

BIOLOGICAL ASSAYS TO STUDY INDUCTION OF RESISTANCE IN *PLATANUS* × *ACERIFOLIA* TO *CERATOCYSTIS FIMBRIATA*

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SUMMARY

A study was undertaken on *Platanus* × *acerifolia* to assess the potential of wounding, salicylic acid, jasmonic acid, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, and the pharmacological inhibitors antimycin and norbornadiene, to induce local resistance to *Ceratocystis fimbriata* f.sp. *platani*, the agent of canker stain. *In vitro* studies were used to determine fungitoxicity and phytotoxicity of the chemicals. Fungitoxicity was characterized by fungal growth and germination of conidia on media supplemented with the chemicals. Results from these experiments indicate a role of salicylic acid and ethylene in promoting *C. fimbriata* conidia germination. Treatments of detached branches and leaf discs with the chemicals were used to determine non-phytotoxic concentrations for the experiments of resistance induction. Leaf and wood disc assays were developed for measuring the induction of resistance. In these assays, pretreatment with the chemicals caused a reduction of disease severity and of fungal growth, respectively, with the exception of jasmonate, which enhanced disease severity, compared to the 48 h wounding/water control. In leaf and wood disc experiments, wounding reduced disease severity and mycelium growth respectively. The fungitoxic effects of inducers and the possibility that a genuine induction of resistance could be triggered in the assays are discussed.

Key words: canker stain, resistance inducers, wounding effect, leaf disc assay, wood disc assay.

INTRODUCTION

Canker stain, caused by *Ceratocystis fimbriata* Ell. and Halst. f.sp. *platani* Walter is by far the most dangerous disease of *Platanus* × *acerifolia* Ait. (Willd.) in Europe, due to its rapid spread and the lethality of the infections in street plantings. *C. fimbriata* can infect the

woody organs through wounds or root anastomosis, then invades and necrotizes xylem tissue by rapidly invading the medullary rays and vessels. The destructiveness of the disease led to its definition as a quarantine pest and to the legal obligation, in Italy, to apply expensive measures aimed to prevent or to eradicate infection foci. Unfortunately, this type of control has failed to produce the desired results. The application of fungicides, based on pressure injection, only temporarily halts the progress of infection inside the tree (Panconesi, 1999).

Obtaining resistant genotypes is probably the most likely way to combat the pathogen, while respecting the environment and human health. Up to this point, research on the control of this disease has proved difficult and slow but some results have been obtained (Vigouroux and Chalvon, 1999; Pilotti *et al.*, 2002). An interesting complement to the work of genetic improvement is to study induced resistance in order to understand the transduction pathways activated during establishment of resistance or disease development. Induced resistance has been studied for many years, mostly on herbaceous species and has been demonstrated in many plant families. Induced resistance is expressed through previous exposure of the plant to diverse stimuli such as pathogen attack or rhizobacterial infections that trigger both local and systemic, long-lasting resistance, effective against subsequent attacks of different types of pathogen (Sticher *et al.*, 1997; Van Loon *et al.*, 1998). This resistance can be called systemic acquired resistance (SAR) or induced systemic resistance (ISR) using, according to the case, the prefix pathogen-induced- or rhizobacteria-induced- (Métraux, 2001). An important characteristic of these types of resistance is their effectiveness towards different races of the pathogen that can usually overcome the highly specific gene-for-gene resistance. The signalling molecules salicylic acid (SA), jasmonic acid (JA) and ethylene have a role, yet to be fully understood, in triggering induced resistance; when applied exogenously they can induce resistance by themselves (Sticher *et al.*, 1997; Van Loon *et al.*, 1998; Vijayan *et al.*, 1998; Métraux, 2001).

In the present study, biological assays, based on leaf and wood discs and detached branches, were developed and the ability of SA, JA, and of the ethylene precursor

1-aminocyclopropane-1-carboxylic acid (ACC) to induce resistance in *P. × acerifolia* to *C. fimbriata*, was tested. The pharmacological inhibitors bicyclo [2.2.1] hepta-2,5-diene (2,5 norbornadiene), an inhibitor of ethylene action (Sisler and Young, 1984; Peck and Kende, 1995), and antimycin, an activator of the alternative respiratory pathway, a specific branch of SA signalling that induces resistance to viruses (Bahr and Bonner, 1973; Murphy *et al.*, 2001), were also tested. The effect of wounding on disease severity was also explored.

MATERIALS AND METHODS

Test organisms. The strain of *C. fimbriata* used in this study was isolated from a naturally infected plane tree. Bioassays were performed on plant tissues from S3, MS20b and MS21 *P. × acerifolia* accessions. S3 and MS20b seem to be less susceptible to canker stain while MS21 appears highly susceptible (Pilotti *et al.*, 2002). For the wounding-assays on detached branches, one year-old sprouts were harvested from the stump of a plane that had been felled.

In vitro fungitoxicity of the chemicals. To test the direct mode of action of the chemicals either on mycelial growth or on conidial germination, two types of study were carried out.

For the first type, *C. fimbriata* was cultured on potato dextrose agar (PDA, potato extract powder 4 g l⁻¹, glucose 20 g l⁻¹, agar 15 g l⁻¹ (Oxoid, Unipath Ltd., Bedford, UK) containing the following chemicals: sodium salicylate (NaSA, Fluka, Sigma-Aldrich, St. Louis, USA), 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma-Aldrich), methyl jasmonate (MeJA, Sigma-Aldrich), bicyclo [2.2.1] hepta-2,5-diene (2,5 norbornadiene, Sigma-Aldrich), antimycin A (Fluka, Sigma-Aldrich). Concentration series were: 0.04, 0.4, 2, 10 and 50 mM for NaSA; 0.2, 1 and 5 mM for ACC; 0.02, 0.2, 1, 5, 75, 125, 250 and 500 mM for MeJA; 0.2 mM for norbornadiene; 0.05 mM for antimycin; 0.2 mM for ACC and norbornadiene in combination. All the chemicals were freshly dissolved in water except antimycin, which was previously prepared as a 25 mM stock containing 25% dimethyl sulfoxide (DMSO), and norbornadiene, dissolved with DMSO at 0.025%. All chemicals were added to the media after autoclaving and cooling; the pH of these media ranged from 5.4 to 5.7. For each concentration of chemical tested, five 25 ml dishes, were inoculated with mycelial plugs cut from the edge of a *C. fimbriata* colony actively growing on PDA. After incubation for 12 days at 25°C in the dark, colony diameters were measured in two directions at right angles, and the mean value determined. Where colony growth was limited to few mm or was absent, viability of the fungus was tested by sub-culturing it on PDA.

