Available online at www.sciencedirect.com
SCIENCE DIRECT
Phytochemistry 66 (2005) 2609–2616
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Camalexin induces detoxification of the phytoalexin brassinin in the plant pathogen *Leptosphaeria maculans*

M. Soledade C. Pedras *, Mukund Jha, Oladapo G. Okeola

Department of Chemistry, University of Saskatchewan, 110 Science Place, Saskatoon, SK, Canada S7N 5C9

Received 9 July 2005; received in revised form 18 August 2005
Available online 2 November 2005

Abstract

The impact of the phytoalexins camalexin and spirobrassinin on brassinin detoxification by *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.], a pathogenic fungus prevalent on crucifers, was investigated. Brassinin is a plant metabolite of great significance due to its dual role both as an effective phytoalexin and as an early biosynthetic precursor of the majority of the phytoalexins produced by plants of the family Brassicaceae (Cruciferae). The rate of detoxification of brassinin in cultures of *L. maculans* increased substantially in the presence of camalexin, whereas spirobrassinin did not appear to have a detectable effect. In addition, the brassinin detoxifying activity of cell-free extracts obtained from cultures incubated with camalexin was substantially higher than that of control cell-free extracts or cultures incubated with spirobrassinin, and correlated positively with brassinin oxidase activity. The discovery of a potent synthetic modulator of brassinin oxidase activity, 3-phenylindole, and comparison with the commercial fungicide thiabendazole is also reported. The overall results indicate that brassinin oxidase production is induced by camalexin and 3-phenylindole but not by spirobrassinin or thiabendazole. Importantly, our work suggests that introduction of the camalexin pathway into plants that produce brassinin might make these plants more susceptible to *L. maculans*.

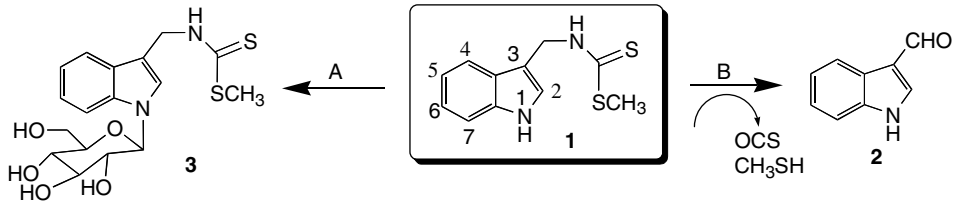
Keywords: Brassicaceae; Brassinin oxidase activity; Dithiocarbamate; Camalexin; *Leptosphaeria maculans*; *Phoma lingam*; Spirobrassinin

1. Introduction

The naturally occurring dithiocarbamate brassinin (**1**) is a plant metabolite of great significance due to its dual role both as an effective phytoalexin and as an early biosynthetic precursor of the majority of the phytoalexins produced by plants of the family Brassicaceae (Cruciferae) (Pedras et al., 2003). Phytoalexins are crucial chemical defenses produced de novo by plants to fight pathogens (Bailey and Mansfield, 1982; Essenberg, 2001). Coincidentally, the dithiocarbamate moiety of brassinin is known to be a potent toxophore in synthetic agrochemicals used to control fungi and weeds (Leroux, 2003; Caldas et al., 2001). Despite the antifungal activity of brassinin (**1**), several crucifer pathogens can detoxify it, which can make the plant more susceptible to microbial colonization. The phytopathogenic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary detoxified brassinin (**1**) to 1-β-D-glucopyranosylbrassinin (**3**) using an inducible glucosyl transferase (Pedras et al., 2004; Pedras and Ahiahonu, 2005), whereas virulent isolates of *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.] transformed **1** into 3-indolecarboxaldehyde (**2**) (Scheme 1). The latter transformation suggested that a putative brassinin oxidase (BO) was involved in this detoxification process, but to date no such enzymes have been reported (Scheme 1) (Pedras et al., 2003). By contrast, the cruciferous phytoalexins camalexin (**5**) and spirobrassinin (**6**) did not appear to be metabolized by *L. maculans* (Pedras et al., 2003). Although the chemical structures of brassinin (**1**), camalexin (**5**), and spirobrassinin (**6**) are rather different, indolyl-3-acetaldoxime (**4**) is their common biosynthetic precursor (Pedras et al., 2003; Glawischnig et al.,

* Corresponding author. Tel.: +1 306 966 4772; fax: +1 306 966 4730.
E-mail address: s.pedras@usask.ca (M. Soledade C. Pedras).

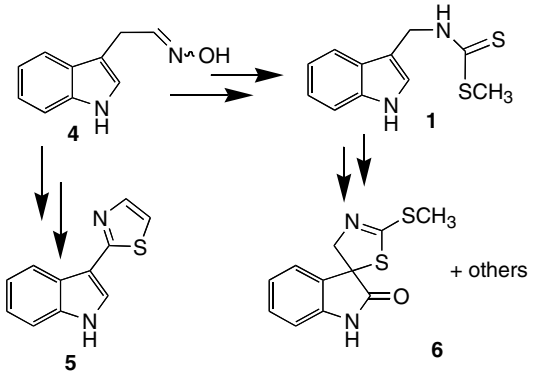
0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.09.013



Scheme 1. Detoxification of brassinin (**1**) by plant pathogenic fungi: (A) *Sclerotinia sclerotiorum*; (B) virulent *Leptosphaeria maculans* (*Phoma lingam*) (Pedras and Ahiahonu, 2005).

