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. $O_2^{\cdot-}$ 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 $MnCl_2$, 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 peroxidase(s) and H_2O_2 accumulation were observed cytochemically, 3 and 4 h post inoculation, respectively. When digitonin, a $O_2^{\cdot-}$ elicitor, was infiltrated into cotyledons of resistant and susceptible cultivars, generation of $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ -generating systems and that cells undergoing the HR in response to an avirulent race of Xcm produce $O_2^{\cdot-}$ 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 Wojtaszek 1997; Lamb and Dixon 1997; Wojtaszek 1997). It has been postulated that highly reactive oxygen intermediates ($O_2^{\cdot-}$, H_2O_2 , $OH^{\cdot}$) 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 Sluzarenko 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 $O_2^{\cdot-}$ 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 mammalian 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

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(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 apoplast 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 $O_2^{\cdot-}$ 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 μ M 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

Fig. 1. Time course of extracellular cytochrome c-reducing activity of cotton cv. Reba B50 3 h after infiltration with water (A, solid circles), *Xanthomonas campestris* pv. *malvacearum* (Xcm) race 18 (B, solid circles), or Xcm race 20 (B, solid squares), or immediately after infiltration with 80 μ M digitonin (C, solid circles). Absorbance at 550 nm of samples was read against a reference cuvette containing 20 μ M cytochrome c + 100 μ M deferoxamine mesylate (DFO) in phosphate-buffered saline. Reducing activities were monitored in presence of 1,000 U/ml of superoxide dismutase (SOD) (open circles), 20 μ M diphenyleneiodonium chloride (DPI; open diamonds), or 50 μ M KCN (open triangles). Values are means $\pm$ SD of three separate experiments with five replicates each.

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 μ M), and insensitive to diphenyleneiodonium chloride (DPI) (20 μ M) (Fig. 1B). Higher concentrations of DPI (up to 100 μ M) also failed to inhibit AOS generation.

Infiltration of cotyledons of both cultivars with 80 μ M 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

Fig. 2. Time course of extracellular cytochrome c-reducing activity of cotton cv. Acala 44. Inoculations, treatments and number of replicates were identical to those in Figure 1.

Figure 3, the incompatible interaction (Reba B50/Xcm race 18) was characterized by a sharp peak of $O_2^{\cdot-}$ production occurring between 2.5 and 3 h after infection. At that time, production of $O_2^{\cdot-}$ was about 40 $\text{nmol g}^{-1} \text{min}^{-1}$. 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_2O_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

Fig. 3. Time course changes in $O_2^{\cdot-}$ production; values of inoculated plants were subtracted from the corresponding water infiltrated controls; (solid circles, Reba B50-Xcm race 18; solid squares, Reba B50-Xcm race 20, Acala 44-Xcm race 18).

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 μ M) 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 μ M), 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. 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 μ m. **B**, After injection of water in resistant cotyledons, few cerium perhydroxide precipitates were seen in mesophyll (m) cell walls (arrows). Bar = 3 μ m.

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 $\pm$ SD of two separate experiments with five replicates each. **B**, Effect of 50 μ M KCN, 5 mM salicylhydroxamic acid (SHAM), 2 mM MnCl₂, and 17 μ M 2,4-dichlorophenol (2,4-DCP) on specific NADH oxidation activity. Crude enzymatic extracts from cotton cvs. 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 injection 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_2O_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_2O_2 by peroxidases was not consistent with production of intermediate superoxide anions characteristic of the plasmalemma 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 gen-

eral 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 $O_2^{\cdot-}$ production was mediated by peroxidase activity.

