Apoplastic Peroxidase Generates Superoxide Anions in Cells of Cotton Cotyledons Undergoing the Hypersensitive Reaction to Xanthomonas campestris pv. malvacearum Race 18


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Cotton cotyledons displayed a hypersensitive reaction (HR) in the resistant cultivar Reba B50 after infiltration with the avirulent race 18 of Xanthomonas campestris pv. malvacearum (Xcm). Generation of active oxygen species during the HR was studied biochemically and cytochemically. \( \text{O}_2^- \) was detected in cotyledon disks by the cytochrome c reduction assay 3 h after inoculation. This activity was inhibited by superoxide dismutase (SOD) and by the peroxidase inhibitors salicylhydroxamic acid (SHAM) and KCN but not by the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI). Strong NADH oxidation activity also was found 3 h after inoculation in crude extracts or in apoplastic washing fluid and was dramatically decreased after treatment with SHAM or KCN. NADH oxidation was activated by 2,4-dichlorophenol and \( \text{MnCl}_2 \), indicating the involvement of a peroxidase. Activity of cationic peroxidase isoforms (pI 9 to 9.5) constitutively expressed in cotyledons was found to be enhanced 3 h after inoculation in the resistant cultivar. Activities of apoplastic peroxidases and \( \text{H}_2\text{O}_2 \) accumulation were observed cytochemically, 3 and 4 h post inoculation, respectively. When digitonin, a \( \text{O}_2^- \) elicitor, was infiltrated into cotyledons of resistant and susceptible cultivars, generation of \( \text{O}_2^- \) radicals was shown to be reduced by SOD and inhibited by SHAM and KCN as observed after infection, and also by DPI. Our results strongly suggest that cotton cotyledons contain two \( \text{O}_2^- \)-generating systems and that cells undergoing the HR in response to an avirulent race of Xcm produce \( \text{O}_2^- \) through the activation of an apoplastic peroxidase.

Among the early events that take place during plant-pathogen interactions, the oxidative burst consisting of a localized, rapid, and transient production of active oxygen species (AOS) has received considerable attention for its role in plant resistance (Sutherland 1991; Medhy 1994; Baker and Orlandi 1995; Bolwell and Wojtazscek 1997; Lamb and Dixon 1997; Wojtazscek 1997). It has been postulated that highly reactive oxygen intermediates (\( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), OH-) could initiate lipid peroxidation of the plant plasma membrane leading at the site of infection to (i) increase cell leakage and hypersensitive cell death, and (ii) release of signal molecules that would be involved in the activation of defense genes in more distant cells (Low and Merida 1996; Anderson et al. 1998). Moreover, cross-linking of cell wall proteins and phenol monomers associated with plant defense has been shown to be promoted by AOS (Tenhaken et al. 1995; Milosevic and Sluzareno 1996; Ogawa et al. 1997). Hydrogen peroxide, produced by challenged cells, has also been demonstrated to act as a diffusible signal for the induction of genes encoding glutathione-S-transferase and glutathione peroxidase in surrounding cells that might protect those cells from the oxidative damage (Levine et al. 1994). More recently, in parsley cells challenged with the oligopeptide elicitor Pep-13, it has been demonstrated that among AOS produced during the oxidative burst only \( \text{O}_2^- \) was an essential element of the signal cascade leading to phytoalexin production (Jabs et al. 1997). Finally, the oxidative burst may also function as a first line of defense in resistant plants, being directly toxic to the pathogen during the earliest stages of infection (Baker and Orlandi 1995).

Studies on enzymatic machineries that produce the oxidative burst in plants have mainly focused on the NADPH oxidase complex (Levine et al. 1994; Desikan et al. 1996; Doke et al. 1996), which is also known to be the primary source of AOS production in mammal neutrophils (Segal and Abo 1993). Although the NADPH complex has not been purified so far from plant cells, Groom et al. (1996) and Keller et al. (1998) recently presented evidence that in rice and Arabidopsis thaliana homologs exist of the neutrophil NADPH oxidase gp91phox subunit gene. This gene encodes a plasma membrane protein containing two putative Ca\(^{2+}\) binding domains and one G protein regulating region (Keller et al. 1998). Several other results from pharmacological, immunological, and reconstitution experiments further strengthen the idea that a complex similar to that found in the mammalian neutrophil is present in plants and regulated via comparable mechanisms.
(Levine et al. 1994; Doke and Miura 1995; Auh and Murphy 1995; Desikan et al. 1996; Dwyer et al. 1995; Simon-Plas et al. 1997). It has also been proposed that certain peroxidases, acting as NAD(P)H oxidases (Halliwell 1978), could be alternative sources of AOS in plants challenged by pathogens (Bolwell et al. 1995) or elicitors (Lindner et al. 1988). Moreover, changes in the activities of enzymes potentially involved in the regulation of AOS accumulation have been shown in hypersensitively reacting bean leaves (Adam et al. 1995). In particular, the activity of several peroxidase isoforms was shown to increase during an incompatible interaction within inoculated and surrounding tissues.