2004), and brassinin (**1**) is a closer biosynthetic precursor of spirobrassinin (**6**, Scheme 2). Nevertheless, most interestingly camalexin (**5**) has not been detected in plants that produce brassinin (**1**) and/or spirobrassinin (**6**) or vice-versa (Pedras et al., 2000). Camalexin (**5**) is produced by various wild species, including *Arabidopsis thaliana* (Pedras et al., 2000).

Considering that detoxification of brassinin (**1**) by fungal pathogens deprives plants of valuable inducible chemical defenses, it is of great interest to understand and inhibit such degradation processes. Ongoing work to design selective and environmentally safer crop protection agents against crucifer pathogens led us to investigate the potential effects of crucifer phytoalexins on brassinin (**1**) detoxification. Results of this work have shown that in *S. sclerotiorum* camalexin (**5**) was able to induce a brassinin detoxifying enzyme, brassinin glucosyltransferase (Pedras et al., 2004), and that camalexin (**5**) was also detoxified via glucosylation (Pedras and Ahiahonu, 2002). Because camalexin (**5**) and spirobrassinin (**6**) are not metabolized by *L. maculans*, and in anticipation that non-degradable phytoalexins might act in synergism, we have investigated their impact on brassinin detoxification. Despite the inhibitory activity of camalexin (**5**) on the growth of *L. maculans*, we have discovered that the rate of detoxification of brassinin (**1**) in fungal cultures increased substantially in the presence of camalexin (**5**), whereas spirobrassinin (**6**) did not appear to affect the rate of brassinin detoxification. In addition, we established that the brassinin detoxifying activity of cell-free extracts obtained from cultures incubated with camalexin (**5**) was substantially higher than that of cell-free extracts obtained from cultures incubated with spirobrassinin (**6**), which in turn was similar to that control cell-free extracts. Importantly, brassinin oxidase activity (BOA) correlated positively with the rate of brassinin transformation in cell cultures and cell-free mycelial extracts of *L. maculans*. Herein we report for the first time results of these studies together with the discovery of a potent synthetic inducer of BOA, 3-phenylindole (**7**), and comparison of its antifungal activity against *L. maculans* with that of the naturally occurring phytoalexins brassinin (**1**), camalexin (**5**) and spirobrassinin (**6**).



Scheme 2. Biosynthetic pathway of crucifer phytoalexins: indolyl-3-acetaldoxime (**4**) is a common precursor of camalexin (**5**) and brassinin (**1**), which is a closer precursor of spirobrassinin (**6**).

2. Results and discussion

2.1. Antifungal activity and kinetics of brassinin biotransformation in cultures of L. maculans

The phytoalexins brassinin (**1**), camalexin (**5**) and spirobrassinin (**6**) were synthesized following previously published procedures (Pedras et al., 2003). The inhibitory activity of each compound was established using mycelial cultures of *L. maculans*, as reported in Section 4. Results of these bioassays, as summarized in Table 1, suggested the range of concentrations to be used in biotransformation experiments with each compound. In addition, antifungal bioassays using a mixture of brassinin (**1**) + camalexin (**5**) did not show the expected synergistic or additive effects; on the contrary, brassinin (**1**) appeared to be less inhibitory of mycelial growth in the presence of camalexin (**5**). For example, brassinin at 0.2 mM caused $52 \pm 4\%$ growth inhibition whereas brassinin (**1**) + camalexin (**5**) at 0.3 mM (0.2 + 0.1 mM, respectively) showed a similar percentage of inhibition ($54 \pm 6\%$), and at 0.25 mM (0.2 + 0.05 mM, respectively) showed only $22 \pm 2\%$ inhibition (Table 1). The commercial fungicide thiabendazole (**8**) displayed substantially higher antifungal activity than any of the phytoalexins and ten times higher activity than 3-phenylindole (**7**).

Subsequently, 48-h-old cultures of *L. maculans* were incubated for 24 h with camalexin (**5**, 0.05 mM) followed by addition of brassinin (**1**, 0.2 mM) and further incubation for 24 h. Similar experiments were carried out using spirobrassinin (**6**, 0.05 mM) instead of camalexin (**5**). Control cultures of *L. maculans* containing brassinin (**1**, 0.2 mM), camalexin (**5**, 0.05 mM) or spirobrassinin (**6**,

Table 1
Effect of brassinin (**1**), camalexin (**5**), spirobrassinin (**6**), 3-phenylindole (**7**) and thiabendazole (**8**) on the inhibition of mycelial growth[a] of *Leptosphaeria maculans* in solid cultures

Compound	Concentration (mM)	Inhibition (%) ± standard deviation[a]
Brassinin (**1**)	0.5	c.i.[b]
	0.2	52 ± 4
	0.1	24 ± 5
Camalexin (**5**)	0.5	c.i.[b]
	0.2	63 ± 2
	0.1	27 ± 4
Spirobrassinin (**6**)	0.5	50 ± 5
	0.2	32 ± 4
	0.1	n.i.[c]
3-Phenylindole (**7**)	0.1	c.i.[b]
	0.05	53 ± 2
	0.01	40 ± 3
Brassinin (**1**) + camalexin (**5**)	0.2 + 0.1	54 ± 6
	0.2 + 0.05	22 ± 2
Brassinin (**1**) + 3-phenylindole (**7**)	0.2 + 0.02	43 ± 5
	0.2 + 0.01	31 ± 4
Thiabendazole (**8**)	0.01	c.i.[b]
	0.005	88 ± 2
	0.001	23 ± 3

[a] Percentage of inhibition = 100 − [(growth on medium containing compound/growth on control medium) × 100] ± standard deviation (average of three independent experiments conducted in triplicate) after 5 days of incubation on solid media (described in Section 4).
[b] c.i. = complete inhibition.
[c] n.i. = no inhibition (growth on control medium and on medium containing compound is similar).