Treatment with digitonin, a steroid glycoalkaloid known to elicit the production of $O_2^{\cdot-}$ in various plant species (Doke and Chain 1985), resulted in an immediate increase in the extracellular reducing activity in both cultivars that was inhibited to about 60% by addition of SOD. This probably reflected that digitonin not only elicited the production of $O_2^{\cdot-}$ but also triggered an oxygen-independent process able to reduce the cytochrome c. In both cultivars, a part of the digitonin-induced activity was inhibited by DPI, suggesting that digitonin was able, as earlier described by Doke and Miura (1995), to stimulate production of $O_2^{\cdot-}$ through elicitation of an NADPH oxidase activity. In addition, KCN was shown to inhibit strongly the digitonin-stimulated reducing activity in both cultivars, suggesting that part of KCN inhibition could be explained by its action on a putative digitonin-elicited peroxidase and also by its effect on the oxygen-independent process. From our data, it appears that in cotton cotyledons, digitonin elicited a complex process leading to the extracellular reduction of cytochrome c. At least one component of this process was not dependent on oxygen (insensitive to the action of SOD), and was inhibited by KCN, while the oxygen-dependent component was partially inhibited by DPI and by

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

Treatments	Crude extracts		Apoplastic fluids ^a	
	NADH oxidation ^b	Guaiacol activity ^{b,c}	NADH oxidation ^b	Guaiacol activity ^{b,c}
Control ^d	0.21 ± 0.05	36.3 ± 7.9	1.14 ± 0.58	257 ± 65.4
Infected cotyledons ^e	0.5 ± 0.16	39.3 ± 7.15	2.67 ± 0.19	255 ± 55.6

^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 μ m. **C,** Electron-dense middle lamella showing peroxidase activity (arrows) surrounding site of attachment of bacteria close to cell wall (w). Bar = 0.3 μ m. **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 μ m. **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 μ m. **F,** Control in which H_2O_2 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 μ m.

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 $O_2^{\cdot-}$. The ability of Acala 44 cotyledons to produce $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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 (Halliwell 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_2O_2 -generating peroxidases having a neutral and a slightly alkaline optimum pH. Moreover, as mentioned by these authors, the transient alkalization that takes place in the apoplast of elicited bean cells could favor the activity of a wall-bound cationic peroxidase, leading to H_2O_2 synthesis. Based on the assumption that $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ through two distinct enzymes, a KCN-sensitive peroxidase with NADH oxidase activity and DPI-sensitive NADPH oxidase. In our experiments, the production of $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ (Kiba et al. 1997). Accordingly, our data indicate that peroxidase $O_2^{\cdot-}$ -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_2O_2 was the AOS produced in this system during the oxidative burst. We also demonstrated by epipolarized microscopical observations that H_2O_2 accumulated in the apoplast of the resistant cotyledon cells infected by Xcm race 18. H_2O_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_2O_2 synthesis could partly result from dismutation of $O_2^{\cdot-}$, the production of which occurred between 2.5 and 3 h after inoculation, it is reasonable to propose an additional source for H_2O_2 , in as much as $O_2^{\cdot-}$ 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_2O_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). H_2O_2 generated during the oxidative burst has been suggested to be required for longer term defense 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 Sluzarenko 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 H_2O_2 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; Dangel et al. 1996; Mittler and Lam 1996). Extracellular generation of O_2^- 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 O_2^- 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 H_2O_2 , 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 apoplast in the plant responses to pathogens. Accordingly, identification of the extracellular peroxidase substrate(s) at that site is of crucial interest. Finally, it has been shown that HR phenotype varies following gene-for-gene interactions (Mansfield et al. 1997). Since cotton cells are able to produce O_2^- by two different enzymes, it would be of critical importance to know whether or not elicitation of the oxidative 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 $\times$ Stoneville 2B), similar to the 101-102B line, contains the B2B3 blight resistance key genes, which confer immunity to all Xcm races, including race 18, except race 20 (Hillocks 1992; Innes 1983). Both cultivars were inoculated by infiltration of the bacterial suspension (10^7 to 10^8 CFU/ml) as described by Dai et al. (1996). So far, three interactions have been tested: one incompatible, Reba B50/Xcm race 18, and two compatible, Reba B50/Xcm race 20 and Acala-44/Xcm race 18. Controls consisted of plants from each cultivar that were infiltrated with sterile water.

