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Bioassays on *Leptographium wingfieldii*, a bark beetle associated fungus, with phenolic compounds of Scots pine phloem

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Abstract

The fungicidal activity of five phenolic metabolites occurring in Scots pine phloem (Cat, Tax, PS, PC and PSM) and of phenolic extracts from unwounded (fresh) and wounded phloem of Scots pine were measured against *Leptographium wingfieldii*, a fungus associated with *Tomicus piniperda*. Bioassays were performed in micro-wells in a standard medium in the absence and presence of Scots pine phloem. In both media, methanol extracts from the unwounded and wounded phloem, PS, PSM and PC inhibited fungal growth. Tax and Cat had no effect or stimulated fungal growth. The presence of Scots pine phloem in the liquid medium reduced the inhibitory effect of phenolic compounds. At low concentrations (10^{-4}–10^{-5} M), the fungus seemed to be able to degrade PS, PSM and PC while, whatever the Cat and Tax concentrations, it did not seem to degrade them. These results suggest that three phenolic compounds have a potential fungitoxicity *in vitro* and that, *in vivo*, these phenols could play a role in the efficiency of the induced reaction to stop fungal growth.

Abbreviations: Cat – (+)-catechin; Tax – Taxifolin; PS – Pinosylvin; PSM – Pinosylvin monomethylether; PC – Pinocembrin.

Introduction

Induced responses of the phloem of conifers is the basic mechanism of resistance to attacks by bark beetles and their associated fungi (Reid et al., 1967; Berryman, 1972; Raffa and Berryman, 1983; Christiansen et al., 1987; Långström et al., 1992; Lieutier, 1993). This reaction is visible as a longitudinal elliptical resin-impregnated zone around each point of attack and seems to be induced by the boring activity of the insect and stimulated by the fungi associated with bark beetles (Lieutier, 1993; Lieutier et al., 1995).

Various factors have been proposed to explain the failure of attacks by bark beetles. Nutritional isolation certainly plays a role because of the physical and chemical barrier constituted by the resinous reaction zone (Wong and Berryman, 1977; Mullick, 1977). The toxicity of the phenolic and terpene compounds located in the reaction zone can also be involved.

In Scots pines, previous investigations have demonstrated that the phloem content of secondary metabolites changes after artificial inoculation with fungi, as well as after bark beetle attacks (Delorme and Lieutier, 1990; Langström et al., 1992; Lieutier et al., 1996a; Bois and Lieutier, 1997). Terpene concentrations increase considerably, but no noticeable changes have been observed in the relative terpene composition of phloem (Delorme and Lieutier, 1990; Långström et al., 1992). Moreover, terpenes are toxic for fungi and for bark beetles (Delorme and Lieutier, 1990). On the other hand, phloem phenolic content considerably changes after artificial introduction of bark beetles or their associated fungi. The stilbenes PS and PSM, as well as the flavonoid PC, not detected in the unwounded phloem accumulate in the reaction zone, while the concentration of others decreases (Lieutier et al., 1996a; Bois and Lieutier, 1997). Moreover, these modifications vary according to the resistance level of Scots pine

clones (Bois and Lieutier, 1997). Thus, phenolic compounds could be involved in the mechanisms of Scots pine resistance to bark beetles and their associated fungi. However, these phenols have never been tested on bark beetles and their associated fungi, although some are known to have a strong inhibitory effect on fungi (Rennerfelt, 1945; Rennerfelt and Nacht, 1955; Coutts, 1970; Loman, 1970 a; Gibbs, 1972; Hart, 1981).

This study is part of a programme aimed at understanding the mechanisms involved in the response of Scots pine (*Pinus sylvestris*) phloem to inoculation with fungi and to bark beetle attacks, and determining whether phenolic compounds could contribute to the failure of the attacks. This paper reports on the interactions between *Leptographium wingfieldii*, a fungus associated with *Tomicus piniperda* (Lieutier et al., 1989) and phenols from Scots pine phloem. This fungus does not seem to play a major role in the establishment of *T. piniperda* populations in Scots pine, probably due to its low frequency on the beetles (Lieutier et al., 1995). However, since it can kill healthy Scots pines after mass inoculations (Solheim and Långström, 1991; Solheim et al., 1993) it is an excellent tool for studying the response of Scots pine phloem to aggressors (Solheim and Långström, 1991; Lieutier et al., 1996b).

