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Accumulation of phytoalexins in leaves of plane tree (*Platanus* spp.) expressing susceptibility or resistance to *Ceratocystis fimbriata* f. sp. *platani*

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Abstract

Inoculation of leaves of resistant *Platanus occidentalis* and susceptible *Platanus acerifolia* leaves with *Ceratocystis fimbriata* f. sp. *platani*, the canker stain disease agent, induced foliar necrosis and biosynthesis of two phytoalexins, scopoletin and umbelliferone. Foliar symptoms keep localized and accumulation of coumarin phytoalexins was rapid for incompatible interactions. Necrosis spread widely and accumulation of these phenolic compounds was much later and lower for compatible interactions. The differential response could be used in a genetic improvement program for resistance against canker stain.

Introduction

Canker stain of plane trees is a widely reported disease [Walter, 1946; Panconesi, 1972; Ferrari and Pichenot, 1974; Vigouroux, 1979] caused by *Ceratocystis fimbriata* f. sp. *platani* (Ellis and Halsted) Walter (CFP). This pathogenic fungus enters through wounds in the roots, trunk and branches and causes foliar withering accompanied by trunk canker. This disease is dangerous and large vigorous trees may be destroyed within 4 to 7 years [Vigouroux, 1979]. Fungicide treatments have not solved the problem [Ferrari and Pichenot, 1979] except for prophylactic treatments which can partially prevent progression of the parasite. The European species, *Platanus acerifolia* (Aït) Wild, is always susceptible to canker stain but young herbaceous seedlings (3 months) are relatively impervious to the extension of CFP [Vigouroux and Rouhani, 1987]. In a previous study, we assessed the biochemical and histological responses of these plants after CFP inoculation. Vascular modification such as tylosis formation, gels, warts and induction of hydroxycoumarin accumulation, scopoletin and umbelliferone have been correlated with fungitoxic activity [Clérivet and El Modafar, 1994; El Modafar *et al.*, 1993]. As far as we know, these hydroxycoumarins have never been reported in trees, apart from scopoletin in *Hevea brasiliensis – Microcyclus ulei* interaction [Giesemann *et al.*, 1986]. Involvement of these defensive reactions have been suggested in the restriction of CFP development in stem tissues around the inoculation site.

Recently, a natural source of resistance was highlighted in the southeastern United States in the American species *Platanus occidentalis* L. [Mc. Craken, nonpublished data], but it cannot be directly exploited since the species is not acclimatized in Europe. However, some cuttings were introduced in France for the development of a resistant hybrid in a genetic improvement programme [Vigouroux, 1992]. This objective requires full knowledge of host-parasite interactions and must take into account all available results on *Platanus acerifolia*-CFP interactions. The aim of this study was to determine if marked differences such as variation in foliar symptoms and phytoalexin accumulation could exist between leaves of susceptible *Platanus acerifolia* and resistant *Platanus occidentalis* after inoculation with the canker stain disease agent.

Materials and methods

Fungal material

The monospore fungus *Ceratocystis fimbriata* f. sp. *platani* (CFP) was cloned from an isolate collected on naturally infected *Platanus acerifolia* in Marsillargues (Hérault, France). The fungus was grown at 25 °C on potato dextrose agar medium.

Plant material

Seven day old leaves were cut off 3 year old potted susceptible *Platanus acerifolia* (CVS L1, L11 and L36) and *Platanus occidentalis* (MO2S1) plants grown from seeds and 3 year old potted resistant *Platanus occidentalis* plants grown from cuttings of resistant American trees (CVS M18 and M11). The relative behaviour of plane CVS against CFP was previously determinated by artificial inoculation, as described previously [Vigouroux, 1992]. Plants were grown in a greenhouse. Harvesting was carried out in May when the temperature was 20 to 25 °C.