In other experiments, cotyledonary leaves were also infiltrated with digitonin solutions (80 μM in water containing 0.5% ethanol). Inoculated plants and those treated with digitonin were assayed for their extracellular cytochrome c reducing activities.

Assay of extracellular cytochrome c reducing activity of cotyledon disks.

The O_2^- generating activity of cotyledon disks was assayed by measuring spectrophotometrically the reduction of exogenously 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 diameter) 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 chelex-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 containing the reaction buffer (PBS + 20 μM cytochrome c + 100 μM DFO). The O_2^- -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 O_2^- production rates were determined by measuring the initial 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 absorptivities for ferricytochrome c ($0.89 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and ferrocyanochrome c ($2.99 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) at 550 nm. KCN (50 μM) or SHAM (5 mM) was added to the reaction 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 harvested 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% polyvinylpyrrolidone. After centrifugation (15 min at $12,000 \times g$), the supernatant was filtered on polyether-sulfone membrane (0.45 μm , Nalgene; 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 followed from 0 to 15 min. Effect of SHAM, KCN, or the peroxidase activators 2,4-DCP (17 μM) or MnCl_2 (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 $6,300 \text{ M}^{-1} \text{ cm}^{-1}$ 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 $70 \times 80 \text{ mm}$ 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-ethylcarbazole (wt/vol), and 0.03% (vol/vol) H_2O_2 . The gel lane corresponding to the isoelectric point marker was cut off and stained with Coomassie blue R-250. In the absence of H_2O_2 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 nKa 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 5 mg of DAB in 10 ml of 0.05 M pH 7.6 Tris-HCl buffer and 0.1 ml of 1% (vol/vol) H_2O_2 . Following incubation, all preparations were postfixed in 2% (wt/vol) OsO_4 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 H_2O_2 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 CeCl_3 in 0.05 M Tris-maleate buffer pH 5; (ii) CeCl_3 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 ($1.5 \mu\text{m}$) 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-amino-9-ethylcarbazole, 3-amino-1,2,4-triazole, BSA, bovine heart cytochrome c, CeCl_3 , digitonin, DFO, DPI, glutaraldehyde, NADH, SHAM, SOD, and thymol were purchased from Sigma (St. Louis, MO). KCN was purchased from Merck (Damstadt, Germany). Chelex-100 resin and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were purchased from Bio-Rad. Ampholytes and protein test substances for pI determination were purchased from Serva-Bioehringer (Ingelheim Bioproducts, Heidelberg, Germany). Epon 812 resin was purchased from J. Delville (St. Germain-en-Laye, France).