0.05 mM) were incubated separately. The biotransformation of brassinin (**1**) was monitored by HPLC analysis (photodiode array detection at 206 and 220 nm, brassinin $R_t = 18.8$ min; 3-indolecarboxaldehyde $R_t = 6.8$ min). Samples were withdrawn after addition of brassinin (**1**) every 6 h, extracted with ethyl acetate, the extracts were concentrated, and were analyzed by HPLC. HPLC data analyses showed that the metabolism of brassinin (**1**) in cultures of *L. maculans* was consistent with previous results (Pedras and Ahiahonu, 2005). Brassinin (**1**, 0.2 mM) was almost completely metabolized to 3-indolecarboxaldehyde (**2**) within 18 h (cultures of *L. maculans* containing no other phytoalexin, Fig. 1). Most unexpectedly, however, in cultures pre-incubated with camalexin (**5**, 0.05 mM) for 24 h, brassinin (**1**, 0.2 mM) was almost completely metabolized within 6 h (Fig. 1). Furthermore, in cultures of *L. maculans* pre-incubated with spirobrassinin (**6**, 0.05 mM) for 24 h, brassinin (**1**, 0.2 mM) was almost completely metabolized within the usual 18 h (Fig. 1). That is, spirobrassinin (**6**) did not affect noticeably the rate of brassinin (**1**) transformation, whereas camalexin (**5**) increased the rate substantially. However, when brassinin (**1**) and camalexin (**5**) were added simultaneously to 48-h-old cultures of *L. maculans*, the increase in the rate of transformation of brassinin was not noticeable.

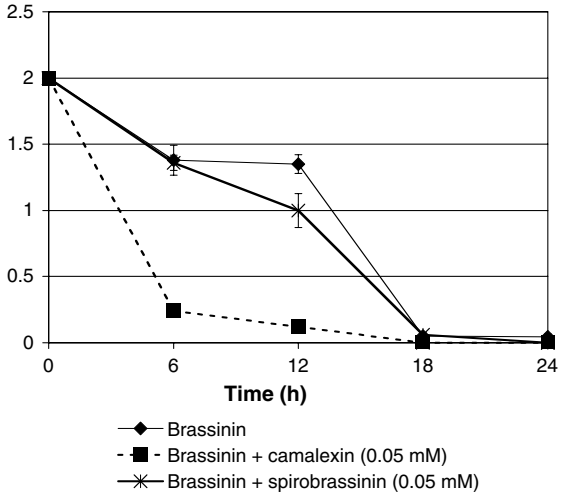


Fig. 1. Progress curves for the transformation of brassinin (**1**, 0.2 mM) in cultures of *Leptosphaeria maculans* (*Phoma lingam*) pre-incubated for 24 h with camalexin (**5**, 0.05 mM) and spirobrassinin (**6**, 0.05 mM) over a 24-h incubation period.

The rather puzzling effect of camalexin (**5**) on the rate of brassinin detoxification was further investigated using higher camalexin concentration (**5**, 0.2 and 0.1 mM). Doubling the concentration of camalexin (**5**) doubled the transformation time of brassinin (**1**) (from 6 to 12 h); a 5-fold increase in the concentration of camalexin (**5**) increased the transformation time to 18 h (Fig. 2). Visual inspection of the cultures containing brassinin (**1**, 0.2 mM) and camalexin (**5**, 0.2 mM) indicated strong growth inhibition of the mycelia, even after 18 h of incubation when almost all brassinin (**1**) had been transformed to the less toxic aldehyde **2**. These results indicated that, despite the strong growth inhibition observed in the presence of a 1:1 mixture of brassinin (**1**) and camalexin (**5**), the fungus could still metabolize brassinin (**1**) within 18 h.

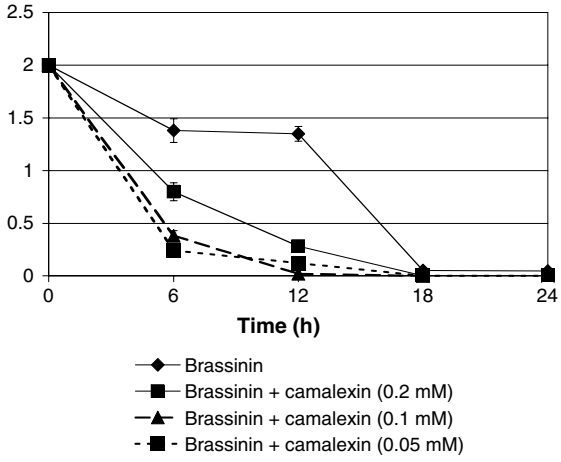


Fig. 2. Progress curves for the transformation of brassinin (**1**, 0.2 mM) in cultures of *Leptosphaeria maculans* (*Phoma lingam*) pre-incubated for 24 h with camalexin (**5**) at different concentrations (0.2, 0.1 and 0.05 mM) over a 24-h incubation period.