Infiltration of avirulent races of the bacterial pathogen *Xanthomonas campestris* *pv. malvacearum* (*Xcm)* into the apoplasma of cotyledons from resistant cultivars of cotton (*Gossypium hirsutum*) induces a hypersensitive reaction (HR). The resistant cultivar Reba B50 contains the resistance gene combination B2-B3 involved in gene-for-gene interactions with the *Xcm* avirulent race 18 (De Feyter et al. 1993; Innes 1983). By contrast, race 18 is virulent on the susceptible cultivar Acala 44 and spreads beyond infection sites, inducing characteristic leaf blight symptoms. Research on the nature of cotton resistance to bacterial blight has mainly focused on sesquiterpenoid phytoalexins, the major antimicrobial molecules produced by cotton plants (Essenberg and Pierce 1994). Flavonoids were also histochemically detected accumulating at sites where the HR developed (Dai et al. 1996). In a recent study, we showed that the activity of several peroxidases was locally stimulated in cells undergoing the HR in resistant cotyledons and systemically induced in whole plants (Martinez et al. 1996), but functional roles for these peroxidases were not elucidated.

In the present paper, we investigate the oxidative burst induced in cotyledons of the resistant cultivar Reba B50 by the *Xcm* avirulent race 18. Our experiments reveal that O$_2^-$ is transiently produced in the extracellular apoplast during the first few hours of the incompatible interaction. Results suggest that the AOS production is generated by the activation of apoplastic peroxidases. We also present data demonstrating that both the resistant Reba B50 and susceptible Acala 44 cultivars contain an NADPH oxidase that remains inactive following infection by *Xcm*.

**RESULTS**

**In vivo characterization of the oxidative bursts in cotton cultivars Reba B50 and Acala 44.**

Cytochrome c reducing activities were estimated with cotyledon disks of the two cultivars Reba B50 (Fig. 1) and Acala 44 (Fig. 2), resistant and susceptible to *Xcm* race 18 respectively, 3 h after either water infiltration or *Xcm* inoculation, or immediately after infiltration with 80 µM digitonin. Controls, infiltrated with water or with ethanol (0.5% in water), showed slight extracellular reducing activities (Figs. 1A and 2A) that were similar at 3 h to those estimated immediately after water treatment (data not shown). Intensities of the reducing activity (estimated by the initial slopes of the curves) differed between the two cultivars since activities in Reba B50 controls were twofold higher than in Acala 44 ones. These activities were 80 to 90% inhibited by the addition of superoxide dismutase (SOD) (1,000 U/ml) to the reaction medium (Figs. 1A and 2A), indicating that superoxide anion was the main component involved in the extracellular reduction of cytochrome c.

Three hours after infiltration of cotyledons from Acala 44 with *Xcm* race 18 (Fig. 1B) or from Reba B50 with *Xcm* race...
20 (Fig. 1B), the reducing activity remained similar to controls, whereas it was enhanced in race 18-infiltrated Reba B50 cotyledons to 300% of controls (Fig. 1B). This activity was 90% inhibited by the addition of SOD, totally blocked by KCN (50 μM), and insensitive to diphenyleneiodonium chloride (DPI) (20 μM) (Fig. 1B). Higher concentrations of DPI (up to 100 μM) also failed to inhibit AOS generation.

Infiltration of cotyledons of both cultivars with 80 μM digitonin immediately stimulated reducing activities (Figs. 1C and 2C) that were about threefold higher in Reba B50 than in Acala 44. Sixty percent of the activity in both cultivars was inhibited by SOD. The digitonin-induced cytochrome c reducing activities of both Reba B50 and Acala 44 were partially sensitive to DPI (33% and 19% inhibition, respectively) and KCN (60% and 80% inhibition, respectively).

**Time course of changes in AOS production during cotton/Xcm interactions.**

The superoxide anion production rates were estimated from the respective cytochrome c reducing activities. As shown in Figure 3, the incompatible interaction (Reba B50/Xcm race 18) was characterized by a sharp peak of O$_2^-$ production occurring between 2.5 and 3 h after infection. At that time, production of O$_2^-$ was about 40 nmol g$^{-1}$ min$^{-1}$. In the compatible interactions, Acala 44/Xcm race 18 and Reba B50/Xcm race 20 (Fig. 3), a low basal production (not significantly different from controls) was observed over a 5-h period.

Hydrogen peroxide was histochemically detected with cerium chloride. Ce$^{3+}$ was added to trap H$_2$O$_2$ as insoluble cerium perhydroxide that appears fluorescent after illumination of sections from the treated fragments with epipolarized ultraviolet light. Two hours after bacterial infiltration, no deposits of refractive particles were seen in the inoculated tissues, while 4 to 6 h after inoculation more fluorescence was seen in walls and in intercellular spaces of cotyledon cells challenged with bacteria (Fig. 4A) than observed in water-infiltrated controls (Fig. 4B). Fluorescence of walls was intense in the spongy mesophyll where the pathogen was inoculated but weak in palisade cells.