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LITERATURE CITED

- Adam, A. L., Bestwick, C. S., Barna, B., and Mansfield, J. W. 1995. Enzymes regulating the accumulation of active oxygen species during the hypersensitive reaction of bean to *Pseudomonas syringae* pv. *phaseolicola*. *Planta* 197:240-249.
- Allan, A. C., and Fluhr, R. 1997. Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell* 9:1559-1572.
- Anderson, M. D., Chen, Z., and Klessig, D. F. 1998. Possible involvement of lipid peroxidation in salicylic acid-mediated induction of PR-1 gene expression. *Phytochemistry* 47:555-566.
- Apostol, I., Heinsteins, P. F., and Low, P. S. 1989. Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. *Plant Physiol.* 90:109-116.
- Auh, C. K., and Murphy, T. M. 1995. Plasma membrane redox enzyme is involved in the synthesis of O_2^- and H_2O_2 by *Phytophthora* elicitor-stimulated rose cells. *Plant Physiol.* 107:1241-1247.
- Baker, C. J., and Orlandi, E. W. 1995. Active oxygen in plant pathogenesis. *Annu. Rev. Phytopathol.* 33:299-321.
- Bestwick, C. S., Brown, I. R., Bennet, M. H. R., and Mansfield, J. W. 1997. Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *phaseolicola*. *Plant Cell* 9:209-211.
- Bolwell, G. P., Butt, V. S., Davies, D. I., and Zimmerlin, A. 1995. The origin of the oxidative burst in plants. *Free Radical Res.* 11:517-532.
- Bolwell, G. P., and Wojtaszek, P. 1997. Mechanisms for the generation of reactive oxygen species in plant defence - a broad perspective. *Physiol. Mol. Plant Pathol.* 51:347-366.
- Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Czaninski, Y., Sachot, R. M., and Catesson, A. M. 1993. Cytochemical localization of hydrogen peroxide in lignifying cell walls. *Ann. Bot.* 72:547-550.
- Dai, G. H., Nicole, M., Martinez, C., Bresson, E., Daniel, J. F., Andary, C., and Geiger, J. P. 1996. Flavonoids accumulate in cell walls, middle lamellae and callose-rich papillae during an incompatible interaction between *Xanthomonas campestris* pv. *malvacearum* race 18 and cotton. *Physiol. Mol. Plant Pathol.* 49:285-306.
- Dangl, J. L., Dietrich, R. A., and Richberg, M. H. 1996. Death don't have no mercy: Cell death programs in plant-microbe interactions.