Materials and methods

Fungal material

The fungus *L. wingfieldii* Morelet was initially isolated from *T. piniperda* L. and its galleries. It was purified by monospore cultures and maintained on malt-agar 3%. Spores of *L. wingfieldii* were obtained from malt-agar cultures incubated at 20 °C in the dark for two weeks. For the bioassays, spore suspensions of $2 \cdot 10^6$ spores ml^{-1} were prepared in sterile, deionized water.

Bioassays

Two liquid media were used: Medium I was the standard medium prepared by adding 15 g malt extract and 5 g bacto peptone to 1 l distilled water. Medium II was medium I to which 0.2% of ground freeze-dried phloem were added. For the bioassays, aliquots of a methanolic solution of Cat, Tax, PS, PSM, PC or phloem phenolic extracts were added aseptically to test tubes containing 2 ml of sterilized medium (I or II depending on the bioassays).

In medium I, the pure phenolic compounds were tested alone or in combination. Concentrations varied from slightly higher to lower than those previously observed in the tree tissue (Lieutier et al., 1996a; Bois and Lieutier, 1997). Tax, PSM and PC were tested at 10^{-5}, 10^{-4} and 10^{-3} M, PS at 10^{-5}, 10^{-4}, $3 \cdot 10^{-4}$ and 10^{-3} M and Cat at 10^{-4}, 10^{-3} and 10^{-2} M. For the combinations, the concentrations tested were those which were partially active when the compounds were tested alone: Cat + PSM and Cat + PC (10^{-3} M Cat, 10^{-4} M PSM or PC), Cat + PS (10^{-3} M Cat, 3.10^{-4} M PS), Tax + PS (10^{-4} M Tax, $3 \cdot 10^{-4}$ M PS), Tax + PSM and Tax + PC (10^{-4} M), PSM + PC ($5 \cdot 10^{-5}$ M), PSM + PS and PC + PS ($5 \cdot 10^{-5}$ M PC or PSM, $1.5 \cdot 10^{-4}$ M PS). In medium II, Tax, PSM and PC were tested at 10^{-5}, 10^{-4} and 10^{-3} M, PS at 10^{-4}, $3 \cdot 10^{-4}$, 10^{-3} M, and Cat at 10^{-4}, 10^{-3}, 10^{-2} M. In the two media, in all bioassays with whole phenolic extracts, we used extracts previously characterised by HPLC (Bois and Lieutier, 1997). These extracts came from 30 mg of ground freeze-dried phloem extracted according to a method described by Lieutier et al. (1996a). For the tests, a 0.5 ml aliquot of the final extract (2 ml) was dried under vacuum and 0.1 ml of methyl alcohol was added to the residue. In all bioassays, the concentration of methanol used to dissolve the different compounds was 1%. Three controls were used for each test: absolute methanol solvent (concentration 1%) with fungus; absolute methanol (concentration 1%) without fungus; methanolic solutions (concentration 1%) of phenols without fungus.

Bioassays were conducted in sterilised micro-wells on polystyrene plates, according to a method previously described by Høiland and Dybdahl (1993). Each plate consisted of 12 columns and 8 rows, each column representing one particular treatment and each row representing one replicate. Each control and each treatment were replicated eight times. Two hundred µl of liquid medium (I or II) with or without phenols were deposited aseptically in each well. Then, 10 µl of the spore suspension was added. For the controls without fungus, 10 µl of deionized water was added.

The plates were stored at 20 °C in an incubator for 4–6 days. Variations in fungal growth were indicated by variations in light absorbency (450 nm) through the micro-wells. The absorbency values were obtained from a microplate reader (MR 5000, Dynatech). Two absorbency measurements were performed, one at the beginning of the experiment, the second when a vigorous fungal growth was observed in the control wells

without phenols (after four days in medium I, six days in medium II). The increase in absorbency was then calculated individually for each well by subtracting the absorbency at the beginning of the experiment from that at the end. The estimated fungal growth in a particular phenolic concentration was assessed by the difference between the increase in absorbency in the wells with fungus and the increase in absorbency in the wells without fungus.

In order to estimate the stability of pure phenolic compounds alone, and fungus effect on these compounds, the media with or without fungus were extracted with ethyl acetate at the end of the experiment. All replicates from a particular concentration were pooled, filtered to remove the mycelium, and then extracted. Phenolic compounds were analysed and quantified by HPLC (310 nm, except for catechin at 280 nm) according to a method previously described (Lieutier et al., 1996a).

Statistical methods

The data were analysed by using SAS software (SAS Institute Inc. 1985, 1986, 1987). Treatments were tested by one way analysis of variance (ANOVA procedure), with Tukey test for multiple comparisons. All means were expressed with their confidence interval at the 95% level. Differences between means were taken into account only when significant at the 95% level.