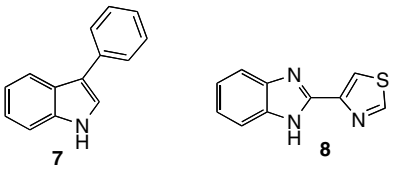


Fig. 3. Chemical structures of 3-phenylindole (**7**) and thiabendazole (**8**).

Next, we determined the potential impact of the thiazole ring of camalexin (**5**) by replacing it with a phenyl ring to afford 3-phenylindole (**7**, Fig. 3) (Rodriguez et al., 2000). Antifungal bioassays against *L. maculans* revealed that 3-phenylindole (**7**) was about five times more potent than brassinin (**1**) and camalexin (**5**), causing complete mycelial growth inhibition at 0.1 mM, as shown in Table 1. Furthermore, HPLC analyses of extracts of cultures of *L. maculans* showed that 3-phenylindole (**7**, 0.01 mM), similar to camalexin (**5**), was not metabolized in cultures of *L. maculans*. Subsequently, to further evaluate the effect of 3-phenylindole (**7**) on the metabolism of brassinin (**1**), similar to experiments described above for camalexin, 48-h-old cultures of *L. maculans* were incubated for 24 h with 3-phenylindole (**7**, 0.01 mM) followed by addition of brassinin (**1**, 0.2 mM) and further incubation for 24 h. Control cultures of *L. maculans* containing brassinin (**1**, 0.2 mM) or 3-phenylindole (**7**, 0.01 mM) were incubated separately. The metabolism of brassinin was monitored every 6 h by HPLC analyses, as described above for camalexin. As reported for camalexin (**5**) (vide supra), there was a dramatic increase in the rate of brassinin (**1**) transformation in the presence of 3-phenylindole (**7**, Figs. 4 and 5). Cultures pre-incubated with 3-phenylindole (**7**) transformed completely brassinin (**1**) in 6 h while this transformation took more than 18 h in cultures containing only brassinin (**1**). Similar to camalexin (**5**), when brassinin (**1**) and 3-phenylindole (**7**) were added simultaneously to 48-h-old cultures of *L. maculans*, the increase in the rate of transformation of brassinin was not noticeable. Similar experiments carried out with thiabendazole (**8**, Fig. 3), a commercial fungicide containing a thiazole ring as well, indicated that it had no effect on the rate of brassinin (**1**) transformation.

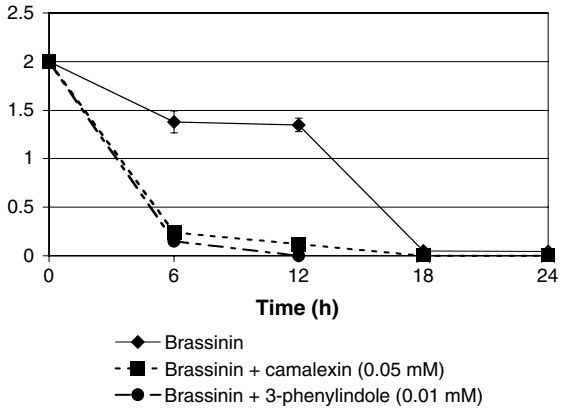


Fig. 4. Progress curves for the transformation of brassinin (**1**, 0.2 mM) in cultures of *Leptosphaeria maculans* (*Phoma lingam*) pre-incubated for 24 h with camalexin (**5**, 0.05 mM) and 3-phenylindole (**7**, 0.01 mM) over a 24-h incubation period.

In further experiments, to compare the effects of camalexin (**5**) and 3-phenylindole (**7**) on the detoxification of brassinin and on the growth of *L. maculans*, the metabolism of brassinin (**1**) in cultures pre-incubated for 24 h with 3-phenylindole (**7**) was monitored every 2 h by HPLC. After 12 h of incubation, cultures were filtered and the mycelia was allowed to dry and weighed. As show in Fig. 6, cultures of *L. maculans* pre-incubated for 24 h with 3-phenylindole (**7**) at 0.01 mM was metabolized faster than in cultures incubated either with 3-phenylindole (**7**) at