Inoculation

Directly after sampling, leaves were placed in glass Petri dishes containing moist Whatman filter paper. Half of the lower leaf surface, relative to the central vein, was inoculated with 10 μl droplets of conidial suspension in sterile distilled water (titrated to 4×10^4 conidia ml^{-1}) prepared from a 24 h old fungus culture. The other part of the leaf was used as control with sterile distilled water droplets (10 μl). Droplets were regularly distributed along the surface of the lamella. Samples were maintained at 25 °C in continuous light (40 μ E s^{-1}, Philips fluorescence tube, light day, 30 W).

Microscopy

For 3 days following inoculation, pieces of leaves and free-hand transverse sections of leaves exposed to distilled water or inoculum droplets were mounted in 0.1 M phosphate buffer at pH 8 and observed under an epifluorescence Leitz Laborlux microscope (340–380 nm) to assess tissue fluoresence.

To detect the presence of the pathogenic fungus on the leaf surface and in the tissues, samples (pieces of leaves and transverse sections) were observed after aniline blue staining [Sitch and Snape, 1987] under a Leitz Laborlux microscope.

For scanning electron microscopy, samples were prepared as previously described [Clérivet and El Modafar, 1994] and viewed under a Cambridge S 360 SEM operated at 20 Kv.

Biochemical techniques

Extraction and identification of hydroxycoumarins were carried out as previously described [El Modafar *et al.*, 1993] from leaf areas exposed to inoculum and control water droplets. They were also directly analysed from droplets (inoculum and control water) recovered on the lower leaf surface after filtration under Millipore membrane (0.22 μm).

Hydroxycoumarins were quantified by spectrofluorimetry at 345 and 460 nm corresponding to excitation and emission wavelength respectively. The results were expressed in nmole of umbelliferone ml^{-1} cm^{-2} (leaf surface). All data correspond to means of 3 replicates with samples of 3 leaves.