Results and discussion

Interactions between *L. wingfieldii* on the one hand, and the whole phenolic extracts from wounded and unwounded phloem and five pure phenolic compounds of Scots pine phloem on the other hand were performed in liquid media, with or without phloem powder. The effect of these compounds on fungal growth was investigated in micro-wells by measuring light absorbency through the micro-wells. The effect of the fungus on the phenolic compounds was investigated at the end of the experiment by extracting the media and analysing them by HPLC.

Effects of pure phenols on fungal growth

At 10^{-2} M, Cat had a stimulating effect on the growth of *L. wingfieldii*, while Tax had no effect whatever the concentration used (Figure 1). This corroborates other studies on the effects of Tax and Cat on fungal growth. Indeed, Tax and its glucoside are already known to stimulate or to have no effect on the growth of litter-decomposing basidiomycetes, while Cat stimulated these fungi (Lindeberg et al., 1980) as well as the *Aspergillus* genus (Weidenbörner et al., 1990).

Growth of *L. wingfieldii* was partially inhibited by concentrations of PSM at 10^{-4} M, PC at 10^{-4}–10^{-3} M and PS at $3 \cdot 10^{-4}$ M (Figure 1). Moreover, a complete inhibition was obtained with PS or PSM at 10^{-3} M. Similarly, these three compounds are already known to inhibit spore germination and fungal growth of various fungi such as *Coniophora puteana*, *Merulius lacrymans*, *Polyporus pinicola*, *Fomes annosus*, *F. pini*, *Peniophora gigantea*, *P. balsameus*, *P. pseudo pini*, *Poria sericeomollis*, *Amylosterum areolatum*, *Melampsora medusae* and *Marssonina brunnea* at concentrations of 10^{-3}–10^{-4} M (Rennerfelt, 1945; Rennerfelt and Nacht, 1955; Coutts, 1970; Loman, 1970a; Gibbs, 1972; Hart, 1981; Shain and Miller, 1982). On the other hand, our results are in disagreement with the results of Rennerfelt and Nacht (1955), Hart and Shrimpton (1979) and Schultz et al. (1994) who observed that the fungal growth of three blue-stain fungi (*Ophiostoma pini*, *Ceratocystis montia* and *C. clavigera*), the group to which *L. wingfieldii* belongs, were particularly tolerant to PS and PSM at concentrations of 10^{-3}–10^{-4} M.

In most cases, the phenolic combinations (Figure 2) tested in our study did not modify the effect of PS, PSM or PC on fungal growth, when compared to the effect of the same compounds used alone in the medium. However, when Cat was added to PSM the effect of PSM on fungal growth was lowered. Our results with PS, PSM and PC are in accordance with those of Schultz et al. (1991), who observed no synergism when various combinations of stilbenes and bibenzyls were tested on fungi. On the other hand, they disagree with those of Loman (1970a) who reported an increase in the inhibitory effect. However, in Loman's experiment, the fungus species differed from those used in the present study, and the total phenol concentrations of inhibitory compounds was twice as high as the concentrations used in the assays with the compounds alone. It seems difficult to conclude about the mechanisms involved in the modulation caused by Cat on PSM effect. It might only be due to an additive effect of the two compounds.