Inoculation of the susceptible cultivar with Xcm race 18 or infiltration with water did not yield any significant fluorescence. Similar results were obtained whether or not aminotriazole (catalase inhibitor) was added to the inoculum.

**NADH oxidation activity in cotton cotyledons challenged with Xcm; time course changes and characterization.**

NADH oxidation activity was assessed according to Halliwell (1978). The oxidation activity of crude cotyledon extracts was measured over 4 h following inoculation of Reba B50 and Acala 44 with Xcm race 18 (Fig. 5A). Constitutive activities, estimated in extracts from control samples, were 0.1 ± 0.02
nKat mg⁻¹ protein for Reba B50 and 0.07 ± 0.01 nKat mg⁻¹ protein for Acala 44. Whereas NADH oxidation activities remained unchanged during the compatible interaction, including that with Xcm race 20 (0.09 ± 0.02 nKat mg⁻¹ protein), the incompatible interaction was characterized by a threefold increase in specific activity by 3 h after inoculation (0.31 ± 0.01 nKat mg⁻¹ protein), which dropped to the basal level after 4 h. As shown in Figure 5B, the activity of extracts obtained 3 h after inoculation of Reba B50 with Xcm race 18 was 75% inhibited when KCN (50 μM) or salicylhydroxamic acid (SHAM) (5 mM) was added to the reaction mixture. In water-infiltrated controls, the activity of extracts was only 42% inhibited, while a slight inhibition (15%) in such activity was observed with Acala 44 extracts. Addition of MnCl₂ (2 mM) or 2,4-dichlorophenol (2,4-DCP) (17 μM), known to stimulate peroxidase activity (Halliwell 1978), strongly enhanced the specific activities of extracts obtained from both cotton cultivars. Activities in extracts from water-infiltrated or Xcm race 18-infected Reba B50 plants were statistically equivalent when treated with Mn²⁺ (0.95 nKat mg⁻¹ protein) or with 2,4-DCP (1.18 nKat mg⁻¹ protein). The specific activities of Acala 44 extracts from both extracts reached the level of 0.45 and 0.49 nKat mg⁻¹ protein when treated with Mn²⁺ or 2,4-DCP, respectively. In a separate set of experiments, both NADH oxidation and guaiacol peroxidase activities were measured at 3 h in the crude extracts and in the apoplastic washing fluids (AWF) obtained from water and Xcm race 18-infiltrated resistant cultivar (Table 1). Both specific activities were higher in AWF than in crude extracts (about five- to sevenfold). Although no difference was spectrophotometrically detected in the guaiacol peroxidase activities between control and infected plants, the NADH oxidation activity was 2.4-fold higher in extracts from inoculated plants (Table 1) and differences in the activity of cationic peroxidase isozymes (pI 9 to 9.5) were observed on isoelectric focusing (IEF) gels (Fig. 6) only 3 h after inoculation of the resistant cultivar. Comparatively, no apparent modification in the strong staining intensity of the acidic peroxidase isoforms was observed from 0 to 12 h after infection. The optimum pH of this NADH oxidase activity was estimated at 5.

Cytochemical localization of peroxidase activity.

Electron microscope observations of sections made in infected resistant cotyledons revealed weak electron-dense areas

![Fig. 4](image-url)  
**Fig. 4.** Epipolarized fluorescence micrographs of the localization of H₂O₂. A, Four hours after infiltration of cotyledons of resistant plants of cotton cv. Reba B50 with the pathogen, intense fluorescence of walls (small arrows) is mainly observed in the spongy mesophyll (m) where the pathogen was inoculated (large arrow). Bar = 3 μm. B, After injection of water in resistant cotyledons, few cerium perhydroxide precipitates were seen in mesophyll (m) cell walls (arrows). Bar = 3 μm.

![Fig. 5](image-url)  
**Fig. 5.** A, Activity of NADH oxidation of crude enzymatic extracts of cotton cv. Reba B50 (solid circles, open circles) and Acala 44 (solid squares, open squares) inoculated with Xanthomonas campestris pv. malvacearum (Xcm) race 18. Open symbols correspond to NADH oxidation activity of water controls. Values are means ± SD of two separate experiments with five replicates each. B, Effect of 50 μM KCN, 5 mM salicylhydroxamic acid (SHAM), 2 mM MnCl₂, and 17 μM 2,4-dichlorophenol (2,4-DCP) on specific NADH oxidation activity. Crude enzymatic extracts from cotton cva. Reba B50 and Acala 44 were prepared 3 h post-inoculation with Xcm race 18 or after infiltration with water (see Materials and Methods). Compounds were added to the reaction buffer just before addition of enzymatic extracts. Values are means of two separate experiments with five replicates each; significant difference values are designated by different letters (P = 0.05).
in the Xcm-infiltrated zone 2 and 6 h after infection. By contrast, 3 h after infiltration, strong electron-dense areas were seen in the spongy mesophyll of the resistant cultivar. The middle lamella-bordering intercellular spaces in the spongy mesophyll, as well as wall areas, were strongly electron-dense (Fig. 7A). At this time, a weak reaction was observed in palisade mesophyll cells (Fig. 7B). Electron-dense areas were sometimes, but not always, seen at sites of attachment of bacteria into the surrounding middle lamella (Fig. 7C). After infiltration with deionized water, cotyledons of resistant plants showed a weak electron density in cell walls (Fig. 7D). In infected cotyledons of susceptible plants, a slight peroxidase activity was found in the tonoplast (Fig. 7E), but not in the middle lamella and the cell walls. Incubation of sections in medium without H$_2$O$_2$ did not induce any electron density (Fig. 7F).