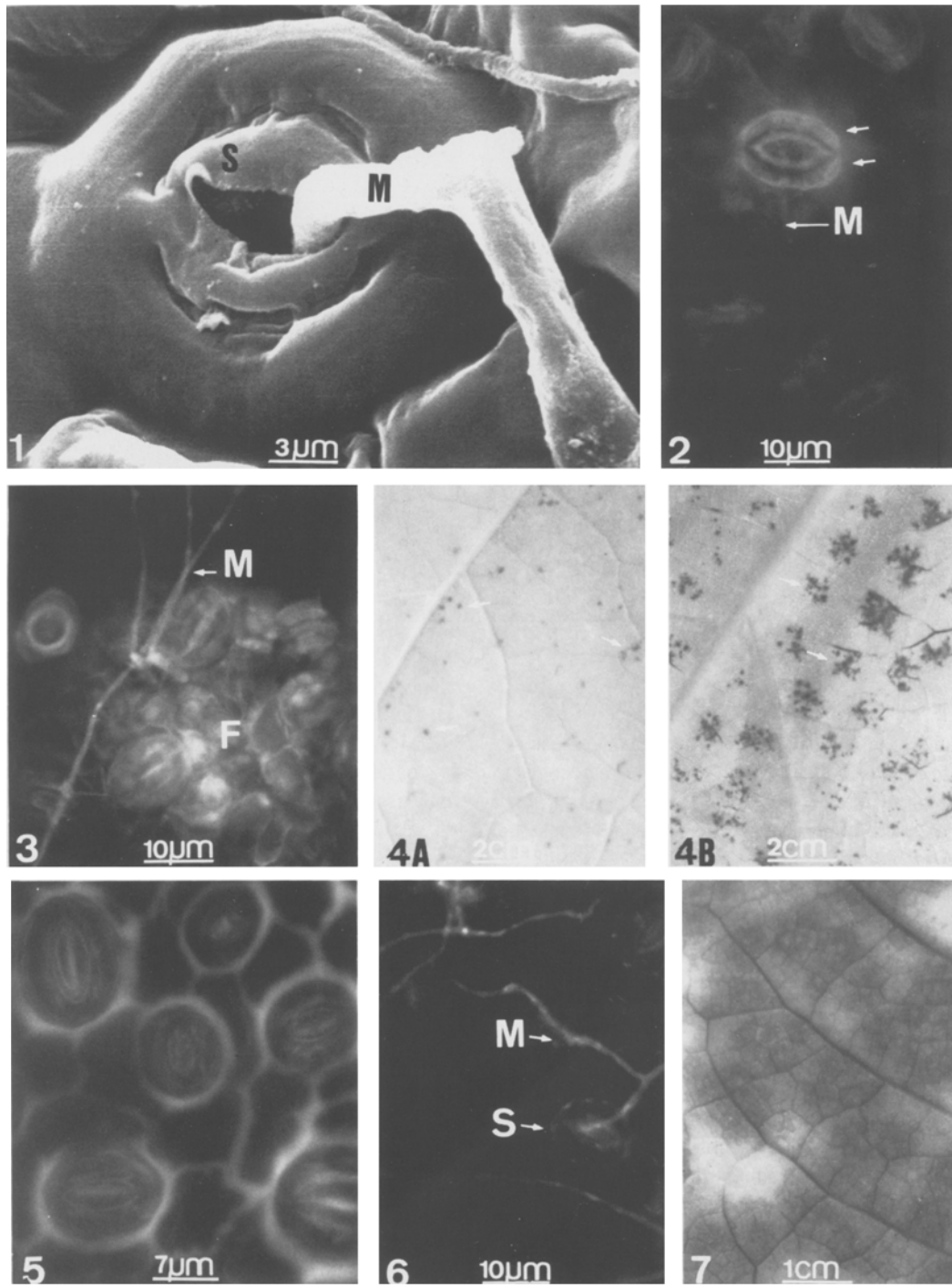
Results

Infection process and symptomatology

Germination of CFP conidia on leaves of susceptible and resistant plane trees was identical. Seven hours after inoculation germ tubes entered through open stomates (Fig. 1) and progressed into the mesophyll. Direct cuticular penetration was never observed.

In leaves of resistant plane trees (*P. occidentalis* M18, M11), penetration was first accompanied by a strong blue fluorescence of stomatal guard cells (Fig. 2) and adjacent epidermal cells (Fig. 3), followed by a localized necrotic process, leading to the appearance of necrotic flecks (Fig. 4A, B). Mycelium development was rapidly stopped in foliar tissues 10 to 16 h after inoculation. This fluorescence decreased 32 h after inoculation, but a new one appeared in the anticlinal walls of epidermal cells and in the mesophyll of the entire leaf area bearing inoculum droplets (Fig. 5). In leaves of susceptible plane trees (*P. occidentalis* MO2S1 and *P. acerifolia*), penetration by hyphae was also accompanied by fluorescence of guard cells, but it was reduced and never extended to the nearby epidermal cells (Fig. 6). Necrosis appeared only 48 h after inoculation and spread rapidly (Fig. 7). Mycelium development was very extensive in the mesophyll.

In leaves of resistant plane trees, direct observation of inoculum droplets under UV (250 and 360 nm) revealed that a faint fluorescence was already perceptible 4 h after inoculation, followed by a rapid increase which reached a peak after 48 h (Fig. 8A). In contrast,