- Plant Cell 8:1793-1807.
- De Feyter, R., Yang, Y., and Gabriel, D. W. 1993. Gene-for-gene interactions between cotton *R* genes and *Xanthomonas campestris* pv. *malvacearum* *avr* genes. Mol. Plant-Microbe Interact. 6:225-237.
- Desikan, R., Wood, L. G., Coffey, M. J., and Neill, S. J. 1996. Generation of active oxygen in elicited cells of *Arabidopsis thaliana* is mediated by a NADPH oxidase-like enzyme. FEBS Lett. 382:231-217.
- Doke, N. 1983. Generation of superoxide anion by potato tuber protoplasts during the hypersensitive response to hyphal wall components of *Phytophthora infestans* and specific inhibition of the reaction by suppressors of hypersensitivity. Physiol. Plant Pathol. 23:359-367.
- Doke, N., and Chain, H. B. 1985. Activation of superoxide generation and enhancement of resistance against compatible races of *Phytophthora infestans* in potato plants treated with digitonin. Physiol. Plant Pathol. 27:323-334.
- Doke, N., and Miura, Y. 1995. *In vitro* activation of NADPH-dependant $O_2^{\cdot-}$ generating system in a plasma membrane-rich fraction of potato tuber tissues by treatment with an elicitor from *Phytophthora infestans* or with digitonin. Physiol. Mol. Plant Pathol. 46:17-28.
- Doke, N., Miura, Y., Sanchez, L. M., Park, H., Noritake, T., Yoshiora, H., and Kawakita, K. 1996. The oxidative burst protects plants against pathogen attack: Mechanism and role as an emergency signal for plant bio-defence - a review. Gene 179:45-51.
- Dwyer, S. C., Legendre, L., Low, P. S., and Leto, T. L. 1995. Plant and human neutrophil oxidative burst complexes contain immunological related proteins. Biochem. Biophys. Acta 1289:231-237.
- Essenberg, M., and Pierce, M. 1994. Sesquiterpenoid phytoalexins synthesized in cotton leaves and cotyledons during the hypersensitive response to *Xanthomonas campestris* pv. *malvacearum*. Pages 183-198 in: Handbook of Phytoalexin Metabolism and Action. M. Daniel and R. P. Purkayastha, eds. Marcel Dekker, Calcutta, India.
- Greenberg, J. T., Guo, A., Klessig, D. F., and Ausubel, F. M. 1994. Programmed cell death in plants: A pathogen-triggered response activated coordinately with multiple defense functions. Cell 77:551-563.
- Groom, Q. J., Torres, M. A., Fordham-Skelton, P., Hammond-Kosak, K. E., Robinson, N. J., and Jones, J. D. G. 1996. *rhoHA*, a rice homologue of the mammalian *gp91phox* respiratory burst oxidase gene. Plant J. 10:515-522.
- Halliwell, B. 1978. Lignin synthesis: The generation of hydrogen peroxide and superoxide by horseradish peroxidase and its stimulation by manganese (II) and phenols. Planta 140:81-88.
- Hammond-Kosak, K. E., and Jones, J. D. G. 1996. Resistance gene-dependant plant defense responses. Plant Cell 8:1773-1791.
- Hillocks, R. J. 1992. Bacterial blight. Pages 39-86 in: Cotton Diseases. R. J. Hillocks, ed. CAB Int., Oxon, UK.
- Hunter, R. E., Brinkerhoff, L. A., and Bird, L. S. 1968. The development of a set of upland cotton lines for differentiating races of *Xanthomonas malvacearum*. Phytopathology 58:830-832.
- Innes, N. L. 1983. Bacterial blight of cotton. Biol. Rev. 58:157-176.
- Jabs, T., Dietrich, R. A., and Dangel, J. L. 1996. Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. Science 273:1853-1856.
- Jabs, T., Tschöpe, M., Colling, C., Hahlbrock, K., and Scheel, D. 1997. Elicitor-stimulated ion fluxes and $O_2^{\cdot-}$ from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. Proc. Nat. Acad. Sci. USA 94:4800-4805.
- Keller, T., Damude, H. G., Werner, D., Doerner, P., Dixon, R. A., and Lamb, C. 1998. A plant homolog of the neutrophil NADPH-oxidase gp 91^{phox} subunit gene encodes a plasma membrane protein with Ca^{++} binding motifs. Plant Cell 10:255-266.
- Kiba, A., Miyake, C., Toyoda, K., Ichinose, Y., Yamada, T., and Shiraishi, T. 1997. Superoxide generation in extracts from isolated plant cell walls is regulated by fungal signal molecules. Phytopathology 87:846-852.
- Kiefer, F., Simon-Plas, F., Maume, B., and Blein, J. P. 1997. Tobacco cells contain a protein, immunologically related to the neutrophil small G protein Rac2, and is involved in elicitor-induced oxidative burst. FEBS Lett. 403:149-153.
- Lamb, C., and Dixon, R. A. 1997. The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48:251-275.