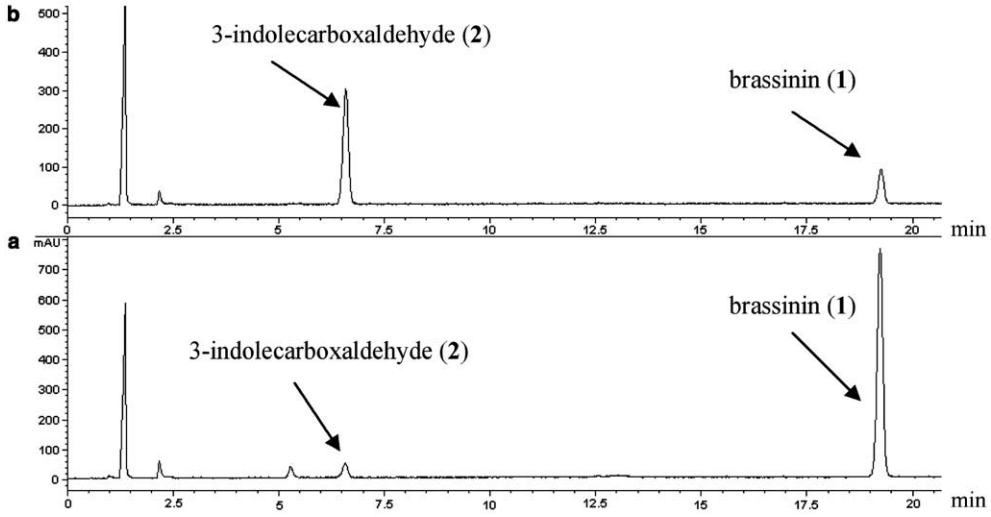


Fig. 5. HPLC chromatograms of extracts of cultures of *Leptosphaeria maculans* (*Phoma lingam*). (a) Transformation of brassinin (**1**, 0.2 mM) to 3-indolecarboxaldehyde (**2**) after 6 h of incubation. (b) Transformation of brassinin (**1**, 0.2 mM) in cultures pre-incubated for 24 h with 3-phenylindole (**7**, 0.02 mM), after 6 h of incubation.

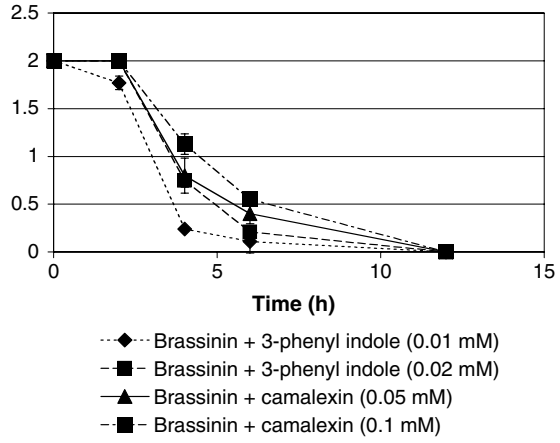


Fig. 6. Progress curves for the transformation of brassinin (**1**, 0.2 mM) in cultures of *Leptosphaeria maculans* (*Phoma lingam*) pre-incubated for 24 h with camalexin (**5**, 0.05,0.1 mM) and 3-phenylindole (**7**, 0.01, 0.02 mM) over a 12-h incubation period.

Table 2
Mycelia (dry weight) of cultures of *Leptosphaeria maculans* (*Phoma lingam*) pre-incubated for 24 h with camalexin (**5**, 0.05,0.1 mM) and 3-phenylindole (**7**, 0.01, 0.02 mM) after a 12-h incubation period

Compound added to cultures	Weight (mg) ± standard deviation
No compound (control culture)	56 ± 3
Brassinin (0.2 mM)	43 ± 3
Camalexin (0.1 mM)	34 ± 5
Camalexin (0.05 mM)	49 ± 4
3-Phenylindole (0.02 mM)	32 ± 6
3-Phenylindole (0.01 mM)	52 ± 2
Brassinin (0.2 mM) + camalexin (0.1 mM)	33 ± 1
Brassinin (0.2 mM) + camalexin (0.05 mM)	48 ± 4
Brassinin (0.2 mM) + 3-phenylindole (0.02 mM)	32 ± 1
Brassinin (0.2 mM) + 3-phenylindole (0.01 mM)	50 ± 2

0.02 mM or camalexin (**5**) at 0.05 or 0.1 mM. As shown in Table 2, the dry weight of mycelia of cultures of *L. maculans* after 12 h of incubation with brassinin (**1**) at 0.2 mM was similar (43 ± 3 mg) to that of cultures incubated with brassinin + camalexin at 0.2 + 0.05 mM, respectively (48 ± 4 mg). Although camalexin (**5**) at 0.1 mM appeared to be more inhibitory (34 ± 5 mg) than brassinin (**1**) at 0.2 mM (43 ± 3 mg), when brassinin (**1**, 0.2 mM) and camalexin (**5**, 0.1 mM) were combined their inhibitory effect (33 ± 1 mg) was similar to that of camalexin alone at 0.1 mM (34 ± 5 mg). A partial explanation for this apparent antagonistic effect might come from the kinetic studies of brassinin (**1**) transformation in the presence of camalexin (**5**). For instance, in cultures pre-incubated with camalexin (**5**, either at 0.1 or 0.05 mM), brassinin (**1**, 0.2 mM) was almost completely metabolized in 6 h, therefore the observed inhibitory effect on mycelia during the exponential cell growth period will mostly be due to the presence of camalexin (**5**). Similar reasoning could be made for 3-phenylindole (**7**) to explain the lower inhibitory activity of the mixture of brassinin (**1**) and 3-phenylindole (**7**) shown in Table 1.

2.2. Transformation of brassinin (*1*) in crude cell-free extracts: evidence for brassinin oxidase activity

The transformation of brassinin (**1**) by virulent isolates of *L. maculans* in liquid cultures was established during exponential cell growth. To determine the enzymatic activity of cell-free extracts, it was important to investigate the metabolism of brassinin (**1**) in mycelial cells in stationary phase cultures. Thus, the metabolism of brassinin (**1**) in mycelia incubated exclusively in water was investigated by HPLC analyses of culture samples over a period of 24 h, as described in Section 4. 3-Indolecarboxaldehyde (**2**) was identified after 3 h of incubation, and brassinin (**1**) was completely metabolized after 6 h of incubation. This result suggested that the enzyme(s) involved in the oxidative transformation of brassinin (**1**), a putative brassinin oxidase (BO), might be constitutive. In agreement with this hypothesis, crude cell-free extracts prepared from mycelia of *L. maculans* transformed brassinin (**1**) to 3-indolecarboxaldehyde (**2**) (HPLC analysis). Brassinin oxidase activity (BOA) was observed when the mycelia were extracted in phosphate buffers containing Triton X-100, which indicated that BO was a membrane bound protein. Furthermore, lower BOA was detected with the addition of glycerol to the extraction buffer. However, the transformation of brassinin in cell-free cultures was extremely slow, suggesting that the putative BO was present in very small amounts and/or the reaction conditions were not adequate. Next, because both camalexin (**5**) and 3-phenylindole (**7**) increased substantially the rate of transformation of brassinin in mycelial cultures, their potential as inducers of BO was determined. Cell-free homogenates resulting from mycelial cultures incubated with camalexin (**5**) or 3-phenylindole (**7**) showed a six and 15-fold increase in BOA relative to controls, whereas cell-free homogenates resulting from cultures incubated with spirobrassinin (**6**) showed