In the second type of study, the effect of the chemicals was studied on conidial germination by pipetting onto 25 ml agar water-plates, four 10 µl-drops of a conidial suspension at 5,000 conidia ml⁻¹ prepared as previously described (Pilotti *et al.*, 2002) in 0.005% (v/v) silwet L-77. The media contained one of the following: 0.05% DMSO, 0.4 mM NaSA, 0.2 mM ACC, 0.2 mM MeJA, 0.2 mM norbornadiene, 0.2 mM ACC + norbornadiene, or antimycin at 0.05 mM, 0.025 mM, 0.005 mM, 0.001 mM. 0.025% DMSO was also used to dissolve MeJA and norbornadiene and antimycin as described above. Two Petri dishes were used for each treatment. The plates were incubated at 25°C in the dark. Germination was assessed using a stereomicroscope after 16 h by counting both ungerminated and germinated conidia. The percentage germination was then calculated.

Toxicity assay on detached branches. To establish the toxicity levels of the chemicals used, one-year-old detached branches were used. The branches were collected before bud burst at the end of March, from the base of a street plane. Five branch segments, 10 cm long, were dipped with their base in solutions containing either NaSA (0.4 and 10 mM) or MeJA (0.2 and 5 mM) or ACC (1 mM). The branches were incubated at room temperature (16-23°C) with a 13/11 h light-dark cycle. Solutions were renewed once a week and toxicity effects were assessed over 20 days. The experiment was repeated twice.

Leaf disc assay. To test the effect on resistance to *C. fimbriata*, a leaf disc assay was developed. In fact, leaf resistance correlates with the resistance of woody organs (El Modafar *et al.*, 1995; Pilotti *et al.*, 2002). Discs of 2.4 cm diameter were punched from young expanding leaves of the fourth or fifth node of branches of nursery-grown cuttings or of the mother tree.

Chemical treatments were made two days before challenge inoculations by immersing randomly distributed discs for 1 h under gentle agitation in a solution containing 0.005% (v/v) silwet L-77, as a wetting agent, and either 0.4 mM NaSA, 0.2 mM MeJA, 0.2 mM ACC, 0.2 mM norbornadiene, 0.05 mM antimycin A, or a combination of ACC and norbornadiene at 0.2 mM. DMSO was added to the solution to dissolve some chemicals, with a final concentration of 0.025% (MeJA and norbornadiene) or 0.05% (antimycin). After incubation in the chemicals, five discs per treatment were placed, abaxial surface uppermost, on plates containing technical agar 12 g l⁻¹ (Oxoid, Unipath Ltd.). Challenge inoculation was carried out after 48 h by immersing the discs for 30 min, under gentle agitation, into a 25 ml conidial suspension of *C. fimbriata* at 10³ conidia µl⁻¹, prepared as previously described (Pilotti *et al.*, 2002), in 0.005% (v/v) silwet L-77. The discs were then newly placed on

freshly prepared agar-water plates.

In order to evaluate the effect of wounding on resistance induction, two types of control were prepared: the first was treated with 0.005% silwet L-77 and 48 h later was challenge-inoculated (48 h-wounding/water-control); the second was cut and challenge-inoculated soon after (water-control).

To evaluate any effect of toxicity-related necrosis due to use of silwet, DMSO, and the other chemicals under study, appropriate uninoculated controls were also prepared. Silwet and DMSO were also checked for any effect on induction of resistance. Plates were incubated at room temperature (18-25°C) with a photoperiod of 14/15 h day and 10/9 h night.

All treatments described above were tested on the S3 accession. Wounding effect was also tested on MS20b and MS21. The experiments were repeated more than twice, as shown in Table 1.

Three to five days after challenge-inoculation, disease severity of leaf discs was assessed according to the following rating (r):

- absence of any alteration (r: 0);
- necrotic margin (1 or 2 mm thick) and up to 30 necrotic spots (r: 0.5);
- necrotic margin (1 or 2 mm thick) and up to 90 necrotic spots (r: 1.25);
- coalescing necroses covering up to 40% of the surface (r: 2);
- coalescing necroses covering 41-60% of the surface (r: 2.75);
- coalescing necroses covering 61-100% of the surface (r: 4).

The infection rate was calculated, for each experiment, according to the McKinney index (McKinney, 1923).

Wood disc assay. In order to evaluate the effects of chemical inducers on wood, one of the target tissue of natural *C. fimbriata* attacks, a wood disc assay was developed. Wood discs, including the bark, about 2.5 cm in diameter and 1.5-1.7 cm thick, were cut from four-year-old branches of the tree S3.

The methodology was similar to that described for leaf discs assays, with some modifications. The discs were immersed for 2 h in solutions containing either NaSA or MeJA or ACC or antimycin A. Seven discs per treatment were placed, with acropetal orientation, in glass dishes of 18.5 cm in diameter, containing 70 ml of agar-water 6 g l⁻¹.

Inoculation was carried out by pipetting 4 µl of a suspension at about 130 conidia/µl, in the middle of the upper sections of the discs, onto the pith.

Pathogen spread was assessed from the third to the sixth day after inoculation, by estimating the spread of fungal mycelium on the wound surface according to the following rating (r):

- absence of mycelium (r: 0);
- very poor presence of fungal hyphae, detectable with a stereo microscope (r: 0.1);
- scattered presence of mycelium, detectable with a stereo microscope (r: 0.6);
- presence of mycelium on the whole disc surface, detectable with a stereo microscope (r: 1);
- presence of dense, greyish mycelium, detectable with the naked eye, covering respectively 5-20% (r: 1.5), 21-40% (r: 2.5), 41-60% (r: 3.5), 61-80% (r: 4.5), 81-100% (r: 5.5) of the disc surface.

The experiments were repeated three times. Mycelial growth rate was calculated for each experiment, according to the McKinney index (McKinney, 1923).

Wounding assay on detached branches. This assay was performed in order to evaluate if wounding induces systemic resistance to fungal infection. Ten pieces of branches 18-23 cm in length and 1.2-1.9 cm in diameter, were cut from the collar of the fallen tree, at the beginning of October, 7 and 3 days before their inoculation, and kept with their base in water. As controls, ten similar branch pieces were collected and inoculated soon after. Inoculation was performed with a toothpick (Pilotti *et al.*, 2002) in the middle of each branch, by inserting the pick, thus pushing about 1,000 conidia for 2 mm through the bark and the wood. The experiment was conducted at room temperature (18-23°C). After 11 days, length and width of necrosis were measured and average values calculated.

