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ISOPEROXIDASES ARE ASSOCIATED WITH RESISTANCE OF COTTON TO *Xanthomonas campestris* pv. *malvacearum*

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ABSTRACT

Resistant (Reba B50) and susceptible (Acala 44) cotton plants were investigated for intratissular growth of bacterial populations and peroxidase (POx) activity, after infection of cotyledons with races 18 or 20 from *Xanthomonas (Axonopodis) campestris pv. malvacearum.* Considerable multiplication of the bacterial population was noticed in the compatible interaction (Acala 44 / Xcm race 18); it was much lower during the incompatible interaction when race 18 was infiltrated into cotyledons of Reba B50. An intermediate level of bacterial growth was obtained when Reba B50 was infiltrated with race known to overcome resistance of this line. High increase in POx activity occurred into the infected cotyledons during incompatible interaction, while the increase was much lower when the interactions were compatible. On leaves, a similar and significant difference in enzyme activity was also observed indicating that the „peroxidase response" was systemically induced in entire resistant plants. Five isoperoxidases were evidenced by IEF in both lines, whether they were infected or not. But only two of them accounted for the increase in activity in infected resitant cotyledons. Microscopy revealed that POx activity, detected at the infection sites two hours after infiltration of the resistant line was mainly located in cell walls and the middle lamella bordering intercellular spaces. Our data indicate that bacterial infection of cotton plants enhanced the activity of two of the preexistent isoperoxidases in resistant plants and suggest that stimulation of POx activity is associated with resistance mechanisms.

INTRODUCTION

Bacterial blight caused by *Xanthomonas* (present name: *Axonopodis*) *campestris pv. malvacearum* (Xcm) is one of the most important disease of cotton recorded in almost every cotton-growing countries of the world (6). On infected cotyledons and leaves of susceptible plant cotton (*Gossypium hirsutum* L.), Xcm produced small dark green water-soaked areas, usually expanding along veins. The disease then spreads upwards leaves, bolls, and stems, finally leading to plant death. Resistance of cotton to Xcm depends on a number of genetical,

anatomical and chemical characteristics that occur both constitutively and as a active defense mechanisms. A high degree of correlation in the expression of resistance of the plants has been dermonstrated for a wide range of genotypes. In *Gossypium* species, the resistance results from 22 so-called B-genes (6). The Reba B50 contains the B_2-B_3 gene combination which induces an hypersensitive reaction (HR) of the plants when infected with Xcm race 18, while the Acala 44 line, with no major genes for resistance, is susceptible to blight.

Work on cotton resistance to bacterial blight has been mainly focused on terpenoid molecules, the major antimicrobial metabolites produced by cotton. Some were idedentified from leaves and cotyledons of inoculated resistant plants, at a sufficient level to inhibit *in vivo* growth of the pathogen at infection sites (3,4,5). *De novo* synthesis of flavonoids was also histochemically evidenced close to the infection sites during the incompatible interaction (1). The associated ultrastructural observation of such phenol producing-cells indicated that these phenolics accumulated in callose-rich papillae and host cell walls.

The role of plant POx during interactions between plant and Xanthomonas is poorly documented, although the induced-HR by this pathogen was more widely studied (12). Venere et al. (13) showed that a POx activity increased within resistant cotton cotyledons syringe-injected with 10^8 viable cells of Xcm. More recently, an increased activity of a cationic POx was associated with an incompatible relation between rice and the vascular bacteria *X. oryzae pv. oryzae* (10). This POx, immunolocalized in xylem vessels was suspected to be involved in ligninsynthesis (14).

In the present study, we investigated POx isoenzymes involved in interactions between two cotton lines (Reba B5 and Acala 44) and two races of Xcm (race 18 and race :20). We evidenced that the activity of two anionic POx increased early during the incompatible interaction, while they did not, or weakly, in the infected susceptible plants. They were also shown to be first restricted to infection sites where the HR developed, and then systemically induced in the whole infected plants.