Table 3
Effect of camalexin (**5**), spirobrassinin (**6**), and 3-phenylindole (**7**) on brassinin (**1**) oxidase activity (BOA) of cell-free extracts of *Leptosphaeria maculans* obtained from mycelia of liquid cultures

Compound	Concentration (mM)	Relative specific activity (induced/non-induced)[a]
Camalexin (**5**)	0.01	2 ± 1
	0.05	6 ± 3
Spirobrassinin (**6**)	0.01	1 ± 0 (identical to control)
	0.05	1 ± 0 (identical to control)
	0.1	2 ± 0
3-Phenylindole (**7**)	0.005	9 ± 4
	0.02	15 ± 7

[a] Relative activity with respect to control cultures (incubated without any compound); results are the means of three independent experiments carried out in duplicate; brassinin at 0.1 mM was used in each enzyme assay.

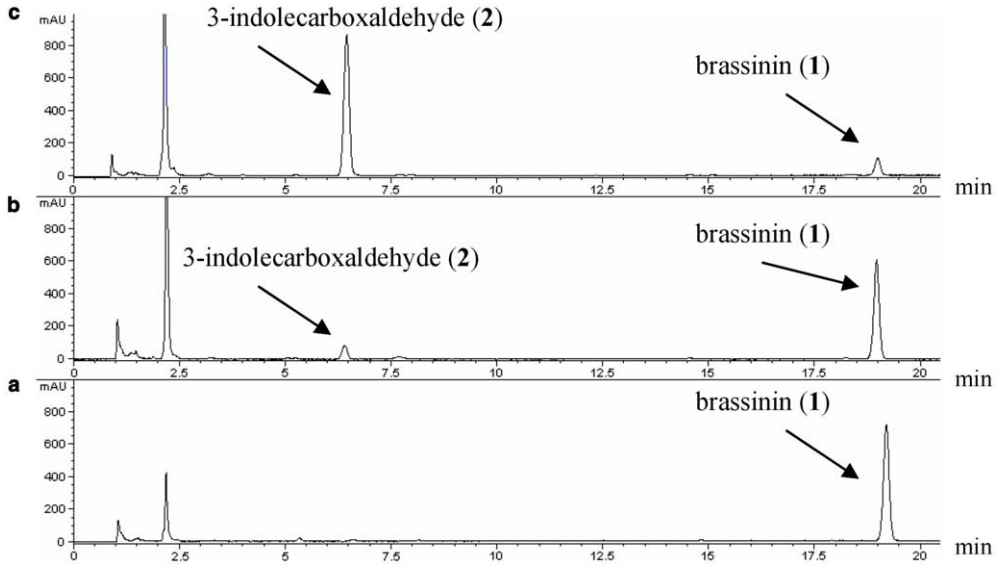


Fig. 7. HPLC chromatograms of ethyl acetate extracts of enzyme assays using brassinin as substrate (**1**, 0.1 mM) to determine brassinin oxidase activity (BOA) in cell-free extracts of mycelia of *Leptosphaeria maculans* (*Phoma lingam*). (a) 0 min incubation; (b) 60 min incubation (no induction); (c) 60 min incubation (induction with 3-phenylindole (**7**) 0.02 mM).

Table 4
Effect of cofactors on brassinin oxidase activity (BOA) of crude cell-free extracts of cultures of *Leptosphaeria maculans* incubated with camalexin (**5**, 0.05 mM)

Cofactor (2.0 mM)	Relative specific activity[a] ± SD
No cofactor	1.0 ± 0.0
NADP[b]	1.0 ± 0.0
NADPH[b]	1.0 ± 0.0
$MgSO_4$	1.0 ± 0.0
$MnSO_4$	1.0 ± 0.0
$CuCl_2$	11 ± 0.4

[a] Results are the means of three independent experiments carried out in duplicate; brassinin at 0.1 mM was used for each enzyme assay.
[b] Final concentration in the assay mixture 0.5 mM.

no increase in BOA (Table 3, Fig. 7). Similar experiments using cell-free homogenates obtained from cultures incubated with thiabendazole (**8**) showed no increase in BOA relative to controls. Because thiabendazole (**8**) did not appear to induce BOA, it is unlikely that induction of BOA is due to the antifungal effect of either camalexin (**5**) or 3-phenylindole (**7**); however the mechanism of this induction process remains to be determined. The potential cofactors required by BO, were determined after incubation of crude cell-free extracts of induced cultures of *L. maculans* with brassinin in the presence of NADP, NADPH, $MgSO_4$, $MnSO_4$ and $CuCl_2$. As shown in Table 4, when $CuCl_2$ was added to the reaction mixture, an 11-fold increase in BOA was obtained relative to the control, indicating the Cu^{2+} is the best co-factor of BO.