Statistical analysis. Multiple-way fully random analysis of variance (ANOVA) and the Newman-Keuls multiple range test were used in order to evaluate the following parameters: growth of *C. fimbriata* on media containing the chemicals at different concentrations; germination rate of conidia on media amended with different chemicals; size of the necrotic area produced in relation to the different wounding times. Percentage values were previously transformed in $\arcsin\sqrt{\%}$.

RESULTS

Fungitoxicity of the chemicals. Multiple range test analysis on the growth rate of *C. fimbriata* showed a continuous range of variation, depending on the chemical and concentration used (Fig. 1). Increasing concentrations of NaSA and MeJA caused evident and progressive reduction of fungal growth. For NaSA, the first significant reduction of growth was recorded at 0.4 mM (34.1%), growth was absent at 10 mM, and mycelial death occurred at 50 mM. For MeJA, the first significant reduction of growth was recorded at 0.02 mM (8.2%) and 0.2 mM (33.9%), but the fungus remained viable up to 500 mM. In contrast, a proportional trend

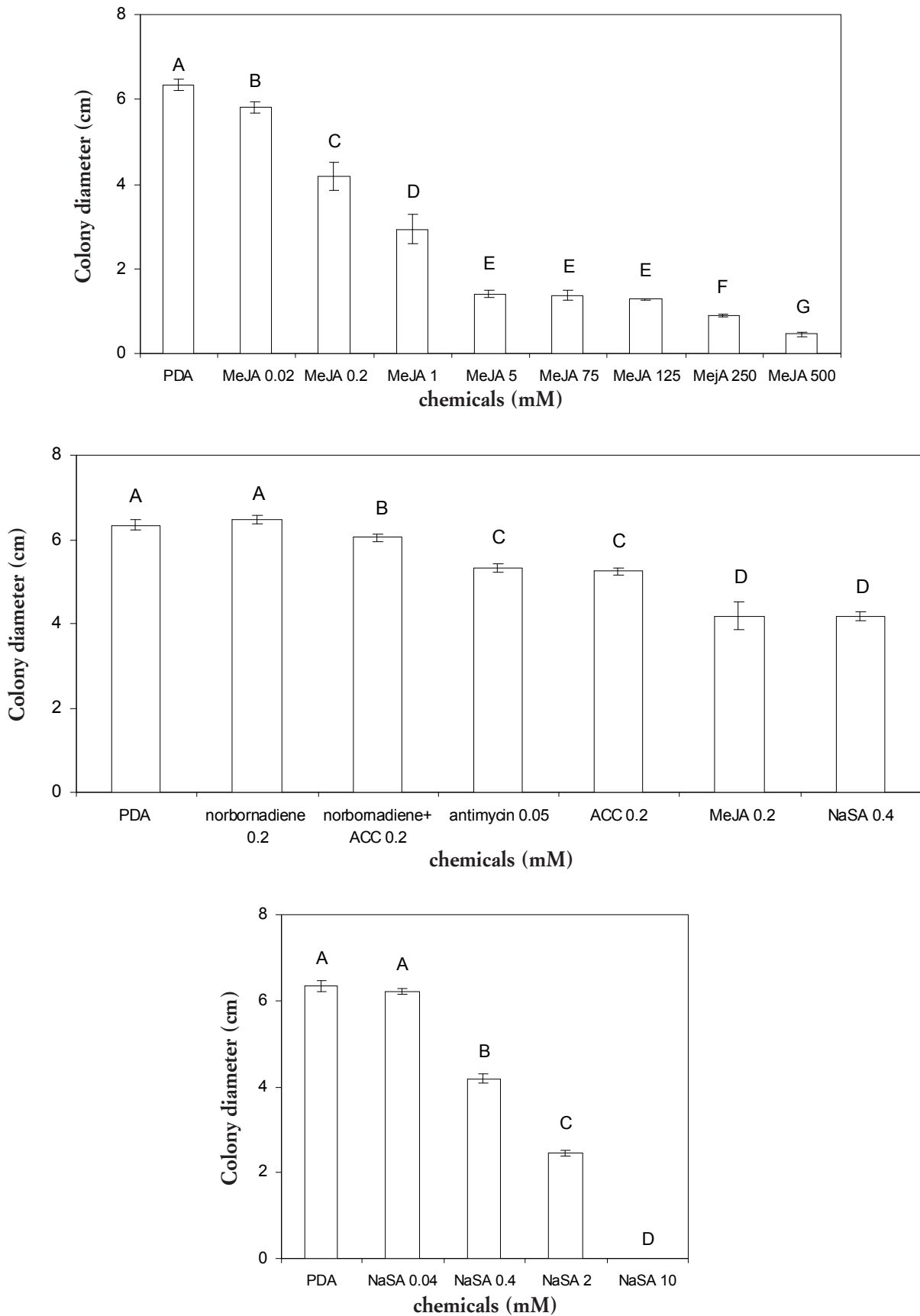


Fig. 1. Effect of chemicals on the *in vitro* growth of a *C. fimbriata* culture after 12 days. Different letters show significant differences among bars (Newman-Keuls, p=0.01). Comparisons have been made inside the concentration series of MeJA and NaSA to show the dose-dependent fungitoxic effect and among the chemicals chosen for the experiments of induction of resistance.

between ACC concentration and growth was observed, with 0.2 mM causing the greatest growth reduction (17.4%). Significant growth reduction was also recorded after treatment with 0.05 mM antimycin (15.9%) and with the combination 0.2 mM ACC and norbornadiene (4.7%). However, treatments with 0.2 mM norbornadiene and 0.04 mM NaSA did not differ significantly from the control.

Conidial germination was tested twice with similar results (Fig. 2). After 16 h, the percentage of germination of the control was 46.2%. ACC and NaSA significantly stimulated germination with percentages of 81.5% and 84.6% respectively. MeJA, norbornadiene and the combination norbornadiene + ACC, significantly decreased germination with percentages of 6.1, 10.6 and 2.4%, respectively. DMSO did not significantly affect germination. Antimycin strongly inhibited germination; germination percentages were 0.9, 5.8, 6.6, 12.1% at concentrations of 0.05, 0.025, 0.005 and 0.001 mM, respectively. Germination in all treatments was significantly lower than controls. Significance was assumed with null hypothesis rejected for $p < 0.01$.

Toxicity assay on sprouting branches. On the branches kept in water, sprouting occurred after 4-8 days. Ten mM NaSA, 5 mM MeJA and 1 mM ACC generally caused an evident delay in growth of new shoots. In addition, ACC also delayed the opening of buds (at 5-13 days). The shoots were chlorotic, with a glassy consistence and produced short internodes and small leaves (Fig. 3A).