MATERIALS and METHODS

Plant material, bacterial strains and inoculation method. The two cotton lines used in this study were the susceptible Acala 44 and the resistant Reba B50. The bacterial pathogen, *Xanthomonas campestris pv. malvacearum* race 18, was isolated in cotton fields in Burkina Faso. Cotyledonnary leaves of 10 days-old cotton plants grown in a greenhouse (30°C ± 1°C, 80% relative humidity) were inoculated by infiltration of a 10^8 colony-forming units (c.f.u) / mL bacterial suspension with an hypodermic needleless syringe. Controls consisted in plants either infiltrated with sterile water, or not infiltrated at all. Plants were also infected with Xcm race 20, to which no resistance was exhibited by both lines Bacterial population countings and POx assays were performed on ten replicates from which mean values and the corresponding standard errors were calculated.

Estimation of the bacterial population throughout the infection process. Bacteria were recovered by triturating in sterile deionized water cotyledon fragments collected close to the inoculation sites. After serial dilutions, 100 µL of each suspension was plated on nutrient broth (YPDA); the developping colonies were numbered 2 days later The bacterial populations were expressed as c.f.u. / cm^2 of leaves. Viable counts were determined by serial dilutions on YPDA plates.

Peroxidase assay. Soluble POx of cotyledon or leaf crude extracts [2 mL sodium acetate buffer (0.05M, pH 6; 25mM mercaptoethanol; 5% (w/v) Polyclar AT) per g of leaves

(f. w.)] was assayed in a 1 mL reaction medium (0.05 M / pH 6 sodium phosphate buffer containing 0.2% guaiacol and 0.03% H_2O_2). The activity, determined by measuring the slope from the linear increase in absorbance at 470 nm, was expressed as ΔO_{470}/min/mg tissues.

Isoelectric focusing (IEF). IEF was basically monitored according to Robertson et al. (11) on vertical gels, the pH gradient of which ranged from 2 to 10. After migration, POx isoforms were stained in a reaction medium containing 0.2% guaiacol, 0.03% 3-amino-9-ethylcarbazole and 0.03% H_2O_2 in 0.05M sodium phosphate buffer pH 6.

Histochemical localization of peroxidase activity. Peroxidase activity was detected in fresh cotyledon sections using 3,3',5,5'-tetramethylbenzidine (7) or guaiacol (8) as substrate. After incubation, the sections were rinsed in distilled water and observed with a light microscope. Control rnaterial was incubated in the medium (a) in the absence of H_2O_2 or (b) containing 0.05 M 3-amino-1,2,4-triazole (AT) in order to inhibit catalase if present.

Ultrastructural localization of peroxidase activity. Cytochemical localization of POx activity at the ultrastructural level was performed using 3,3'-diaminobenzedine tetrahydrochloride as the substrate (9). Following incubation, samples were fixed, dehydrated and embbeded in Epon 812. Sections were examinedin a Jeol 100X electron microscope operating at 80Kv.

RESULTS

Symptoms. Necrotic lesions that corresponded to the hypersensitive reaction were visually detected on cotyledons of the resistant line Reba B50, 8 to 10 h after inoculation with Xcm race 18. In the susceptible line Acala 44 water-soaked lesions developed along veins of cotyledons 8 days after infection, and spread towards the stem and leaves causing the plant death 10 to 15 days after inoculation.
When Reba B50 was inoculated with Race 20 of Xcm known to overcome cotton resistance, symptoms similar to that observed on the susceptible line were seen. No necrotic lesions were differentiated on such infected plants.

Evolution of the bacterial population within infected cotyledons. During the three days following inoculation, the bacterial multiplication was nearly similar in both incompatible (Reba B50 / Xcm race 18) and compatible (Acala 44 / Xcm race 18, Reba B50 / Xcm race 20) interactions (Table 1). Nevertheless, whereas the race 18 population levelled at that time into the cotyledons of the cv Reba B50, the same pathogen was continuing to multiply into the susceptible Acala 44 until plant death (l0 days after inoculation in this experiment). No differences were observed in the time course of the infection process between bacterial populations of race 18 and race 20 when inoculated on the susceptible and the resistant line, respectively. These results are in agreement with symptoms exhibited by the cotyledons of the different lines when inoculated by Xcm either race 18 or race 20, and confirm the above classification as compatible or incompatible host/pathogen couples.

