44 respectively, were resolved (Fig. 4(B)). In both cases an increase in intensity up to 120 hr, the last time point in the study, can be seen. The initial, transient peaks at 12 hr, as observed in Fig. 3(A) and (B), are however not prominent due to the low sensitivity of the staining techniques.

In conclusion, the ability of cell wall derived elicitors from *V. dahliae* to induce defence responses in leaf disks from a resistant cotton cultivar was demonstrated. The tissue responded to the elicitor by increased phytoalexin synthesis and lignin deposition. The transient, coordinated induction of chitinase and β-1,3-glucanase activity was observed and specific isoforms of chitinase and 1,3-β-glucanase were identified

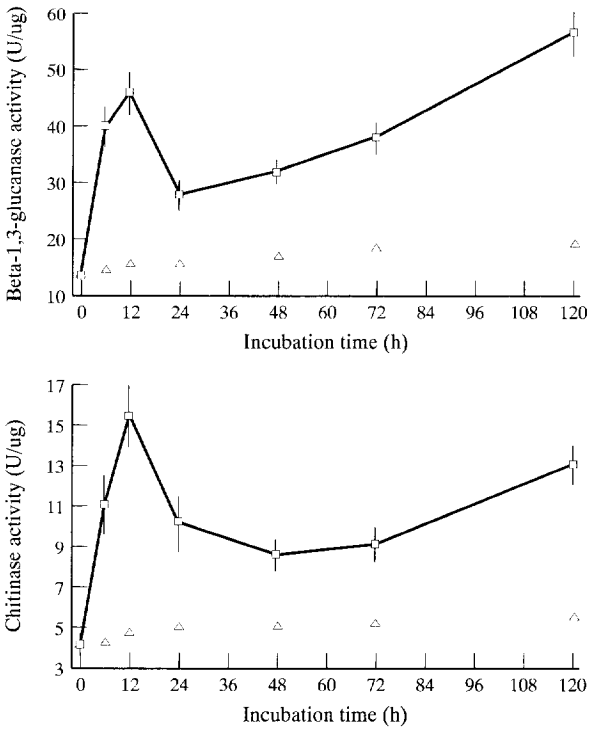

Fig. 3. Time course for the induction of (A) 1,3-β-glucanase activity and (B) chitinase activity in cotton leaf disks by cell wall derived elicitors (30 μg ml^{-1}) of *V. dahliae*. β-1,3-Glucanase activity is expressed as pmol glucose-equivalents released min^{-1} μg protein^{-1}, and chitinase activity as cpm released min^{-1} μg protein^{-1}. Controls (△) were treated with sterile distilled water; vertical bars indicate standard deviation.

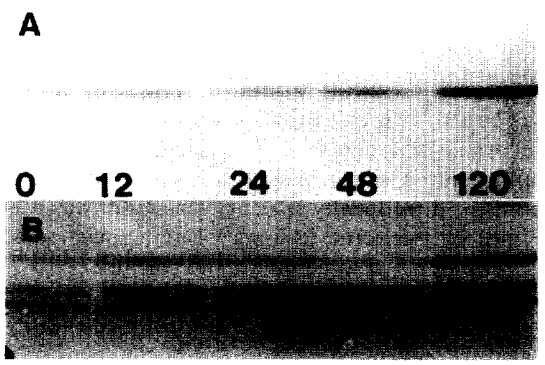

Fig. 4. Activity staining of (A) 1,3-β-glucanase activity and (B) chitinase activities in intercellular fluid of cotton leaf disks; lane 1: control; lanes 2–5: extracts after 12, 24, 48 and 120 hr of elicitor treatment. Proteins (5 μg per lane), were separated by 10% anodic PAGE.

in the intercellular fluid. The induced defence responses may proceed from the same recognition event(s) or be interlinked through common precursors and may work together in a complementary manner towards resistance [6]. The obtained results support the view that individual plant defence reactions have complementary roles and contribute to the overall expression of disease resistance.

EXPERIMENTAL

Plant material and fungal isolate. Seeds of the cotton cultivar OR_{19} (*Verticillium*-resistant) were obtained from the Tobacco and Cotton Research Institute, South Africa. The seeds were germinated and the plants were maintained in a greenhouse at 25–30°. The leaves of uniformly developed 8-week-old plants were used in all experiments. A pathogenic isolate of *V. dahliae* was obtained from infected $Acala_{1517/70}$ cotton stems [22].

Elicitor preparation. Fungal mycelia were grown in liquid cultures, cultivated for 7 days in the dark at 25° in 1 l flasks, each containing 100 ml of medium [23]. Cell walls and elicitors were prepared from mycelia [24]. The carbohydrate content of the cell walls and the elicitor fraction was quantified by the anthrone assay with glucose as a standard [25]. Protein content was determined by the Coomassie blue dye binding method [26].