Ten mM NaSA and 5 mM MeJA caused necrosis of the bark and xylem and finally the death of the branches. In particular 5 mM MeJA also caused shoot with-

ing soon after bud opening. For NaSA and MeJA at the lowest concentrations (0.4 and 0.2 mM, respectively) no toxic effects were observed, except limited marginal necrosis of the young leaves, observed in NaSA-treated branches in the last days of the observation period, and a mild decrease of growth for MeJA (Fig. 3B, C).

Leaf disc assay. Inoculation of *C. fimbriata* on leaf discs had the same effect as inoculation on entire leaves (Pilotti *et al.*, 2002): necrotic flecks appeared from the third day and progressively increased in size and number until coalescence; within five to eight days, spreading necrosis affected the entire leaf disc surface (Fig. 4). Necrosis also started from the disc border and generally did not extend more than 2 mm. Perithecium development was always associated with the spreading necrosis, representing a marker of fungal growth within the leaf tissue.

Symptoms of phytotoxicity were not observed on the chemical-treated controls; silwet and DMSO showed no influence on induction of resistance/susceptibility (data not shown).

Application to the leaf discs of NaSA, ACC, antimycin, norbornadiene, and the combination of ACC and norbornadiene, caused a reduction of disease severity 3 to 4 days after inoculation, in comparison with 48 h-wounding/water-control and the water-control (Fig. 5A). MeJA clearly increased disease severity compared with the 48 h-wounding/water-control. Wounding effects caused reduction of necrosis in the three plane accessions tested (Fig. 5B, Table 1).

After 7 to 10 days, the leaf discs in all treatments were completely necrotized with abundant development of perithecia.

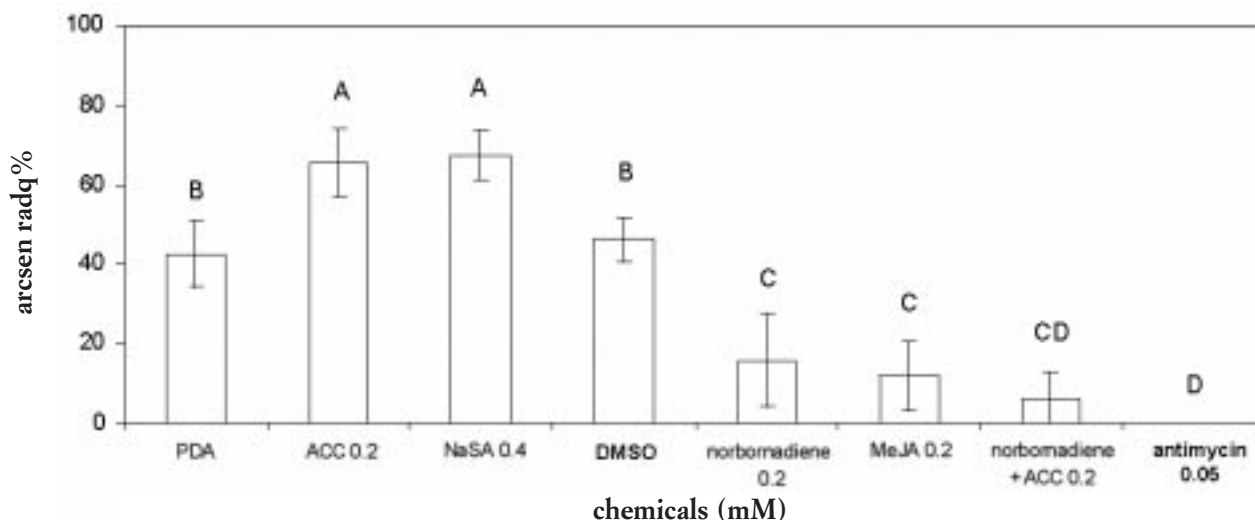


Fig. 2. Effect of chemicals on the *in vitro* germination of *C. fimbriata* conidia after 16 hours. Different letters show significant differences among bars (Newman-Keuls, $p=0.01$). Comparisons have been made among the chemicals chosen for the experiments of induction of resistance.