**DISCUSSION**

An important feature of plant resistance responses toward invading pathogens is thought to be the rapid production of AOS, a phenomenon known as the "oxidative burst" (Baker and Orlandi 1995). There is evidence that AOS generators may closely resemble those involved in the respiratory burst in mammalian phagocytes (Medhy 1994), with similar features such as (i) involvement of a plasma membrane-bound NADPH oxidase activity (Desikan et al. 1996; Murphy and Auh 1996; Ogawa et al. 1997; Simon-Plas et al. 1997), (ii) existence of the rbohA gene homologue of the mammalian gp91phox (Groom et al. 1996; Keller et al. 1998), and (iii) immunologically related peptides (Levine et al. 1994; Dwyer et al. 1995; Kiefer et al. 1997). Several other enzymes have been proposed as the source of AOS production during plant pathogenesis, including lipoxygenases, oxalate-oxidase, xanthine-oxidase, amine-oxidases, and peroxidases (Vera-Estrella et al. 1992; Adam et al. 1995; Baker and Orlandi 1995; Allan and Fluhr 1997; Lamb and Dixon 1997). Bolwell et al. (1995) demonstrated that generation of H$_2$O$_2$ by peroxidases was not consistent with production of intermediate superoxide anions characteristic of the plasmamembrane NADPH oxidase. Although some experimental data indicate that several sources of AOS are potentially functional in a given plant cell (Allan and Fluhr 1997), it is reasonable to hypothesize that this could be a general feature. However, there is insufficient evidence to understand how "crosstalk" may operate between the plant and the pathogen that leads to the activation of a particular AOS production pathway. It cannot be excluded that different AOS sources could be sequentially or simultaneously elicited during a plant-pathogen interaction, as emphasized by Wojtaszek (1997).

In the present work, several lines of evidence demonstrate that superoxide anions and hydrogen peroxide were produced during the incompatible interaction between cotton cotyledons and Xcm, but not during the susceptible interactions. In response to inoculation of Reba B50 cotyledons with Xcm race 18, a threefold increase in extracellular cytochrome c reducing activity was observed relative to water controls at 3 h post-inoculation, whereas activity in Acala 44 cotyledons remained unchanged. Since the reducing activity in Reba B50 cotyledons was inhibited 90% by SOD, totally blocked by KCN, and insensitive to DPI, we postulated that the O$_2^-$ production was mediated by peroxidase activity.

Table 1. Comparison of NADH oxidation levels and total guaiacol activities between crude extracts and apoplastic fluids from the resistant cultivar

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Crude extracts</th>
<th>Apoplastic fluids</th>
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<tbody>
<tr>
<td></td>
<td>NADH oxidation</td>
<td>Guaiacol activity</td>
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<tr>
<td>Control$^c$</td>
<td>0.21 ± 0.05</td>
<td>36.3 ± 7.9</td>
</tr>
<tr>
<td>Infected cotyledons$^d$</td>
<td>0.5 ± 0.16</td>
<td>39.3 ± 7.15</td>
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$^a$Apoplastic fluids were extracted as described in Materials and Methods.

$^b$Activity expressed as nKat per mg$^{-1}$ of protein ± SD as mean of 10 replicates (five plants).

$^c$Guaiacol was used as the peroxidase substrate.

$^d$Cotyledons were infiltrated with water.

$^e$Cotyledons were infiltrated with *Xanthomonas campestris* pv. *malvacearum* (Xcm) race 18.