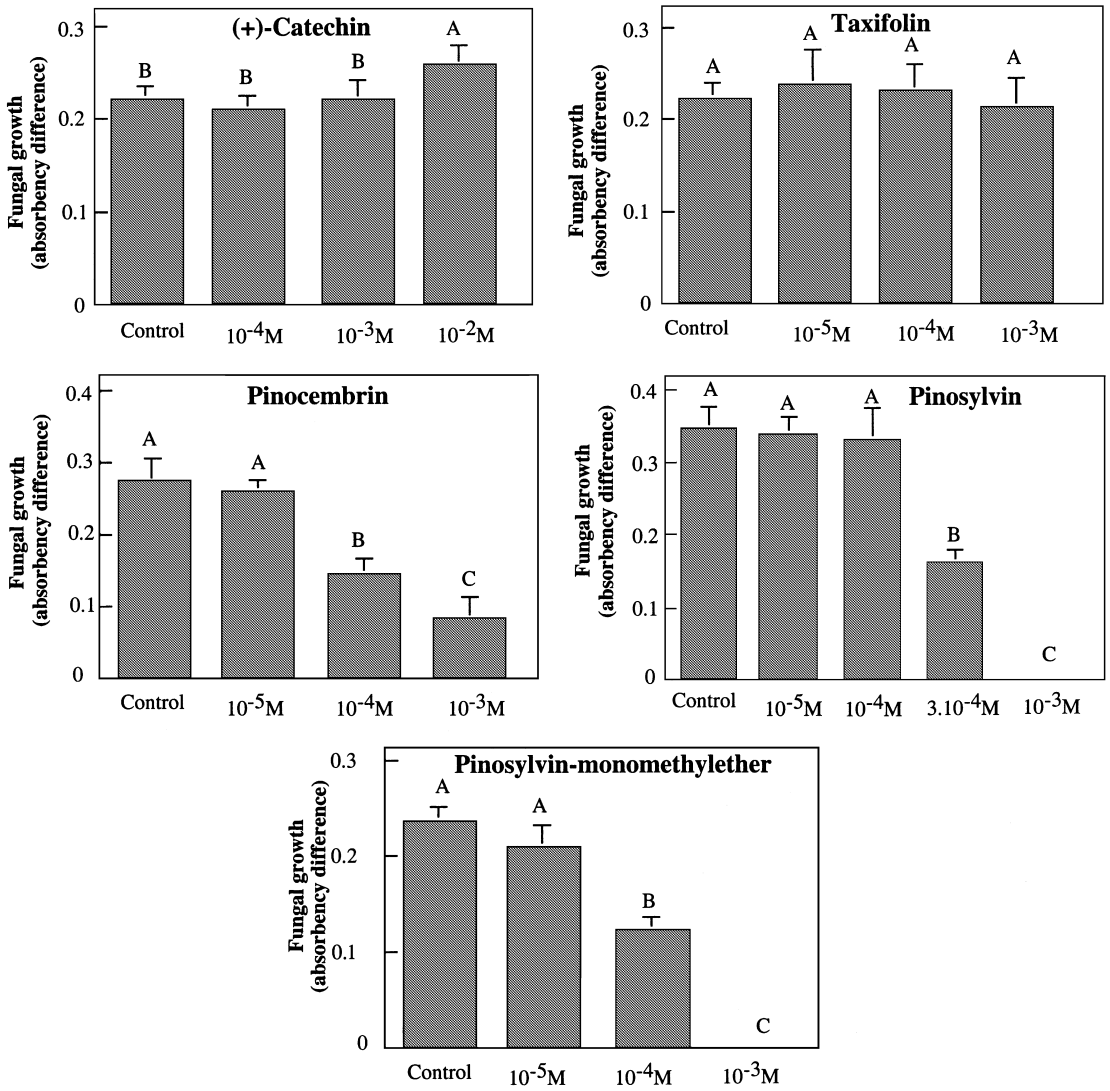

Figure 1. Growth of *L. wingfieldii* in medium I (standard liquid malt medium) containing different concentrations of (+)-catechin, taxifolin, pinocembrin, pinosylvin and pinosylvin monomethylether. Control is medium I plus methanol alone (1%). Values are means ± confidence intervals ($n = 8$ for each sample). Values followed by the same letter do not significantly differ ($p = 0.05$).

Effects of phloem addition to assays

The fungus seemed to grow better in medium II than in a medium I. This could be due to the presence of phloem in medium II.

In the medium containing phloem powder, Cat lost its stimulatory effect on fungal growth, and Tax between 10^{-4} and 10^{-3}M stimulated it (Figure 3). PS, PSM and PC inhibited *L. wingfiledii* but the effective inhibitory concentrations were higher than in the medium without phloem (Figure 3). Schultz et al. (1991) have already reported that tests in woody substrate required higher levels of stilbenes. According to Loman (1970a), Hart and Hillis (1974) and Hart and Shrimpton (1979) the antifungal property of most stilbenes decreases by 90–99%, when bioassays are conducted on woody substrate, which is not completely in accordance with our results. The differences in the inhibitory concentrations between the two media may be explained by an induction and a synthesis of fungal enzymes depending on the substrate (Loman, 1970a; Hart and Hillis, 1974). According to these authors,