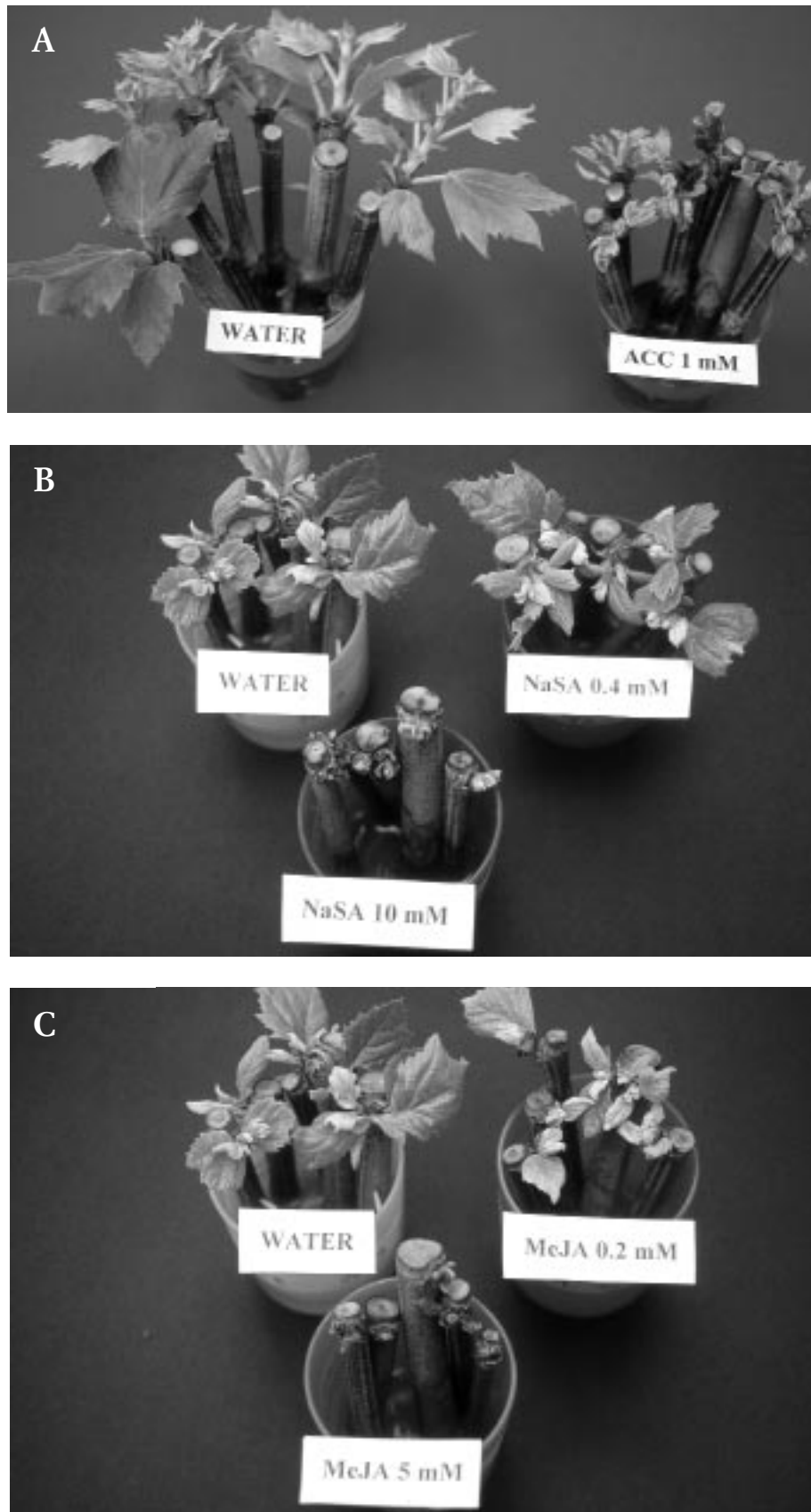


Fig. 3. Phytotoxic effects of the chemical solutions on detached branches of *P. × acerifolia*. **A)** ACC 1 mM caused a delay in the opening of the buds and the production of vitrescent sprouts with short internodes and little leaves. **B)** NaSA 0.4 mM and NaSA 10 mM, caused respectively no relevant phytotoxic effect and death of the branches. **C)** MeJA 0.2 mM and MeJA 5 mM caused, respectively, no relevant phytotoxic effect and death of the branches soon after the opening of the buds.

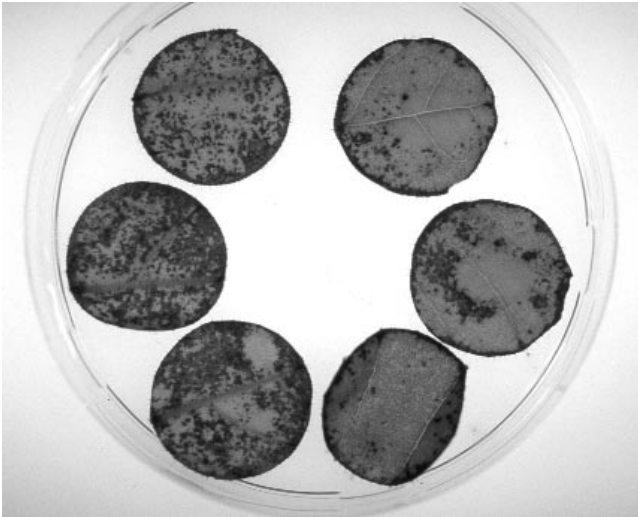


Fig. 4. Inoculation of *C. fimbriata* on leaf discs of *P. x acerifolia* caused the appearance of necrotic flecks increasing, with time, their size and their number until coalescence. The infection was more severe when the discs were placed on the plate with the abaxial surface uppermost (three discs on the left) in comparison with those placed with the adaxial surface uppermost (three discs on the right) (data not shown in the text).

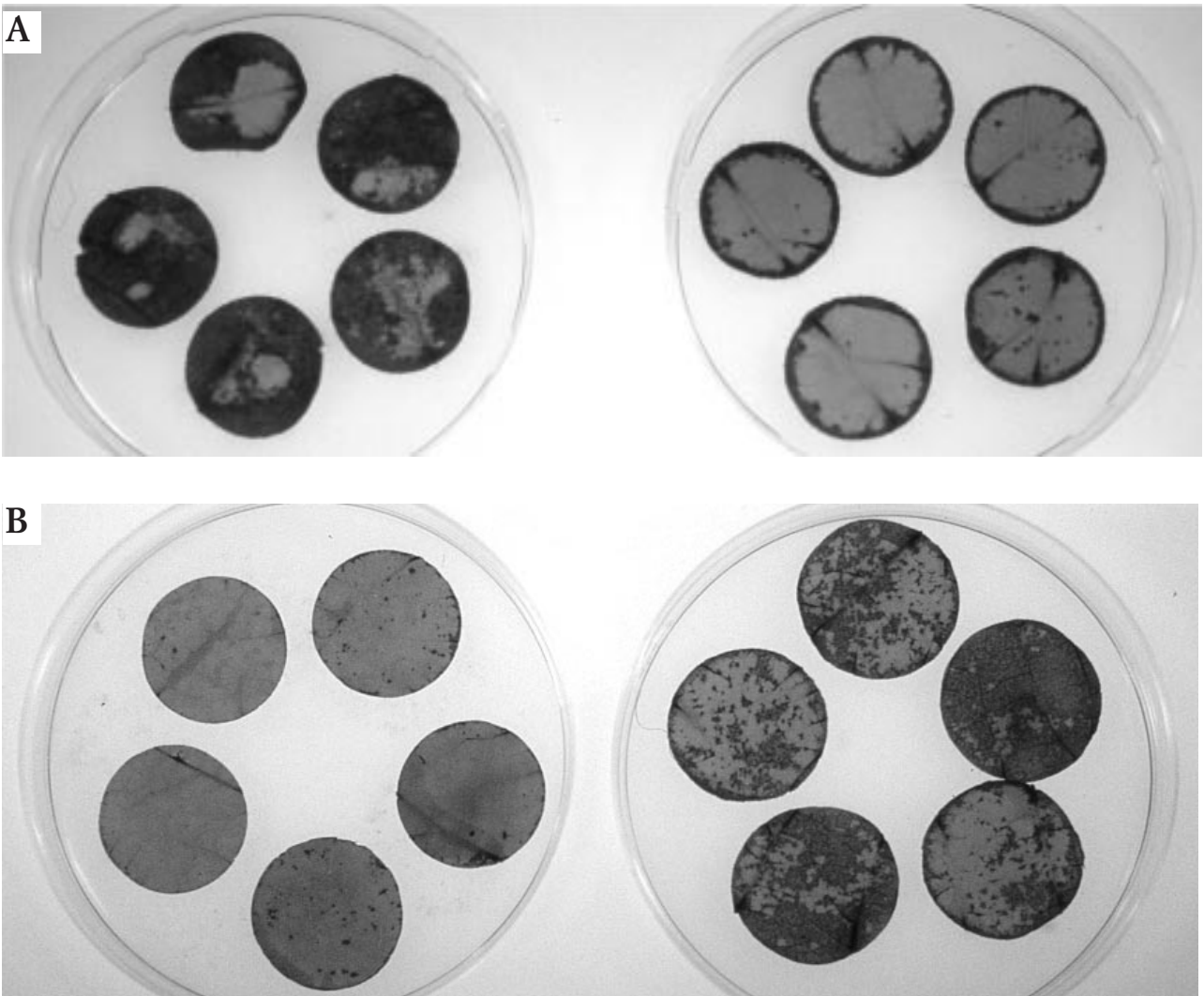


Fig. 5. Effects of some inducing treatments on the inoculation of leaf discs with *C. fimbriata*. Disease severity was recorded at the third-fourth day after inoculation. **A)** ACC 0.2 mM caused a reduction of disease severity (plate on the right), in comparison with 48 h-wounding/water-control (plate on the left). **B)** Leaf discs previously cut and water-treated and inoculated with *C. fimbriata* 48 h later (48 h-wounding/water-control namely wounding effect) (plate on the left) showed a reduction of disease severity in comparison with leaf discs cut and inoculated soon after (water-control) (plate on the right).