Treatment of cotton leaves and leaf disks with elicitor. Leaves were injected with sterile solutions (0–100 μg ml^{-1}) of elicitor, and used in a bio-assay to evaluate the effect of the elicitor. Symptoms of chlorosis and necrosis were evaluated after one week. Disks (11 mm diameter, 30 per time point in each experiment) were cut with a cork borer from young expanded leaves of 8-week-old cotton plants. Leaf disks were washed twice with distilled water to remove wound metabolites and floated with their adaxial surfaces down on 10 ml of elicitor solution under sterile conditions in Petri dishes. The elicitor solution consisted of 30 μg of elicitor ml^{-1} sterile distilled H_2O which corresponds to 21 μg glucose-equivalents ml^{-1}. Control disks were

floated on sterile distilled water. Petri dishes with leaf disks were incubated for 0, 6, 12, 24, 48, 72 and 120 hr at 25° under fluorescent light. All experiments were performed in triplicate.

Preparation of leaf extracts. Selective low pH extracts of PR-proteins were prepared by homogenizing leaf disks in a Waring blender at 4° in two volumes (2 ml g^{-1}) of 0.05 M sodium acetate (pH 5.2) containing 15 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM diethyl dithiocarbamate and 10% (w/w) Polyclar AT. After filtration through a double layer of miracloth, the extracts were centrifuged at $12\,000 \times g$ for 20 min at 4°. The supernatants were used for the determination of changes in inducible proteins and enzyme activities. Protein was measured as described [26].

Preparation of intercellular fluid. Intercellular fluid (IF) was extracted by vacuum infiltration and low-speed centrifugation [20], with minor modifications. Treated leaf disks were infiltrated *in vacuo*, with gentle agitation for 5 min with a large excess of the following cold (4°) mixture: 25 mM Tris–HCl, pH 7.8 containing 10 mM $MgCl_2$: 10 mM $CaCl_2$, 0.5 mM PMSF and 5 mM 2-mercaptoethanol. The leaf disks were blotted dry and placed in small 5 ml disposable syringes fitted with syringe caps. After low speed centrifugation at $2\,000 \times g$ for 15 min at 4°, the extracts were recovered in the syringe caps and were used immediately or frozen at −20°.

Electrophoretic analyses. Discontinuous electrophoresis under denaturing conditions (SDS–PAGE) on slab gels (8×7) cm was performed [27]. The same system was used for non-denaturing conditions for acidic and neutral proteins (anodic PAGE) except that sodium dodecyl sulphate (SDS) was omitted. The gels were run for *ca.* 2 hr at 4° with constant current settings at 20 mA gel^{-1}. Proteins were detected with silver staining according to the protocol supplied by Amersham. Isoelectric focussing (IEF) was performed in a vertical polyacrylamide minigel system [28]. Polyacrylamide gels (5.2%, 0.75 mm thick, pH 3.5–10.0) was focused for 1.5 hr at 4° with constant voltage of 200 V followed by a constant voltage of 400 V for another 1.5 hr. After completion, the IEF gels were cut and the section containing the standard marker proteins were stained with Coomassie brilliant blue R-250. Gel sections were also stained for 1,3-β-glucanase and chitinase activity (described below) for the determination of pI values of the enzymes.

Enzyme activity determinations. Assays for chitinase activity in the low pH extracts of PR-proteins were performed in triplicate. Regenerated [^{3}H]-chitin was used as a substrate [29]. The reaction mixture contained 100 mM potassium phosphate buffer pH 5.2, 0.02% NaN_3, 1.2×10^5 c.p.m. of [^{6}H]-chitin and leaf extract in a total volume of 250 μl. After incubation at 37° for 30 min (the linear part of the progression curve), 0.25 ml of 10% trichloroacetic acid was added, and the suspension was centrifuged at $4\,000 \times g$ for 5 min. The radioactivity of the supernatant was determined by liquid scintillation counting. Controls were prepared for each extract by using a boiled aliquot in the reaction mixture. Chitinase activity was calculated [30], and is expressed as cpm in the released soluble products μg $protein^{-1}$ min^{-1}. 1,3-β-Glucanase activity of protein extracts was assayed in triplicate by measuring the rate of reducing sugar production with laminarin as the substrate [8]. The release of product was a linear function of enzyme concentration. Glucanase activity was expressed as pmol glucose-equivalents liberated min^{-1} μg $protein^{-1}$.

Enzyme staining. Chitinase activity staining in gels was performed using Calcofluor White M2R to stain unhydrolysed glycol chitin that was used as substrate [31]. Lytic zones were visualized by placing the gels on a UV transilluminator and photographed with Polaroid type 55 film with UV-haze and orange filters. Glycol chitin was obtained by acetylation of glycol chitosan [29, 31]. For the detection of 1,3-β-glucanase activity the 2,3,5-triphenyltetrazolium chloride method was used to detect reducing sugars released by the enzyme from the substrate, laminarin [32].

Lignin determination. Following extraction with methanol to remove free phenolic compounds and alkaline hydrolysis of cell wall-bound phenolic esters, total lignin present in 1 g leaf tissue was extracted and determined as the thioglycol derivatives as 280 nm [33].

Phytoalexin analysis. Sesquiterpenoids were extracted from 1 g of leaf disks after 0, 24 and 48 hr of elicitation and analysed by TLC for the presence of induced cadalenes and lacinilenes [12].

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