**Fig. 6.** Analytical isoelectric focusing (IEF) according to time (0 to 5 h; lanes 0 to 5) of samples from resistant cotton cv. Reba B50 infected with *Xanthomonas campestris* pv. *malvacearum* (Xcm) race 18. Cationic peroxidase isozymes (pI 9 to 9.5) were separated on an IEF gel (pH 3 to 11) followed by guaiacol staining. Gel pH is shown in right margin. Increase in activity is observed 3 h following inoculation (lane 3).
Fig. 7. A and B, Transmission electron micrographs of the localization of peroxidase activity, 3 h after infiltration of cotyledons of resistant cotton cv. Reba B50 plants with Xanthomonas campestris pv. malvacearum (Xcm) race 18. Strong electron-dense areas are seen in the middle lamella (arrows) of these spongy mesophyll cells (c), indicating presence of peroxidase activity (A). This activity is also located in plasma membrane (arrowheads). In the palisade parenchyma cells (p) (B), a weak peroxidase activity is seen in the plasma membrane (arrows) and the cell wall (ch = chloroplast). Bar = (A) 0.5 and (B) 1 µm. C, Electron-dense middle lamella showing peroxidase activity (arrows) surrounding site of attachment of bacteria close to cell wall (w). Bar = 0.3 µm. D, Control; Transmission electron micrograph of localization of peroxidase activity, 3 h after infiltration of cotyledons of resistant cotton cv. Reba B50 with dionized water. A peroxidase activity is seen in the tonoplast of a spongy mesophyll cell as indicated by the weak electron density (arrows). No strong electron density is seen in primary cell wall (cw) and middle lamella. Bar = 0.5 µm. E, Transmission electron micrograph of localization of peroxidase activity, 3 h after infiltration of cotyledons of susceptible cotton cv. Acala 44 with Xcm. Slight peroxidase activity is detected in vacuole membrane (arrows). No significant increase in electron density is visible in primary cell wall (cw) of this spongy mesophyll cell, ch = chloroplast. Bar = 0.5 µm. F, Control in which H2O2 was omitted in the reaction volume for assessing activity. Transmission electron micrograph of localization of peroxidase activity, 3 h after infiltration of cotyledons of resistant cv. Reba B 50 with Xcm. A weak electron density only is observed in cytoplasm (arrows) and middle lamella (ml), but not in cell wall (cw). Bar = 1 µm.
KCN. This suggests that the latter process depended on the digitonin-mediated activation of both an NADPH oxidase and a peroxidase, indicating that both cotton cultivars display the two enzymatic systems, each of them being able to produce extracellular O$_2^-$.

The ability of Acala 44 cotyledons to produce O$_2^-$ in response to digitonin was, however, significantly less than that of Reba B50. These observations are in accordance with previous work reporting that plant cultivars without major gene(s) for resistance to their pathogens also carry the system for the oxidative burst (Doke et al. 1996).

Following inoculation with Xcm race 18, cotyledons from Reba B50 plants were characterized by a sharp burst of O$_2^-$ between 2 and 3 h through the activation of a putative peroxidase, while the susceptible cultivar Acala 44 was unable to elicit any AOS production. Similarly, the other compatible interaction, Reba B50/Xcm race 20, did not result in the development of an oxidative burst. Whereas crude enzymatic extracts prepared from cotyledons of Acala 44 inoculated with Xcm race 18 or of Reba B50 inoculated with Xcm race 20 did not show any increase in the specific activity of NADH oxidase, those from cotyledons collected from Xcm race 18-infected Reba B50 plants showed a threefold higher activity, 3 h after infection. The time course of increase in NADH oxidase activity correlated that of AOS production. Activity was blocked by the peroxidase inhibitors KCN or SHAM but was strongly stimulated by Mn$^{2+}$ ions or 2,4-DCP, which were both described as powerful stimulating agents of peroxidase (Halliiwell 1978). Two striking points are worth emphasis: (i) in the presence of peroxidase stimulators the oxidation of NADH was statistically similar between Reba B50 samples from inoculated and control plants; and (ii) in the same conditions of treatment, these activities in Acala 44 were high and even higher than that after 3 h post-inoculation in Reba B50 samples. Since in the presence of these activating agents, (i) the specific NADH oxidase activity increased immediately after their addition in the reaction medium, and (ii) samples prepared from inoculated and control plants showed no difference in this activity, we proposed that elicitation of the putative AOS-producing peroxidase was regulated at the post-transcriptional level.

It is also suggested from our data that the NADH oxidase activity is localized extracellularly, since we showed that its specific activity was higher in AWF than in crude extracts. AWF also possessed a very high and constitutive guaiacol peroxidase activity. This was confirmed on IEF gels that showed strong and constant guaiacol staining in the acidic zone of the gel (not shown) and a low staining supported by cationic peroxidase isozymes (pI 9 to 9.5), the activity of which clearly increased at 3 h only in extracts from race 18-inoculated resistant cotyledons. Our cytochemical investigations with DAB as a peroxidase substrate also revealed that, 3 h after inoculation, the activity of some extracellular peroxidases was strongly elicited in plant cell walls and middle lamellae during the incompatible interaction. These observations further strengthen our biochemical data, being in accordance with the assumption of Bolwell et al. (1995) that AOS-generating peroxidases are wall bound. However, taking into account the high constitutive and extracellular guaiacol-peroxidase activity present, we must assume that DAB would be a better substrate for cationic peroxidases, and subsequently we must assess their activity at the ultrastructural level. Our preliminary investigations revealed an optimum pH of 5 for the NADH oxidase activity. Interestingly, Nair and Showalter (1996) have recently isolated in carrot roots a cell wall cationic peroxidase (pI > 9.3), the activity of which was induced by wounding. They showed that this wound-inducible peroxidase has an optimum pH at 4.9 and suggested that it could be regulated at post-transcriptional level. These observations are, however, different from the report by Bolwell et al. (1995) of two bean H$_2$O$_2$-generating peroxidases having a neutral and a slightly alkaline optimum pH. Moreover, as mentioned by these authors, the transient alkalinization that takes place in the apoplasm of elicited bean cells could favor the activity of a wall-bound cationic peroxidase, leading to H$_2$O$_2$ synthesis. Based on the assumption that O$_2^-$ production in cotton/Xcm incompatible interaction is generated by the activity of an extracellular cationic peroxidase having an acidic optimum pH, investigation of extracellular pH modifications in infected cotton mesophyll cells should be revealing. Purification of the cotton wall-bound cationic peroxidase isozymes will confirm in vitro their potent role in planta.