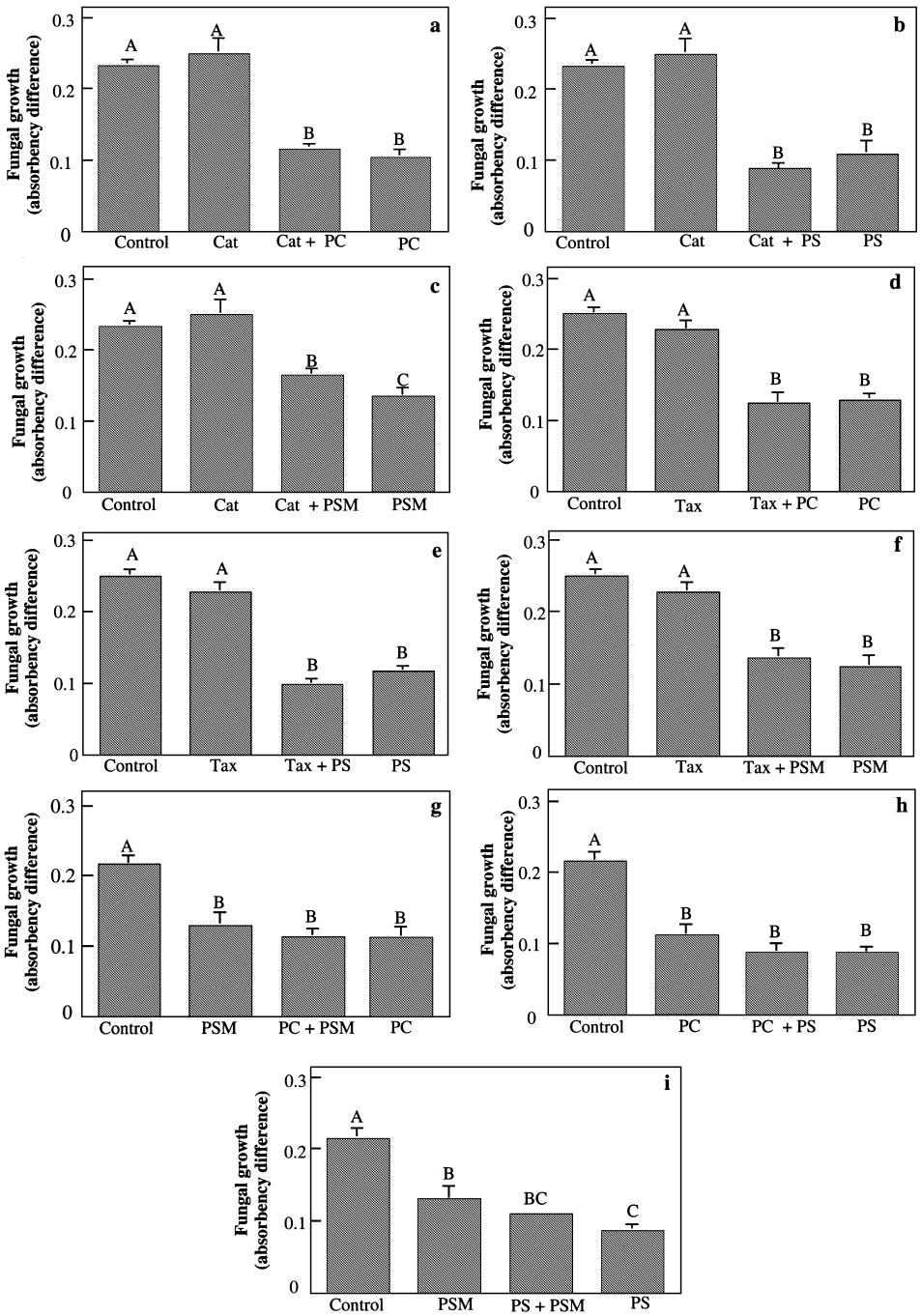

Figure 2. Growth of *L. wingfieldii* in the medium I (standard liquid malt medium) containing different combinations of phenolic compounds. (+)-catechin, taxifolin, pinocembrin, pinosylvin, and pinosylvin monomethylether. Control is medium I plus methanol alone (1%). (a, c) Cat: 10^{-3} M, PC, PSM: 10^{-4} M; (b) Cat, Cat + PS: 10^{-3} M, PS: $3 \cdot 10^{-4}$ M; (d, f) Tax, PSM, PC (10^{-4} M); (e) Tax, Tax + PS: 10^{-4} M, PS: $3 \cdot 10^{-4}$ M; (g) PSM, PC: 10^{-4} M, PSM + PC: $5 \cdot 10^{-5}$ M; (h) PC: 10^{-4} M, PS: $3 \cdot 10^{-4}$ M, PS + PC: $1.5 \cdot 10^{-5}$ M + $5 \cdot 10^{-5}$ M; (i) PSM: 10^{-4} M, PS: $3 \cdot 10^{-4}$ M, PSM + PS: $5 \cdot 10^{-5}$ M + $1.5 \cdot 10^{-5}$ M. Values represent means ± confidence intervals ($n = 8$ for each sample). Values followed by the same letter do not significantly differ ($p = 0.05$).