Figs. 1–7. (1)Mycelium penetration (M) through a stomata (S). Scanning electron microscopy observation, 7 h after inoculation; (2) Mycelium penetration (M) with strong stomatic cell fluorescence (arrows). Resistant *P. occidentalis* M18, 7 h after inoculation. Surface view under epifluorescence (340–380 nm); (3) Fluorescence of epidermal cells (F) near mycelium (M) penetration site. Resistant *P. occidentalis* M18, 16 h after inoculation. Surface view under epifluorescence (340–380 nm) after aniline blue staining; (4) Localized necrosis (arrows). Resistant *P. occidentalis* M18. A: 24 h after inoculation, B: 120 h after inoculation; (5) Intercellular fluorescence of epidermal cells. Resistant *P. occidentalis* M18, 32 h after inoculation. Surface view under epifluorescence (340–380 nm); (6) Mycelium penetration (M) through a stomata (S) with low stomatic cell fluorescence Susceptible *P. occidentalis* MO2S1, 7 h after inoculation. Surface view under epifluorescence (340–380 nm); (7) Diffuse necrosis. Susceptible *P. occidentalis* MO2S1. 120 h after inoculation.

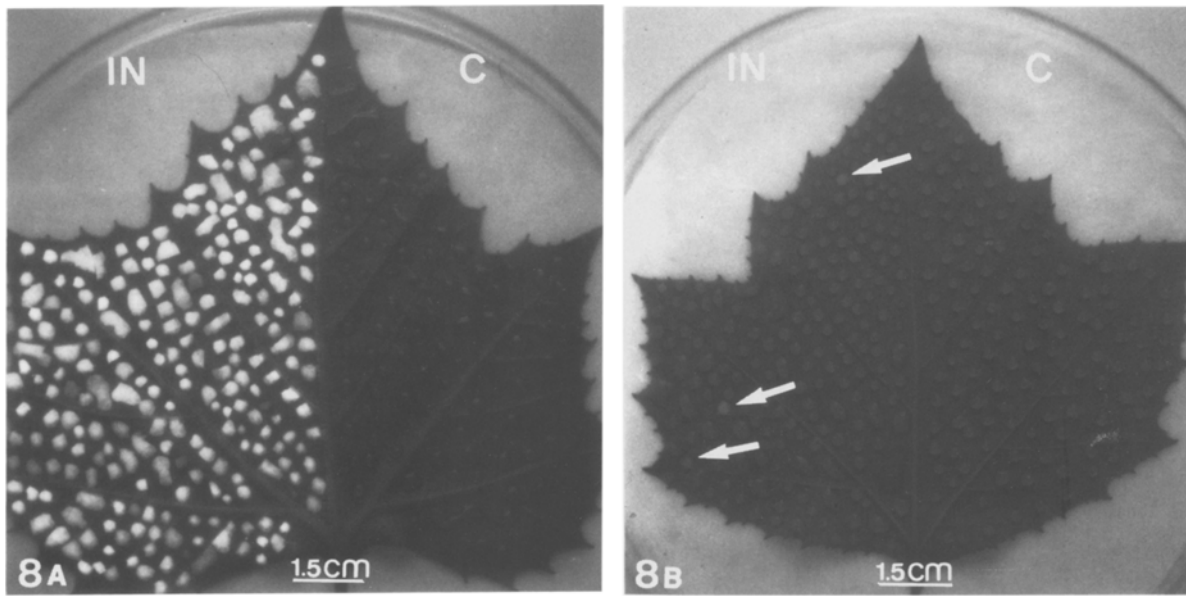


Fig. 8. Fluorescence of inoculum droplets. (A) resistant *P. occidentalis* M18; (B) susceptible *P. occidentalis* MO2S1. Direct observation under 254 nm. IN: inoculated part, C: control part (water droplets).

in leaves of susceptible plants, fluorescence of inoculum droplets was slightly perceptible only 48 h after inoculation (Fig. 8B). In any case, no fluorescence was observed in control water droplets.