3. Conclusion

Detoxification of brassinin (**1**) by *L. maculans* can deprive the plant of crucial inducible chemical defenses. Hence, it is of great interest to understand and inhibit such degradation processes. Because camalexin (**5**) and spirobrassinin (**6**) are not metabolized by *L. maculans*, and in anticipation that non-degradable phytoalexins might act in synergism, we have investigated their impact on brassinin detoxification. Bioassay results on solid media indicated that the inhibitory activity of brassinin (**1**) and camalexin (**5**) on *L. maculans* were similar, whereas spirobrassinin (**6**) was substantially less inhibitory (Table 1). In addition, as shown in Table 2, the weight of the dry mycelia obtained from liquid cultures showed that 3-phenylindole (**7**) at 0.02 mM was a substantially stronger growth inhibitor than brassinin (**1**) at 10 times higher concentration (0.2 mM) or camalexin (**7**) at 2.5 times higher concentration (0.05 mM), but comparable to camalexin (**7**) at five times higher concentration (0.1 mM). As shown in Figs. 1–3, the biotransformation of brassinin (**1**) in cultures of *L. maculans* occurred much faster when cultures were pre-incubated with camalexin (**5**, 0.05–0.2 mM) or 3-phenylindole (**7**) for 24 h. In addition, the rates of brassinin (**1**) detoxification in cultures of *L. maculans* and those of cell-free mycelial extracts were consistent. That is, the brassinin detoxifying activity of cell-free extracts obtained from cultures incubated with camalexin (**5**) or 3-phenylindole (**7**) was substantially higher than that of control cultures (no amendments). However, when brassinin (**1**) and camalexin (**5**) or 3-phenylindole (**7**) were added simultaneously to 48-h-old cultures of *L. maculans*, the increase in the rate of transformation of brassinin (**1**) was not noticeable, suggesting a 12–24-h induction period for production of the enzyme(s) involved in this transformation. Furthermore, the brassinin (**1**) detoxifying activity of cell-free extracts obtained from cultures incubated with spirobrassinin (**6**)

was similar to that of control cultures. The substantial increase in the rate of brassinin (**1**) transformation in culture is most likely due to the higher amounts of the enzyme(s) produced by *L. maculans*. It thus follows that this enzyme(s), the putative BO is induced by camalexin (**5**) and 3-phenylindole (**7**). As shown in Table 3, camalexin (**5**) was a better inducer of BO than spirobrassinin (**5**), and 3-phenylindole (**7**) was an even better inducer of BO than camalexin (**5**, Fig. 7). On the other hand, neither spirobrassinin (**6**) nor thiabendazole (**8**) displayed such an effect. These conclusions are supported by the relative rates of brassinin transformation in cell-free culture homogenates.

In summary, our results demonstrate that *L. maculans* produces enzyme(s) able to transform brassinin (**1**) to 3-indolecarboxaldehyde (**2**), a detoxification process. Most interestingly, despite the inhibitory activity of camalexin (**5**) on *L. maculans* (Table 1), we discovered that the rate of detoxification of brassinin (**1**) in fungal cultures increased substantially in the presence of camalexin (**5**), whereas spirobrassinin (**6**) or thiabendazole (**8**) did not appear to affect the rate of brassinin detoxification. The enzyme(s) responsible for brassinin oxidation, BO, was shown to have higher activity in the presence of Cu (II), suggesting that Cu (II) might be a co-factor. Enzyme assays with crude cell-free extracts from non-induced cultures of *L. maculans* showed substantially lower BOA than those assays carried out with cell-free extracts of cultures induced with camalexin (**5**) or 3-phenylindole (**7**). Furthermore, similar to the results observed in mycelial cultures, spirobrassinin (**6**) did not appear to induce BOA. It is noteworthy that the replacement of the thiazole moiety of camalexin (**5**) with a phenyl group to yield 3-phenylindole (**7**) caused an even larger increase on the production of BO, whereas replacement of the indole ring with the benzimidazole moiety to yield thiabendazole (**8**) did not affect BO production but increased the antifungal activity. The discovery of a good inducer of BOA is expected to greatly facilitate the isolation of BO from *L. maculans*. BO is essential to probe detoxification mechanisms of this plant pathogen and allow the rational design of effective inhibitors of BOA.

Importantly, our work suggests that introduction of the camalexin (**5**) pathway into plants that produce brassinin (**1**) might make these plants more susceptible to *L. maculans*. That is, although in principle the overall effect of adding two metabolites that have roles as plant defenses might make the plant more disease resistant or tolerant, the overall effect of adding new metabolite(s) may be very different. Clearly, the potential impact of introducing novel secondary metabolic pathways into diverse species can be rather unpredictable, this work sets an interesting paradigm for metabolite control of plant–pathogen interactions. Finally, *L. maculans*, detoxified brassinin (**1**) via oxidation of the side chain of the indole ring (at C-3), whereas *S. sclerotiorum* detoxified brassinin through *N*-glucosylation. These different detoxification reactions suggest that these plant pathogenic fungi may have evolved rather different but selective detoxifying enzymes and thus these fungal enzymes might be virulence traits, as in the case of other plant pathogenic fungi (VanEtten et al., 2001).