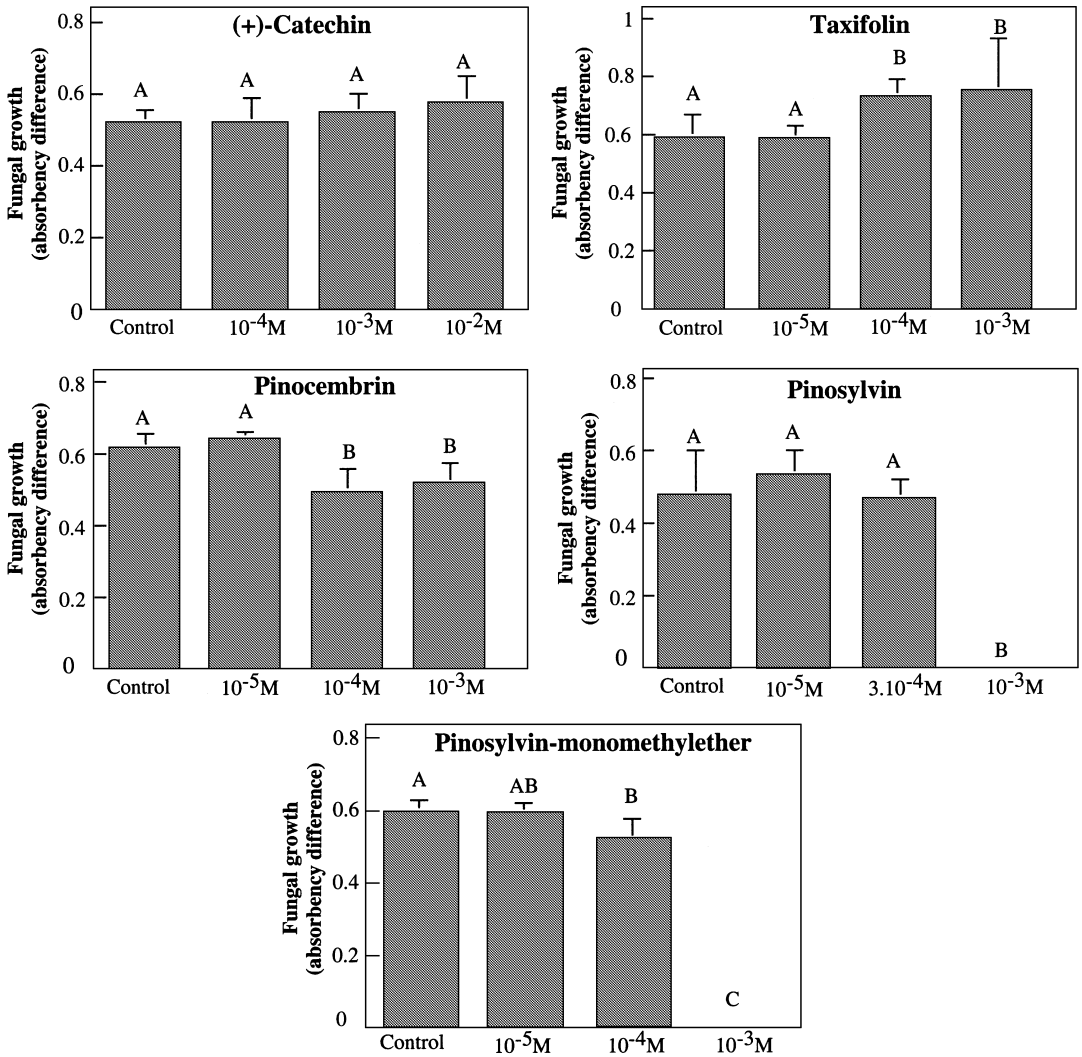

Figure 3. Growth of *L. wingfieldii* in the medium II (medium I + phloem powder) containing different concentrations of (+)-Catechin, taxifolin, pinocembrin, pinosylvin, and pinosylvin monomethylether. Control is medium II plus methanol alone (1%). Values represent means ± confidence intervals ($n = 8$ for each sample). Values followed by the same letter do not significantly differ ($p = 0.05$).

the use of a substrate containing wood reduces the time interval for maximum fungal enzyme activity which can degrade phenols, in comparison with a standard medium (malt and agar). Moreover, Hart and Shrimpton (1979) reported that in a standard medium (malt and agar), stilbenes are not bound to other constituents and hence are free to interact with the fungus, while with wood in the medium, stilbenes bound with lignin, which can reduce their toxicity. According to these authors, cellulose was ineffective as a binding agent. As lignin lacks in phloem, in our bioassays, we used mainly cellulose. The differences observed between the two media could thus very likely be due to the differences in the synthesis of the fungal enzymes. It is also possible that phloem compounds still present after autoclaving stimulated fungal growth or inhibited the effect of the tested chemicals. However, such phloem compounds were certainly not phenolics since whole phenolic extracts themselves inhibited fungal growth (see below and Figure 4).