Effect of bacterial infection on peroxidase activity. Preliminary experiments showed (a) that the recovery in activity was similar - and the recovery in POx isoforms was identical (as shown by IEF separation) - whether extraction buffer contained 0.5M NaCl or not, and (b) that no significant evolution in the POx activity occurred in control (not inoculated or water-infiltrated cotyledons).

Table 1 : Time course of peroxidase activity in leaves and cotyledons and bacterial population during infection

	TIME AFTER INOCULATION (days)	SYMPTOMS	PEROXIDASE ACTIVITY (U/g)* cotyledons	PEROXIDASE ACTIVITY (U/g)* leaves	Cfu/cm2 cotyledon* X 10^6
Reba B50 / Xcm race 18	0	HR t=8h	748 +/- 91	129 +/- 38	2.2 +/- 0.5
	1	HR	1631 +/- 82	135 +/- 23	6.6 +/- 0.8
	2		4048 +/- 1012	354 +/- 12	70 +/- 10
	3		6890 +/- 370	500 +/- 67	150 +/- 12
	4		7120 +/- 204	700 +/- 64	200 +/- 14
	7		8004 +/- 144	1110 +/- 104	250 +/- 28
	8		9728 +/- 130	1360 +/- 137	239 +/- 31
	9		6100 +/- 132	1800 +/- 116	150 +/- 17
	10	Dead of cotyledons	°	1778 +/- 104	°
Reba B50 / Xcm race 20	0	°	735 +/- 128	129 +/- 31	2 +/- 0.5
	1	°	1402 +/- 79.2	110 +/- 18	7 +/- 0.7
	2	°	1300 +/- 175	119 +/- 47	90 +/- 9
	3	°	2300 +/- 280	420 +/- 52	415 +/- 35
	4	Water soaked lesions	2450 +/- 210	602 +/- 39	3000 +/- 25
	7	"	2616 +/- 116	756 +/- 64	3700 +/- 24
	8	"	2636 +/- 80	1056 +/- 104	3980 +/- 15
	9	"	2024 +/- 76	804 +/- 50	3500 +/- 37
	10	Dead of cotyledons	°	708 +/- 51	°
Acala 44 / Xcm race 18	0	°	453 +/- 110	44 +/- 5	2 +/- 0.06
	1	°	132 +/- 32	29 +/- 13	8.2 +/- 0.5
	2	°	1083 +/- 140	90 +/- 38	99 +/- 10
	3	°	1114 +/- 72	91 +/- 23	510 +/- 35
	4	Water soaked lesions	1076 +/- 65	101 +/- 24	3700 +/- 52
	7	"	832 +/- 46	80 +/- 14	4830 +/- 32
	8	"	804 +/- 28	51 +/- 10	5210 +/- 56
	9	"	804 +/- 24	55 +/- 5	4890 +/- 28
	10	Dead of cotyledons	°	43 +/- 16	°

* Mean of ten replicates and standard error

When infected with Xcm race 18, the POx activity increased considerably in the resistant Reba B50 cotyledons during nearly the whole time course (0 to 8 days) of the infection process (Table 1). The maximum (13 fold increase) was reached 8 days after inoculation. When infected with race 20, the increase was lower but also significant (3.6 fold); the maximum was reached at 7-8 days. Finally, when Acala 44 cotyledons were infected with race 18, a weak increase was noticed (2.3 fold) which levelled 2-3 days after inoculation and then decreased slowly. In paralell (same plants and same time-course) the POx activity was measured into leaves (rank 1, just above the cotyledons), indicating a very similar evolution. The main difference as compared with the cotyledons consists in (a) a much lower POx activity (about 5 fold in Reba B50 and 10 fold in Acala leaves), and (b) a delay in the „peroxidase reaction" that started about 24 to 48 h later.