Phytoalexin accumulation

Scopoletin and umbelliferone were isolated from inoculum droplets and tissues after fungal penetration. These compounds were not detected in control droplets or in healthy parts of the leaves. Spectrofluorimetric quantification of phytoalexin in inoculum and control droplets, and in tissue exposed to droplets was carried out 7, 12, 16, 32, 48 and 78 h after inoculation (Table 1). In leaves of resistant plane trees (M11 and M18), phytoalexins could be quantified 7 h after inoculation and at the same time in inoculum droplets. They rapidly increased in foliar extracts until 32 h after inoculation, reaching a high level of accumulation: 297 $\pm$ 112 nmol ml^{-1} cm^{-2} in M18, 211 $\pm$ 93 in M11 followed by a decreasing. Phytoalexins were abundantly secreted into inoculum droplets with maximum accumulation 48 h after inoculation (Table 1). On the other hand, phytoalexins were detected only 48 to 72 h after inoculation in leaves of susceptible plane trees (L1, L11, L36 and MO2S1) and in inoculum droplets. In every case, the level of accumulation was much lower than in resistant plants: approximately 30-fold in inoculum droplets and 120-fold in leaf tissue (Table 1). For the same trees, a previous study [El Modafar, 1993] showed that the phytoalexin response of mature leaves also allowed the distinction between resistant and susceptible plane trees though the accumulation of phytoalexins was lower in older leaves than in young ones.

Discussion and conclusion

Inoculation of plane tree leaves with a CFP conidia suspension induced highly differential symptomatological and biochemical responses which seem related to host resistance and susceptibility to canker stain disease agent.

In leaves of susceptible plane trees (*P. acerifolia* and *P. occidentalis* MO2S1), necrosis spread rapidly, foliar tissues were markedly invaded by the fungus and scopoletin and umbelliferone accumulation was late and low. On the other hand, leaves of resistant plane trees (*Platanus occidentalis* M11, M18) showed a hypersensitive reaction characterized by (i) localized necrosis of cells around the penetration site of the fungus (ii) early, fast and substantial induction of scopoletin and umbelliferone accumulation (iii) halted fungus development. The phytoalexin accumulation in inoculum droplets showed the localisation of phytoalexin synthesis in the restricted area exposed to inoc-

Table 1. Quantification of phytoalexin accumulation in inoculum droplets (ID) and foliar extract (FE) of resistant and susceptible plane tree leaves inoculated by *Ceratocystis fimbriata* f. sp. *platani*

Time		Resistant plant			Susceptible plant		
		M18	M11	MO2S1	L1	L11	L36
7	ID	27 ± 10	10 ± 5	–	–	–	–
	FE	6 ± 3	3 ± 1	–	–	–	–
12	ID	81 ± 32	12 ± 4	–	–	–	–
	FE	12 ± 4	7 ± 3	–	–	–	–
16	ID	124 ± 28	87 ± 31	–	–	–	–
	FE	94 ± 30	83 ± 30	–	–	–	–
32	ID	288 ± 72	157 ± 62	–	–	–	–
	FE	297 ± 112	211 ± 93	–	–	–	–
48	ID	312 ± 92	282 ± 86	16 ± 8	5 ± 3	13 ± 5	6 ± 3
	FE	248 ± 108	173 ± 78	7 ± 3	1 ± 0.6	1 ± 0.6	2 ± 1
72	ID	279 ± 98	296 ± 71	12 ± 4	11 ± 5	9 ± 4	10 ± 4
	FE	32 ± 15	66 ± 31	2 ± 1	1 ± 0.8	–	–

Results are expressed in umbelliferone equivalent in nmol ml^{-1} cm^{-2} of leaf surface covered by inoculum droplets: unmeasurable fluorescence.

ulum, in cells at the contact of infectious germ tubes and in those elicited by soluble elicitors secreted into the droplets during spore germination. Indeed, when spore suspension is replaced by droplets of culture filtrate without conidia (24 h old), a fluorescent reaction is induced. The halted fungus development in incompatible interaction could be related to the fungitoxicity of coumarins as we have yet shown. Accumulation level in the leaves (and in inoculum droplets) 32 to 48 h after inoculation were well above the minimum active dose of fungitoxicity for CFP [El Modafar *et al.*, 1993]. The drastic decreasing of phytoalexin accumulation in foliar tissue 72 h after inoculation could be related to the halted elicitation process by the fungus associated to the fungitoxic effect of phenolic compounds or to the oxydation of coumarins linked to browning of tissue exposed to spore suspension.