The present data strongly suggest that cotton cotyledons can generate extracellular O$_2^-$ through two distinct enzymes, a KCN-sensitive peroxidase with NADH oxidase activity and DPI-sensitive NADPH oxidase. In our experiments, the production of O$_2^-$ during the incompatible interaction was exclusively mediated through the activation of apoplastic peroxidase(s), the DPI-sensitive NADPH oxidase remaining silent. To our knowledge, this is the first time that peroxidase activity is reported to contribute to O$_2^-$ production in planta, despite the presence of an NADPH oxidase. Nevertheless, the presence of both types of enzyme has already been demonstrated in tobacco tissues (Allan and Fluhr 1997); additionally, it was recently shown with isolated plant cell walls from pea and cowpea that wall-bound peroxidase activity leads to the production of O$_2^-$ (Kiba et al. 1997). Accordingly, our data indicate that peroxidase O$_2^-$-generating activity can now be considered an alternative mechanism to the plasma membrane NADPH oxidase, as already suggested by Bestwick et al. (1997), Lamb and Dixon (1997), and Wojtaszek (1997).

Bolwell et al. (1995) have stated that peroxidase-dependent production of AOS in French bean cells infected with Colletotrichum lindemuthianum was neither inhibited by SOD nor measurable by the reduction of cytochrome c, concluding that H$_2$O$_2$ was the AOS produced in this system during the oxidative burst. We also demonstrated by epipolarized microscopical observations that H$_2$O$_2$ accumulated in the apoplast of the resistant cotyledon cells infected by Xcm race 18. H$_2$O$_2$ was visible at this site from 4 h and was still present at 6 h. Persistence of this diffusible AOS at the extracellular level during at least 2 h could be interpreted as resulting from synthesis still active at that time. Although H$_2$O$_2$ synthesis could partly result from dismutation of O$_2^-$, the production of which occurred between 2.5 and 3 h after inoculation, it is reasonable to propose an additional source for H$_2$O$_2$ in as much as O$_2^-$ was no longer produced after 4 h. Correlatively, NADH oxidase activity returned to its basal level at that time. In a non-host lettuce/Pseudomonas syringae system, Bestwick et al. (1997) hypothesized that H$_2$O$_2$ that was not rapidly metabolized plays an important role in the plasma membrane breakdown at sites of bacterial attachment. Although we have found that bacteria could be attached to cell walls or the middle la-
mella that displayed peroxidase activity, we were not able to
demonstrate in the cotton/Xcm system a close relationship
between sites of peroxidase activity and AOS production, and
a possible AOS toxicity toward the pathogen as reported by
Bestwick et al. (1997). H2O2 generated during the oxidative
burst has been suggested to be required for longer term de-
fense responses such as lignification (Apostol et al. 1989;
Levine et al. 1994). The link between AOS metabolism, cell
wall peroxidase activity, and lignification has been reported in
various incompatible systems (Milosevic and Shuarezko
1996; Ogawa et al. 1997; J. M. Chittor, J. E. Leach, and F. F.
White, unpublished), including Xanthomonas sp.-infected rice
(Young et al. 1995; Leach et al. 1996). In our model, although
peroxidase activity and H2O2 were detected in cell walls
within the necrotic lesions, no lignin or suberin was found at
these sites by histochemical methods (Dai et al. 1996).

AOS from the oxidative burst have been postulated to play a
central role in triggering HR cell death and defense genes in
challenged cells (Greenberg et al. 1994; Hammond-Kosak and
Jones 1996; Levine et al. 1994; Tenhaken et al. 1995; Dangl et
al. 1996; Mittler and Lam 1996). Extracellular generation of
O2- by cell wall peroxidases in cotton cotyledons during the
incompatible interaction with Xcm, despite the presence of a
DPI-sensitive NADPH oxidase, opens interesting avenues on
the role that AOS may play in elicitation of HR cell death and
plant resistance. Jabs et al. (1996) demonstrated that O2- was
necessary and sufficient to initiate lesions and cell death in a
mutant A. thaliana plant. Superoxide anions were also shown to
be able to trigger phytoalexin synthesis in parsley cells (Jabs et
al. 1997). In this respect, the place of AOS, including H2O2, in
cotton resistance is currently under investigation with emphasis
on local and systemic responses. In addition, the involvement of
extracellular peroxidase in the production of AOS during the
oxidative burst raises the question of the key role played by the
cell wall and the apoplasma in the plant responses to pathogens.
Accordingly, identification of the extracellular peroxidase sub-
strate(s) at that site is of crucial interest. Finally, it has been
shown that HR phenotype varies following gene-for-gene in-
teractions (Mansfield et al. 1997). Since cotton cells are able to
produce O2- by two different enzymes, it would be of critical
importance to know whether or not elicitation of the oxida-
tive burst via a particular route is pathogen-dependent and a
direct result of specific elicitor/receptor binding.