- Leach, J. E., Young, S. A., Chittoor, J. M., Zhu, W., and White, F. F. 1996. Induction of defense responses in rice. Pages 115-128 in: Molecular Aspects of Pathogenicity and Resistance: Requirement for Signal Transduction. D. Mills, H. Kunoh, N. T. Keen, and S. Mayama, eds. American Phytopathological Society, St. Paul, MN.
- Levine, A., Tehnaken, R., Dixon, R., and Lamb, C. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 6:427-437.
- Lindner, W. A., Hoffmann, C., and Grisebach, H. 1988. Rapid elicitor-induced chemiluminescence in soybean cell suspension cultures. Phytochemistry 27:2501-2503.
- Liu, L., Eriksson, K. E., and Dean, J. F. D. 1995. Localization of hydrogen peroxide production in *Pisum sativum* L. using epi-polarized microscopy to follow cerium perhydroxide deposition. Plant Physiol. 107: 501-506.
- Low, P. S., and Merida, J. R. 1996. The oxidative burst in plant defense: Function and signal transduction. Physiol. Plant. 96:533-542.
- Mansfield, J. W., Bennet, M. H. R., Bestwick, C. S., and Woods-Tör, A. 1997. Phenotypic expression of gene-for-gene interaction involving fungal and bacterial pathogens: Variation from recognition to response. Pages 265-291 in: The Gene-for-Gene Relationship in Plant-Parasite Interactions. I. R. Crute, E. B. Holub, and J. J. Burdon, eds. CAB Int., Oxon, UK.
- Martinez, C., Geiger, J. P., Bresson, E., Daniel, J. F., Dai, G. H., Andary, C., and Nicole, M. 1996. Isoperoxidases are associated with resistance of cotton to *Xanthomonas campestris* pv. *malvacearum*. race 18. Pages 327-332 in: Plant Peroxidases: Biochemistry and Physiology. O. Obinger, U. Burner, R. Ebermann, C. Penel, and C. Greppin, eds. University of Agriculture, Vienna, Austria.
- Medhy, M. C. 1994. Active oxygen species in plant defense against pathogens. Plant Physiol. 105:467-472.
- Milosevic, N., and Sluzarenko, A. J. 1996. Active oxygen metabolism and lignification in the hypersensitive response in bean. Physiol. Mol. Plant Pathol. 49:143-158.
- Mittler, R., and Lam, E. 1996. Sacrifice in the face of foes: Pathogen-induced programmed cell death in plants. Trends Microbiol. 4:10-15.
- Murphy, T. M., and Auh, C. K. 1996. The superoxide synthases of plasma membrane preparations from cultured rose cells. Plant Physiol. 110:621-629.
- Nair, A. R., and Showalter, A. M. 1996. Purification and characterization of a wound-inducible cell wall peroxidase from carrot roots. Biochem. Biophys. Res. Commun. 226:254-260.
- Ogawa, K., Kanematsu, S., and Asada, K. 1997. Generation of superoxide anion and localization of CuZn-superoxide dismutase in the vascular tissue of spinach hypocotyls: Their association with lignification. Plant Cell Physiol. 38:1118-1126.
- Polle, A., Otter, T., and Seifert, F. 1994. Apoplastic peroxidases and lignification in needles of Norway spruce (*Picea abies* L.). Plant Physiol. 106:53-60.
- Robertson, E. F., Dannelly, H. K., Malloy, P. J., and Reeves, H. C. 1987. Rapid isoelectric focusing in a vertical polyacrylamide minigel system. Anal. Biochem. 167:290-294.
- Sanders, S. P., Harrison, S. J., Kuppusamy, H. P., Sylvester, J. T., and Zweier, J. L. 1994. A comparative study of EPR spin trapping and cytochrome C reduction techniques for the measurement of superoxide anions. Free Radical Biol. Med. 16: 753-761.
- Segal, A. W., and Abo, A. 1993. The biochemical basis of the NADPH oxidase of phagocytes. Trends Biochem. Sci. 18:43-47.
- Simon-Plas, F., Restérucci, C., Milat, M. L. F., Humbert, C., Montillet, J. L., and Blein, J. P. 1997. AOS production in tobacco cells elicited by cryptogin. Plant Cell Environ. 20:1573-1579.
- Sutherland, M. W. 1991. The generation of oxygen radicals during host plant responses to infection. Physiol. Mol. Plant Pathol. 39:79-93.
- Tenhaken, R., Levine, A., Brisson, L., Dixon, R., and Lamb, C. 1995. Function of the oxidative burst in hypersensitive disease resistance. Proc. Natl. Acad. Sci. USA 92:4158-4163.
- Vera-Estrella, R., Blumvald, E., and Higgins, V. 1992. Effect of specific elicitors of *Cladosporium fulvum* on tomato suspension cells. Evidence for the involvement of active oxygen species. Plant Physiol. 99:1028-1215.
- Wojtaszek, P. 1997. Oxidative burst: An early plant response to pathogen infection. Biochem. J. 322:681-692.
- Young, S. A., Guo, A., Guikema, J. A., White, F. F., and Leach, J. E. 1995. Rice cationic peroxidase accumulates in xylem vessels during incompatible interaction with *Xanthomonas oryzae* pv. *oryzae*. Plant Physiol. 107:1333-1341.