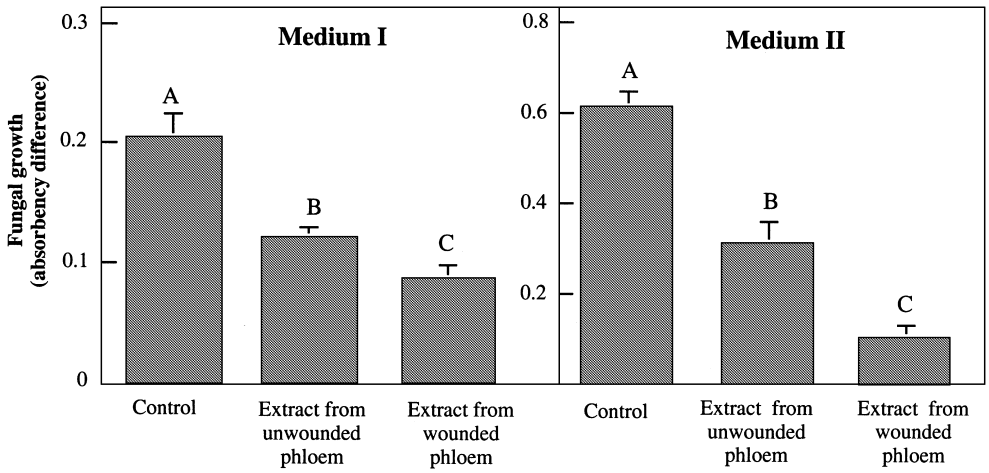

Figure 4. Growth of *L. wingfieldii* in medium I and in medium II, each containing whole phenolic extracts (methanolic solutions) from 30 mg of freeze-dried and ground unwounded or wounded phloem. Control is medium I plus methanol alone (1%) or medium II plus methanol alone (1%). Values represent means ± confidence intervals ($n = 8$ for each sample). Values followed by the same letter do not significantly differ ($p = 0.05$).

Variations in phenolic concentrations (*Table 1*)

At the end of experiment, with or without fungus, whatever the compounds introduced, HPLC analyses never revealed new phenolic compounds.

Whatever the liquid medium, in controls without fungus, all phenols were still present at the end of the bioassays, but their concentration was lower than that introduced at the beginning of the experiment. Probably, this can be explained by adsorption of these compounds on the micro-well walls, but degradation may also occur.

In presence of the fungus, additional phenomena could be involved, due to the presence of the fungus, since at concentrations partially inhibitive or not inhibitive of PS, PSM and PC, phenolic concentrations were lower when the fungus was present than when it was not. Although no new compounds were detected, such metabolites could stay inside fungal tissue or could not be phenolics. At low concentrations of PS, PSM and PC, the compounds introduced were not detected at the end of the experiment and *L. wingfieldii* was not inhibited. This non-detection could be due to a direct or an indirect effect of the fungus on the phenolics. Adsorption on the fungal cell walls, binding with fungal extracellular enzymes (Farkas and Kiraly, 1972; Johansson et al., 1976), or uptake of phenolics by the fungus could occurr. The degradation of these compounds by the fungal enzymes could also occur. Indeed, such a degradation of stilbenes and PC has previously been observed (Loman, 1970b; Sbaghi et al., 1996).

In all cases, on the other hand, Tax and Cat were still present at the end of the experiment, in similar concentration with fungus than without fungi. Thus, *L. wingfieldii* did not seem to degrade Tax and Cat and these two compounds did not seem to be adsorbed or uptaked by the fungus.