MATERIALS AND METHODS

Biological materials, inoculation, and treatment of plants.

Two cotton cultivars (G. hirsutum) were used in this study.
The susceptible Acala 44 cultivar possesses no known major
genes for resistance to Xcm (Hunter et al. 1968; De Feyter et
al. 1993). The Reba B50 cultivar (Allen x Stonewell 2B), similar to the 101-102B line, contains the B2B3 blight resis-
tance key genes, which confer immunity to all Xcm races, in-
Both cultivars were inoculated by infiltration of the bacterial
suspension (107 to 108 CFU/ml) as described by Dai et al.
(1996). So far, three interactions have been tested: one in-
compatible, Reba B50/Xcm race 18, and two compatible, Reba
B50/Xcm race 20 and Acala-44/Xcm race 18. Controls con-
isted of plants from each cultivar that were infiltrated with sterile
water.

In other experiments, cotyledonary leaves were also infil-
trated with digitonin solutions (80 µM in water containing
0.5% ethanol). Inoculated plants and those treated with digi-
tonin were assayed for their extracellular cytochrome c redu-
cing activities.

Assay of extracellular cytochrome c reducing activity
of cotyledon disks.

The O2- generating activity of cotyledon disks was assayed by
measuring spectrophotometrically the reduction of exoge-
nously supplied cytochrome c (Doke 1983). Inoculated plants
were assayed at different times after bacterial or water (control)
infiltration, whereas digitonin-treated ones were tested immediately. Ten cotyledon disks (each 1 cm in diam-
eter) were immersed by gently shaking in 20 ml of a reaction
mixture consisting of 20 µM bovine heart cytochrome c and
100 µM deferoxamine mesylate (DFO) in 0.05 M pH 7.8
phosphate-buffered saline (PBS). Traces of metal impurities
were previously removed by treatment of PBS with chelox-
100 (100 to 200 mesh) according to Sanders et al. (1994).
Absorbance of the sample was measured at regular intervals at
550 nm with a double-beam spectrophotometer (Uvikon 922;
Kontron, Marseille, France), against a reference cuvette con-
taining the reaction buffer (PBS + 20 µM cytochrome c + 100
µM DFO). The O2- mediated reduction of cytochrome c was
estimated by adding 1,000 U ml-1 of SOD in the reaction
buffer just before addition of cotyledon disks. The O2- reduc-
ction rates were determined by measuring the average slopes
of the curves (increase in absorbance at 550 nm during the
first 8 to 10 min) taking into account the inhibition scores with
SOD and molar absorbivities for ferricytochrome c (0.89 104
M-1 cm-1) and ferrocytochrome c (2.99 104 M-1 cm-1) at 550
nm. KCN (50 µM) or SHAM (5 mM) was added to the reac-
tion buffer before immersion of cotyledon disks to tentatively
evaluate the role of peroxidases as extracellular generators of
cytochrome c reducing compounds. Similarly, DPI (20 µM)
was used as inhibitor of an NADPH oxidase.

Preparation of crude extracts and AWF
from cotton cotyledons.

Cotyledons from infected plants and controls were har-
ested 0, 1, 2, 3, 4, 12, and 24 h after inoculation. They were
crushed and homogenized in 0.05 M pH 6 sodium acetate
buffer (2 ml per g of fresh weight) containing 25 mM β-
mercapto-ethanol, and 5% polyvinylpolypyrrolidone. After
centrifugation (15 min at 12,000 × g), the supernatant was
filtered on polyether-sulfone membrane (0.45 µm, Nalgéne;
Hereford, England). AWFs were prepared according to Polle
et al. (1994), where sodium buffer was used instead of MES
(morpholinoethanesulfonic acid) buffer.

Assay of NADH oxidase.

Enzymatic activities were determined on crude extracts or
on AWF, 3 h after inoculation or water infiltration. NADH
oxidation activity was measured by monitoring the decrease
in absorbance at 340 nm. The reaction mixture (1 ml final
volume) consisted of 50 mM pH 5 sodium acetate buffer and
0.15 mM NADH (Halliwell 1978). The reaction was initiated
by adding the enzymatic extract (50 to 200 µl) and was fol-
lowed from 0 to 15 min. Effect of SHAM, KCN, or the peroxi-
dase activators 2,4-DCP (17 µM) or MnCl2 (2 mM) was tested.
after addition in the reaction mixture; NADH oxidation activity was then immediately assessed. Activities were calculated with a molar extinction coefficient of 6,300 M⁻¹ cm⁻¹ for NADH. Protein concentration was determined according to Bradford (1976) with bovine serum albumin (BSA) as a standard.