Characterization of peroxidase isoenzymes by IEF. Four major soluble acidic POx isoenzymes were evidenced after IEF on polyacrylamide gel. They all were present in both non-inoculated and inoculated plants of the resistant as well as the susceptible lines. Their pI values were 3.6 and 4.2, 4.7 and 5.6, respectively. A very weak band was also be observe located at the 5.2 pH zone of the gel. In the Xcm race 18 infected resistant plants, the activity of the 3.6 and 4.2 pI isoforms (and those only) increased considerably during the infection, accounting for the increase in total peroxidase activity shown in the corresponding cotyledon extract. In the infected susceptible plants no similar differential stimulation was observed. When leaf extracts were subject to IEF separation, comparable isoperoxidase patterns were observed, but specific increase in the activity of the 3.6 and 4.2 pI isoforms was not detected, even in leaves from resistant plants.

Histochemical detection of peroxidase activity. Using guaiacol as substrate, POx activity was detected in the spongy mesophyll cells of resistant cotyledons, at the infection sites, as early as 4h after infiltration of resistant cotyledons. In sections from infected cotyledons of the susceptible cv, no significant reaction was found. No staining was observed in sections when the incubation medium failed in hydrogen peroxide.

Cytochemical localization of peroxidase activity at the ultrastructural level. Electron microscopy on sections of infected resistant cotyledons revealed that activity was detectable 2h after infection mainly in spongy mesophyll cells, and weakly in palissade mesophyll cells. The middle lamella bordering intercellular spaces was also strongly electron-dense. In infected cotyledons of susceptible plants, a weak POx activity was observed in the tonoplast, but not in the middle lamella and the cell walls. No electron-dense areas were shown when sections were incubated in hydrogen peroxide-less medium.

DISCUSSION

In a previous work, Venere (13) has shown that POx activity increased in *Xanthomonas*-infected cotyledons of resistant plants. An associated decline in bacterial number recovered from the inoculated tissues several hours after inoculation was also evidenced. Similar results were recorded in our study of the incompatible host-pathogen system Reba B50/Xcm race 18. In the compatible interaction, POx activity enhancement did not occur, and bacterial population increased during the whole time course of infection. Comparison of POx isoforrn patterns monitored by IEF electrophoretic gels during time course experiments indicated that among the five acidic forms that were characterized within cotyledons of the susceptible and the resistant varietes, intensity of the activity of two major isozymes (pI 3.6 and 4.2) enhanced in the resistant line after infection. POx activity was shown by cytochemistry to be restricted to the HR areas in the early stages of the infection process. A systemic enhancement of POx activity was also biochemically evidenced in the infected plant. Such a local and systemic enhancement of POx activity has already been reported after treatment of plants with different pathogens or chemicals (2).

Of interest was (a) that infection of Reba B50 with Xm race 20 (that has overcome resistance of this line), induced into the cotyledons an intermediate enhancement of POx activity as compared both with compatible Acala 44/Xcm race 18 interaction and the incompatible Reba B50/Xcm race 18, but without specific stimulation of the 3.6 and 4.2 pI isoforms, and (b) that no specific isoperoxidase was induced when systemic POx enhancement occurred in leaves. These observations suggest that the „peroxidase reaction" may be under two kinds of control, one depending resulting from activation of the B_2-B_3 resistance gene(s)

of Reba B50 by the *avr* gene(s) of Xcm race 18 leading, among others, to early induction of two specific isoperoxidases, and another one resulting from signaling that triggers systemic and more general defense reactions.

Investigations are in progress, namely (a) to purify the 3.6 and 4.2 pI isoforms and produce specific antibodies in order to cytolocalize these isoenzymes *in situ* and to follow their production during the time course of infection, and (b) to verify if the systemic induction of the POx reaction is associated with a systemic acquired resistance (SAR).

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