Effect of the whole phloem phenolic extract on fungal growth

In the two media, phenolic extracts from wounded and unwounded phloem inhibited fungal growth (Figure 4). The comparison between phenolic extracts from wounded and unwounded phloem has previously shown that PS, PSM and PC are present in the wounded phloem, while they are not detected in the unwounded phloem (Bois and Lieutier, 1997). The present experiment showed that these three compounds inhibited fungal growth (Figure 1). This suggests that the higher toxicity of the extract from the wounded phloem is due to these three compounds. However, because the extract from unwounded phloem also inhibited fungal growth, other compounds not assayed in our experiment probably interfered. Indeed, only some phenolic compounds of Scots pine phloem were tested in our study.

Table 1. Phenolic compounds (mg ml^{-1}) detected in the media at the end of the bioassay

Phenolic compounds	Concentration introduced		Concentrations at the end of the experiment (mg ml^{-1})			
			Medium I		Medium II	
	(mol l^{-1})	(mg ml^{-1})	without fungus	with fungus	without fungus	with fungus
CAT	10^{-4}	0.029	0.017	0.016	0.030	0.027
	10^{-3}	0.29	0.14	0.14	0.18	0.18
	10^{-2}	2.90	1.71	1.64	1.89	1.74
TAX	10^{-5}	0.003	0.001	0.001	0.003	0.003
	10^{-4}	0.03	0.020	0.019	0.019	0.012
	10^{-3}	0.30	0.21	0.20	0.09	0.08
PS	10^{-5}	0.002	0.001	—	/	/
	10^{-4}	0.021	0.006	—	0.008	—
	$3 \cdot 10^{-4}$	0.063	0.042	0.001	0.026	—
	10^{-3}	0.21	0.06	0.06	0.06	0.05
PSM	10^{-5}	0.002	0.002	—	0.001	—
	10^{-4}	0.022	0.02	0.003	0.02	0.001
	10^{-3}	0.22	0.05	0.05	0.05	0.05
PC	10^{-5}	0.002	0.001	—	0.002	—
	10^{-4}	0.025	0.025	0.011	0.025	0.002
	10^{-3}	0.25	0.07	0.06	0.14	0.13

— – not detected; / – not tested.

Other phenolic compounds, such as acetophenone glycoside, para coumaric acid ester and tannins, which have already been detected in Scots pine phloem (Lieutier et al., 1996a; Bois and Lieutier, 1997) might also inhibit the growth of *L. wingfieldii*.

Conclusions

In vitro, at high concentrations, PS, PSM and PC were toxic for *L. wingfieldii* and the fungus seemed able to degrade them at low concentrations. On the contrary, it did not degrade Cat and Tax, compounds which either stimulated or had no effect on fungal growth. All these compounds are met by the fungus in the tree (Bois and Lieutier, 1997), but *L. wingfieldii* thus appeared able to act only on those inhibiting its growth. Similar conclusions have already been reported in other systems (Hart, 1981). This can be interpreted as an adaptative reaction of the fungus to the presence of these compounds and is in favour of a toxic effect of PS, PSM and PC *in vivo*. However, other compounds not tested in our assays are also present in the phloem of the tree (Lieutier et al., 1996; Bois and Lieutier, 1997), and could modify the effect of PS, PSM and PC. In our assays, in combination with Tax or Cat, PS, PSM and PC retained their *in vitro* toxic effect. Moreover, the whole phenolic phloem extracts were toxic to *L. wingfieldii*, demonstrating that the *in vitro* inhibitory effects were predominant over the stimulating effects. In addition, although the toxic effect of the unwounded phloem extract demonstrates that other phenolic compounds interfere, the higher toxicity of the wounded phloem suggests that PS, PSM and PC were toxic even in the presence of all the other compounds. The presence of Scots pine phloem in the medium reduced the *in vitro* toxic properties of PS, PSM and PC, but these compounds were still toxic at concentrations comparable to those met in the tree tissues, at least for PSM and PC (Bois and Lieutier, 1997).

The results of our assays are in agreement with the *in vivo* variations in the phloem phenol content of Scots pines after inoculation with *L. wingfieldii* (Bois and Lieutier, 1997). In that previous study, we have observed that, in response to fungal inoculations, PS, PSM and PC accumulated in Scots pine phloem and that their concentrations stabilised after 14 days. As a consequence of the above conclusions and because these compounds were toxic *in vitro* at concentrations lower or equal to those measured *in vivo* (Bois and Lieutier, 1997), we hypothesise that PS, PSM and PC are toxic for *L. wingfieldii in vivo*. Their accumulation

would play a role in stopping fungal growth in the tree. The stabilization of their concentrations 14 days after a fungus inoculation would be the result of a balance between their degradation rate by the fungus and their accumulation rate in the tree. Their efficiency in the phloem induced reaction would depend on the stabilization level.

Acknowledgments

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