IEF.

Crude extracts were separated under nondenaturing conditions, in 70 × 80 mm glass sheaths (Bio-Rad Laboratories, Hercules, CA), by IEF according to Robertson et al. (1987). The pH gradient of the gel ranged from 3 to 11. Ampholytes 3/10 and 9/11 (1/4, vol/vol) were used at a final concentration of 12% (vol/vol). The anode solution consisted of 20 mM acetic acid and the cathode solution of 25 mM NaOH. After migration, peroxidases were localized by incubating gels in a staining buffer consisting of 0.05 M pH 6 sodium phosphate buffer containing guaiacol 0.2% (wt/vol), 0.01% 3-amino-9-ethylcarbazol (wt/vol), and 0.03% (vol/vol) H₂O₂. The gel lane corresponding to the isoelectric point marker was cut off and stained with Coomassie blue R-250. In the absence of H₂O₂ no staining was observed. The experiment, carried out 1, 2, 3, 4, 5, and 6 h after Xcm or water infiltration, was performed five times, with three infected plants and three plants per controls at each time.

Peroxidase assay and protein determination.

Assays of peroxidase activities of the extracts were carried out with guaiacol as the hydrogen donor. Activities were estimated from increase in absorbance at 470 nm/min. Total activity was expressed in nKat per mg of proteins. Protein determination was performed by the method of Bradford (1976) BSA as a standard. Estimation of proteins and peroxidase activities was performed on cotyledon proteic extracts sampled 0, 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72 h after inoculation.

Ultrastructural localization of peroxidase activity.

Fragments of cotyledons were sampled 2, 3, and 6 h post-inoculation and fixed in 2.5% (vol/vol) glutaraldehyde in 0.05 M phosphate buffer (pH 6.8) for 1 h, washed in buffer for 30 min, and incubated at 37°C for 3 h in a solution of 3,3',diaminobenzidine tetrahydrochloride (DAB) consisting of 0.1% DAB in 10 ml of 0.05 M pH 7.6 Tris-HCl buffer and 0.1 ml of 1% (vol/vol) H₂O₂. Following incubation, all preparations were postfixed in 2% (wt/vol) OsO₄ for 1 h, washed in distilled water, dehydrated in graded ethanol series, and embedded in Epon 812 (TAAB, England) before being examined in an Elmiskop 100C (Jeol, Tokyo) operating at 80 kV. The peroxidase activity was detected as strongly electron-dense deposits. Control material was incubated in the medium (i) in the absence of H₂O₂ or (ii) containing 0.05 M 3-amino-1,2,4-triazole (AT) in order to inhibit catalase if present; if this last treatment fails to reduce staining, peroxidase is assumed to be involved.

Histochemical localization of hydrogen peroxide.

Small fragments were collected 2, 4, and 6 h post-inoculation from infected or healthy cotyledons of both cultivars. They were incubated 2 h in one of the following media: (i) 5 mM CeCl₃ in 0.05 M Tris-maleate buffer pH 5; (ii) CeCl₃ solution containing 0.02 M aminotriazole in order to inhibit catalase; or (iii) Tris-maleate buffer pH 5 alone (Czaninski et al. 1993). After being washed with buffer, samples were fixed, dehydrated, and embedded in Epon 812. Semi-thin sections (1.5 μm) were examined with a Leitz Diaplan microscope (Leitz, Paris) with epipolarized fluorescence light (Liu et al. 1995). Refractive particles of cerium perhydroxide deposits appeared fluorescent when observed with the polarization filter block.

Chemical and reagents.

2,4-DCP, 3-aminoc-9-ethylcarbazol, 3-amino-1,2,4-triazole, BSA, bovine heart cytochrome c, CeCl₃, digitonin, DFO, DPI, glutaraldehyde, NADH, SHAM, SOD, and thymol were purchased from Sigma (St. Louis, MO). KCN was purchased from Merck (Darmstadt, Germany). Chelex-100 resin and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were purchased from Bio-Rad. Amphyloses and protein test substances for PI determination were purchased from Serva-Boehringer (Ingelheim Bioproducts, Heidelberg, Germany). Epon 812 resin was purchased from J. Delville (St. Germain-en-Laye, France).

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LITERATURE CITED


Alston, I., Heinsteins, P. F., and Low, P. 1993. After being washed with buffer, samples were fixed, dehydrated, and embedded in Epon 812. Semi-thin sections (1.5 μm) were examined with a Leitz Diaplan microscope (Leitz, Paris) with epipolarized fluorescence light (Liu et al. 1995). Refractive particles of cerium perhydroxide deposits appeared fluorescent when observed with the polarization filter block.