In contrast, in leaves of susceptible plane trees (*P. acerifolia* and *P. occidentalis* MO2S1) the level of phytoalexin accumulation was relatively low. In these conditions, the phytoalexins are not fungitoxic, they even can be degraded by the fungus [El Modafar *et al.*, 1993]. CFP development would therefore not be affected, leading to gradual invasion of foliar tissues. So, as in other systems [Coleman *et al.*, 1992; Nieman and Steijl, 1994] if phytoalexins can be involved as one of the causes of the incompatibility in CFP interaction, the timing and intensity of phytoalexin synthesis and accumulation, added to other unidentified defense reaction, could be critical in determining the outcome of compatible or incompatible interactions. The resistance in plane trees could be, at least partially, related to the synthetic capacity, speed and intensity of accumulation of the antifungal coumarins, scopoletin and umbelliferone. In contrast, defense responses in susceptible plane trees might not be initiated rapidly enough and the pathogen could be able to degrade fungitoxic phenolic compounds or suppress activation responses, as shown in other interactions [Darvill and Albersheim, 1984; Yamada *et al.*, 1989; Lamb *et al.*, 1989; Shiraishi *et al.*, 1991; Lee *et al.*, 1992; Jakobek *et al.*, 1993].

Induction of phytoalexin formation is thought to be initiated by interaction of fungal conidia or hyphae with the plant cell [Darvill and Albersheim, 1984]. On a molecular level, this interaction requires a fungal elicitor molecule which is probably recognized by a receptor in the plant cell wall or membrane [Dixon, 1986; Templeton and Lamb, 1988; Dixon and Lamb, 1990; Keen, 1992]. Histochemical studies have clearly shown the succession of two distinct 'waves' of fluorescence characterized by their appearance dynamics and spatial localization. The first wave, localized mainly in guard cells during mycelial penetration,

probably corresponds to a cellular response to the initial host parasite interaction. When spore suspension is replaced by droplets of culture filtrate (24 h old) without conidia, the second wave of fluorescence is the only one observed. So, in *Platanus* spp–CFP interactions, it is thus highly likely that soluble fungal elicitors are produced during conidia germination as in *Phytophthora megaspora* f. sp. *glycinea–Glycine max* [Waldmuller *et al.*, 1992]. However, implication of endogenous elicitors can be considered since CFP may produce pectinases and cellulases [D'Ambra *et al.*, 1975, 1977] whose activity on cell walls is well known to release molecules with elicitor activity [Ebel, 1986; Dixon, 1986; Rouxel, 1989; Yoshikawa *et al.*, 1993; Ebel and Cosio, 1994]. The interaction could ultimately lead to activation of host genes which code for enzymes involved in the biosynthesis of phenolic compounds toxic against the invading pathogen [Dixon *et al.*, 1992], such as scopoletin and umbelliferone in plane trees.

Thus, in the CFP–*Platanus* spp. pathosystem, detached leaves could be used (i) to identify CFP elicitors and evaluate their activity on the phytoalexin induction (ii) to know whether gene activation is required for scopoletin and umbelliferone accumulation (iii) to study virulence of various CFP isolates. This could provide new knowledges for a better understanding of the mechanisms of CFP virulence and plane tree defense responses. In view of a genetic improvement program for plane trees, the highly differential leaf responses allow to expect a strong link with the actual resistance of mature plane trees and the possibility to use simple detached leaf assay in the screening process Nevertheless, further investigations are probably necessary to take into account the possibility of variability in leaf responses due to plant age and different environmental and culture conditions of plane trees. First hybridizations conducted for the on going genetic improvement program were already realized, whose progeny will constitute good support to estimate the correlation level between the leaf responses and the actual tree resistance.

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