Table 1. Infection rate of leaf discs pre-treated with different chemical inducers at the indicated concentrations (mM) and inoculated 48 h later with a conidia suspension of *C. fimbriata* (1,000 conidia/μl). Disease severity was recorded after 3/5 days according to the key described in the text and the infection rate was calculated according to McKinney index (%). Chemical treatments were tested repeatedly on the accession S3, wounding effect was tested, repeatedly, on the accessions S3, MS20b and MS21.

Chemicals and concentrations	Accessions													
	S3						MS20b				MS21			
	d+3	d+4	d+4	d+3	d+4	d+5	d+5	d+4	d+3	d+3	d+3	d+3	d+3	d+3
NaSA (0.4)	5.0	17.2	20.0	7.5	28.8	-	-	35.0	-	-	-	-	-	-
ACC (0.2)	7.5	12.5	21.3	21.3	-	-	-	50.0	-	-	-	-	-	-
MeJA (0.2)	35.0	62.5	-	83.8	-	42.5	52.5	-	-	-	-	-	-	-
Antimycin (0.05)	-	-	-	5.0	-	-	-	43.8	-	-	-	-	-	-
Norbonardiene (0.2)	12.5	16.3	-	8.75	10.0	-	-	-	-	-	-	-	-	-
Norbonardiene + ACC (0.2)	7.5	2.5	-	17.5	5.0	-	-	31.3	-	-	-	-	-	-
48 hr-wounding/water	22.5	35.0	35.0	48.8	53.8	27.5	48.8	76.3	20.0	31.3	27.5	42.5	80.0	
Water	25.0	62.5	-	62.5	12.5	100.0	100.0	81.3	100.0	77.5	100.0	100.0	100.0	

Wood disc assay. After treatment of water-treated wood discs with conidial suspension, the mycelium grew vigorously from the second day and formed, within four days, a greyish dense colony that could be detected by naked eye.

Application of the chemicals to the wood discs generally reduced mycelial growth compared to the 48 h-wounding/water-control, with some exceptions (Fig. 6A, B, C). NaSA and ACC reduced mycelial growth in two of the three trials (Fig. 7); antimycin was rather per-

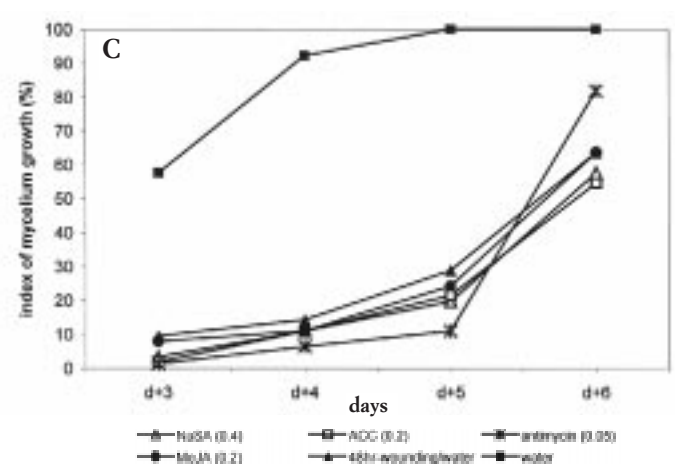
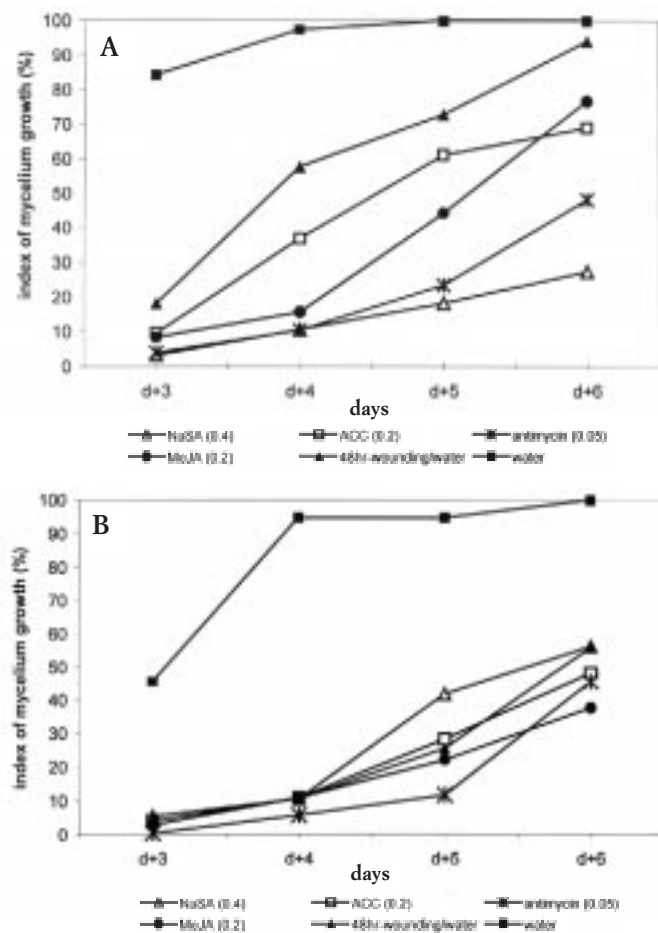


Fig. 6. Effect of pre-treatment with different chemical inducers, at different concentrations (mM), on the inoculation of wood discs with *C. fimbriata*. Comparisons were made with 48 h-wounding/water-control and water-control to show the wounding effect. Mycelial growth was recorded after 3, 4, 5 and 6 days, according to the key described in the text and the mycelium growth rate was calculated according to McKinney index. The experiment was repeated three times (A, B, C).

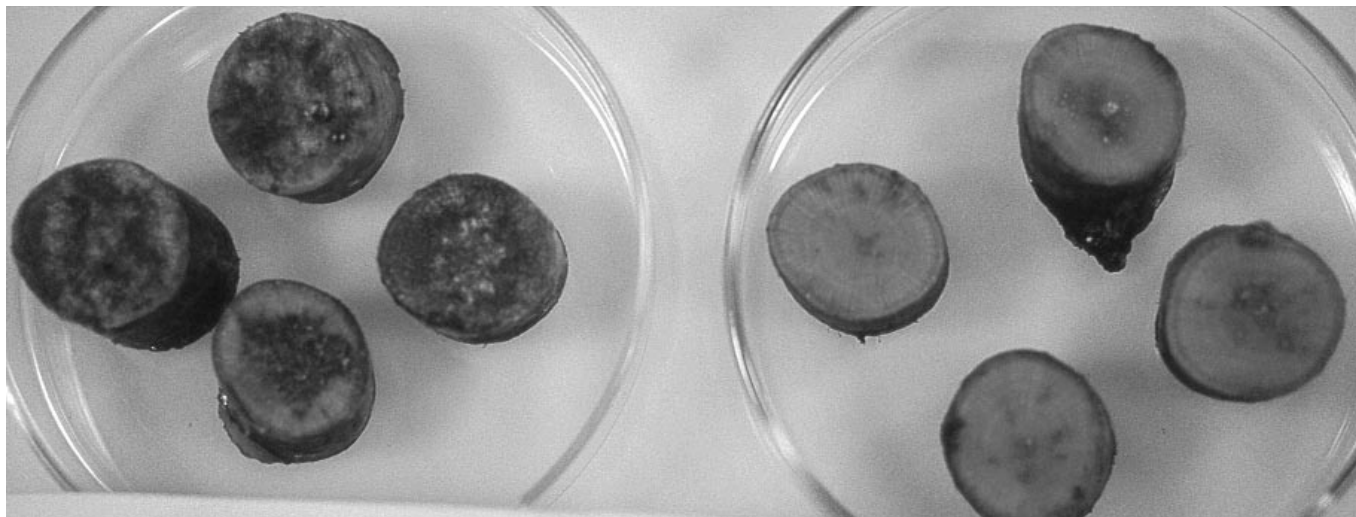


Fig. 7. Wood discs previously treated with NaSA 0.4 mM and inoculated with *C. fimbriata*, showed a reduction of mycelium growth, particularly evident in the first trial (plate on the right), in comparison with 48 h-wounding/water-control (plate on the left)

sistent in showing an action against the fungus, while MeJA with the exception of the first trial, showed no trend clearly distinguishable from the control. Wounding had a more dramatic effect; this was also underlined by the fact that differences between the indexes of mycelial growth of the two controls were much more greater than those between the 48 h-wounding/water-control and chemical treatments (Fig. 6A, B, C).

As with the leaf disc assays, the differences among the treatments in the wood disc assays progressively disappeared with time.

Wounding assay on detached branches. Sprouting of the buds did not occur during the trial period, due most probably to dormancy. No significant differences were recorded among the treatments in relation to necrosis length. Instead, necrosis width was progressively reduced, increasing the time of induction by the wound, with the value obtained at seven days being significantly lower than the control (Fig. 8).

All differences were rejected with null hypothesis for $p < 0.01$.

DISCUSSION

Over many years, synthetic inducers of resistance have been screened (Gottstein and Kuc, 1989; Métraux *et al.*, 1991; Chérif *et al.*, 1992; Mercier *et al.*, 1993; Cohen, 1994; Reuveni *et al.*, 1994; Reglinski *et al.* 1997; Frey and Carver, 1998; Islam *et al.*, 1998; Cohen *et al.*, 1999; Hong *et al.* 1999; Akinwunmi and Lucas, 2001; Oka and Cohen, 2001, Pajot *et al.*, 2001; Seebold *et al.*, 2001). However, in forest species, this type of research is difficult for many reasons that hamper standardization

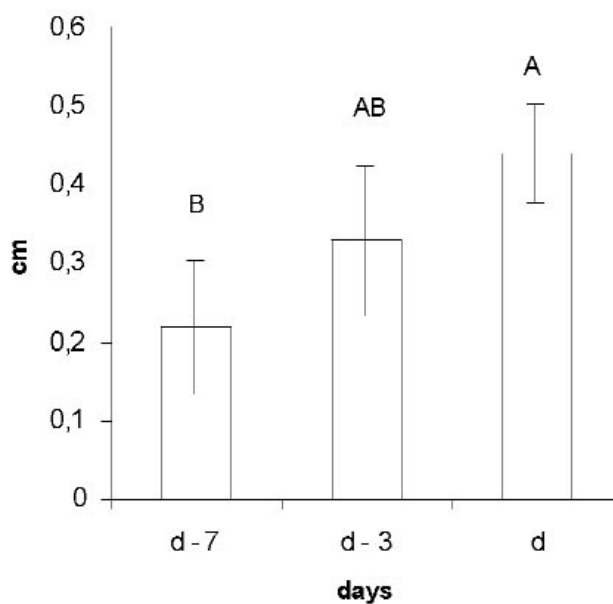


Fig. 8. Effect of wounding on necrosis breadth, caused by the inoculation of *C. fimbriata* on detached branches. Induction by the cut lasted 7 and 3 days and was compared with a control in which the cut and the inoculation were performed in the same day.

of the experiments: difficulties in finding and/or propagating genetically homogeneous material; slow development of symptoms of wood-affecting diseases; lack of well-characterised mutants affected in response to inducers (Shapiro, 2000). Further difficulties arise if the disease is lethal. In this context, it is challenging to identify single or multiple factors able to induce an effective resistance.

NaSA and MeJA showed an interesting inhibition of

Table 2. Effect of the chemicals on *in vitro* mycelium growth, *in vitro* conidia germination, and on the results of artificial inoculation with *C. fimbriata* (C.F.) on leaf and wood discs.

Chemicals and concentrations	Mycelial growth (%) ^a	Conidia germination (%) ^a	Results of C.F. inoculation ^a	
			Leaf disc	Wood disc
NaSA 0.4	-34.1	+83.1	-	-
ACC 0.2	-17.4	+76.4	-	-
MeJA 0.2	-33.9	- 86.8	+	±
Norbornadiene	+2.0	- 77.1	-	-
Norbornadiene + ACC 0.2	-4.7	- 94.8	-	-
Antimycin	-15.9	- 98.1	-	-

^a Percentage values indicate percentage reduction (-) or increase (+) of *C. fimbriata* growth or conidia germination, respectively on PDA and agar-water supplemented with the chemicals, in comparison with values obtained on the unamended control medium. The results of *C. fimbriata* inoculation are indicated by - in the case of a delay of necrosis development or mycelial growth, by + in the case of an acceleration, by ± when no differences were noticed in comparison with 48 h-wounding/water control.

fungal growth *in vitro*: NaSA acted as a fungicide at the concentration of 50 mM, while MeJA was strongly fungistatic between 5 and 500 mM.

The concentrations of chemical used for the inducing treatments were those commonly used to induce resistance. In this work, all treatments significantly decreased fungal growth *in vitro*, with the exception of norbornadiene. However, reduction of growth by antimycin, ACC and the combination ACC + norbornadiene, can be considered negligible. The growth rate obtained with the combination ACC + norbornadiene was intermediate between PDA or norbornadiene and ACC. This might be explained by the norbornadiene-induced inhibition of ethylene action (Sisler and Young, 1984; Peck and Kende, 1995).

The inhibitory effects of MeJA and antimycin on mycelial growth were paralleled by even more evident effects on conidial germination. In contrast, NaSA and ACC stimulated conidial germination. Norbornadiene, alone or in combination with ACC, significantly decreased conidial germination, again confirming its potential as a fungal inhibitor of ethylene action. The effects of SA, ACC and norbornadiene indicate that SA and ethylene play a role in promoting *C. fimbriata* conidial germination. As to ethylene, this has been reported for other pathogenic fungi, such as *Colletotricum* spp. (Flaishman and Kolattukudy, 1994) and *Botrytis cinerea* (Pers. ex Fr.) (Kepczynsky and Kepczynska, 1977; Kepczynska 1993). As to *C. fimbriata*, the amount of growth of different isolates, on artificial media and on sweet potato and carrot root, positively correlated with the ethylene concentrations detected above the growing fungus (Chalutz and DeVay, 1969).

The data obtained in this work indicate that a direct mode of action, in the biological assays, might be likely and preponderant for antimycin and norbornadiene.

Further studies would have to be done to elucidate whether, besides their fungitoxic action, they also operate through induction of host disease resistance.

With regard to NaSA and ACC, the consistent stimulation of conidial germination probably counteracts their inhibition of mycelial growth during infection. Moreover, it is reported that in leaf discs of kiwi fruit treated with 0.4 mM SA, SA metabolites declined rapidly after 19-43 h, suggesting rapid absorption and metabolism of SA in leaves (Reglinski *et al.*, 1997).

In both disc assays, the chemicals tested decreased necrosis/mycelial growth, with the exceptions discussed above. In particular MeJA enhanced the process on leaf discs. The results of biological assays and *in vitro* studies on the fungus are summarized in Table 2 which shows the complexity of this matter.

Considering the opposite direct effects of NaSA and ACC on mycelial growth and conidial germination, it remains difficult to determine the final effect on the results of disc inoculations. However it seems that a direct fungitoxic effect is likely for NaSA, but stimulation of plant defences cannot be excluded for either compound.

Compared to the 48 h-wounding/water control, MeJA increased necrosis development on leaf discs, despite its direct inhibitory effect on the fungus. It is too early to rationalize this result, but some observations can be made. By partially suppressing the wounding effect, JA could possibly play a role in dampening resistance signalling or in inducing susceptibility, at least in leaf tissue. On the other hand an apparent paradox must be noted: JA is generally one of the substances involved in wounding signalling (Bostock, 1999; Turner *et al.*, 2002). However our finding seems to fit with the results of research that demonstrated an increase in JA, during the response of a cell-suspension culture of susceptible *P. × acerifolia* to treatment with the *C. fimbriata* 66 KDa glycoprotein

elicitor (Alami *et al.*, 1999). These speculations would encourage further studies on JA.

The role of SA and ACC on induced resistance can be interpreted by the existence of either distinct or interacting pathways of signals that enhance the resistance of plane to *C. fimbriata*. This interpretation can be supported by the knowledge that emerges from the literature namely the existence of at least two different signalling pathways controlling pathogen-induced-SAR and rhizobacteria-induced-ISR. The first is based on endogenous SA (Gaffney *et al.*, 1993; Delaney *et al.*, 1994), the second is based on the sensitivity to JA and ethylene (Pieterse *et al.*, 1998, 2000). The two types of induced resistance can be active against the same pathogen (Van Wees *et al.*, 1999). Finally, a positive co-ordination of ethylene and SA has been demonstrated in the susceptible disease response of tomato to a bacterial infection and in the ozone response of *Arabidopsis* (Overmyer *et al.*, 2000; Rao *et al.*, 2000; O'Donnel *et al.*, 2001).

In this work SA and MeJA showed opposite actions, not only as to the outcome of leaf disc inoculations but also as to the influence on conidial germination. This seems to be consistent with the well-known reciprocal inhibition occurring between SA and JA signalling pathways. However this also is a complex matter because some evidence indicates that SA and JA can also act synergistically (Bostock, 1999; Genoud and Métraux, 1999; Overmyer *et al.*, 2000; Rao *et al.*, 2000; Shapiro, 2000; Turner *et al.*, 2002; Kachroo *et al.*, 2003; Spoel *et al.*, 2003).

Enhancement of disease resistance by wounding was observed locally in the disc assays, and systemically in detached branches. The mechanism by which wounding acts in plane is unknown and it would be important to ascertain if wounding can induce genuine systemic resistance. In plane trees, histological modifications such as suberization, lignification of cell walls, tyloses, are associated with branch wounds at least 10 to 20 days old. Related to this, a progressive decrease of receptivity to *C. fimbriata* by wounds was also observed (Montecchio *et al.*, 1994). A resistance response to *Ceratocystis polonica* (Siem.) C. Moreau, in Norway spruce, was induced by wounding, and further amplified by pre-inoculation at a lower density with the same fungus (Christiansen *et al.*, 1999). Generally, it is well known that plants quickly react to wounding, locally and systemically, by activating diverse mediators and genes involved in healing and pathogen defence (Pena-Cortés *et al.*, 1995; O'Donnell *et al.*, 1996; Koiwa *et al.*, 1997; Wasternack and Parthier, 1997; Narváez-Vasquez *et al.*, 1999; Seo *et al.*, 1999; Meskiene and Hirt, 2000).

This paper describes a number of bioassays that can be used to determine the effect of various treatments on the susceptibility of plane to *C. fimbriata*. Known inducers of resistance (SA, JA, ACC) were all found to have a direct effect on *C. fimbriata*. An additional effect of these compounds on induced defences of plane is how-

ever not excluded and needs now to be tested. Wounding led to an increase in resistance and was the only treatment that acted via the host metabolism. This offers the potential for further studies to determine the histological and molecular basis of the defence mechanism.

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