4. Experimental

4.1. General procedures

All chemicals were purchased from Sigma–Aldrich Canada Ltd., Oakville, ON. All solvents were HPLC grade and used as such, except for CH_2Cl_2 and $CHCl_3$ which were redistilled. Organic solvents were removed under reduced pressure in a rotary evaporator. All synthetic compounds were purified using flash column chromatography (silica gel); satisfactory spectroscopic data identical to those previously reported were obtained for all compounds.

4.2. Fungal cultures and antifungal assays

L. maculans (virulent isolate BJ-125, IBCN collection, AAFC) was grown on V8 agar under continuous light at 23 ± 1 °C; after 15 days, the fungal spores were collected and stored at −20 °C (Pedras and Khan, 1996). Liquid cultures were initiated by inoculating minimal media (Pedras and Biesenthal, 1998) with fungal spores at different concentrations described below in Erlenmeyer flasks followed by incubation on a shaker under constant light at 23 ± 1 °C.

For antifungal assays, isolate BJ-125 was grown on potato dextrose agar (PDA) plates at 23 ± 1 °C under constant light for 7 days. Stock solutions of each compound in DMSO (0.05 M) were used to prepare assay solutions in PDA media (concentrations shown in Table 1); control solutions contained 1% DMSO in PDA (Pedras and Okanga, 1999). Sterile tissue culture plates (6-well, 35 mm diameter) containing test solutions and control solutions (2.5 ml/well) were inoculated with mycelial plugs placed upside down on the center of each plate (6 mm cut from 7-day-old PDA plates) and incubated under constant light for 5 days. All bioassays experiments were carried out in triplicate, at least two times.

4.3. Metabolism and HPLC analysis

For co-metabolism studies spores (10^6 spores per ml) of *L. maculans* were incubated in Erlenmeyer flasks (125 ml) containing 50 ml of minimal medium; after 48 h of incubation, camalexin (**5**, 0.2, 0.1, 0.05 mM), spirobrassinin (**6**, 0.05), 3-phenylindole (**7**, 0.02, 0.01 mM) or thiabendazole (**8**, 0.01, 0.005 mM) were added to each flask and to uninoculated medium (to establish the chemical stability under these experimental conditions). The flasks were incubated for an additional 24-h period at 120 rpm; brassinin (**1**, 0.2 mM) was then added to each flask and samples (2 ml) were withdrawn at every 6-h intervals. The samples were either immediately extracted with ethyl acetate or frozen for later

extraction. The extracts were dissolved in acetonitrile and were analyzed by HPLC. HPLC analysis was carried out with a high performance liquid chromatograph equipped with quaternary pump, automatic injector, and photodiode array detector (wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, i.d. 4.6 × 200 mm), equipped with an in-line filter. Mobile phase: 75% H_2O–25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient, and a flow rate 1.0 ml/min.

For metabolism studies with brassinin (**1**) and mycelia in water, liquid minimal media (Pedras and Biesenthal, 1998) (100 ml media in 250 ml Erlenmeyer flasks) were inoculated with fungal spores (10^7 spores per ml). After 48 h of incubation, mycelia were filtered off, washed in sterile distilled water and re-suspended in 100 ml sterile distilled water in 250 ml Erlenmeyer flask, to which brassinin (**1**) in DMSO was administered (final concentration 0.05 mM), followed by incubation at 23 ± 1 °C. Samples were withdrawn at 3–12 h intervals, and extracted with ethyl acetate. The aqueous phase was concentrated and analyzed by HPLC.

4.4. Preparation of crude cell-free extracts, protein and enzyme assays

L. maculans was grown in liquid cultures as reported above (10^7 spores per ml); after 48 h the inducer compound dissolved in DMSO (0.05, 0.02, 0.01 mM in culture solution) was added to cultures and the cultures incubated for additional 24 h. The fungal mycelium was removed by filtration, washed with water, the remaining water squeezed out between cheese cloth and the mycelial pad (22–30 g of mycelia per liter of medium) frozen immediately. Frozen mycelia was mixed with ice cold standard buffer (1 g of mycelia in 0.6 ml of buffer, 20 mM diethanolamine, pH 8.4, containing 1 mM dithiothreitol, and 0.1% Triton X-100) and ground using a mortar and pestle until a homogenous mixture was obtained (Frebort et al., 1997). The mixture was then centrifuged at 58,545*g* for 40 min to obtain the cell-free homogenate and the pellet was discarded.

The Bradford protein assay was used to quantify proteins in cell-free extracts using bovine serum albumin standard curves.

Enzyme assays were carried out at 23 ± 1 °C, using brassinin (**1**). The specific activity of cell-free extracts was defined as the number of moles of 3-indolecarboxaldehyde (**2**) formed per min per mg of protein. The standard assay mixture contained the cell homogenate (900 μL, 3–8 mg protein). The reaction was started by adding brassinin (**1**) in DMSO (100 mM, final concentration in assay media 0.1 mM) and incubated for 60 min with constant shaking at 23 ± 1 °C. The reaction was stopped by extracting with ethyl acetate, the solvent was removed under reduced pressure, the extract dissolved in acetonitrile and analyzed by HPLC. Quantification of products was carried out by HPLC using standard calibration curves (average $r^2 \geqslant 0.99$).

Acknowledgments

Support for the authors' work was obtained from the Natural Sciences and Engineering Research Council of Canada (Strategic Grant to M.S.C.P.) and the University of Saskatchewan (Graduate Scholarship to M.J.). We thank G. Séguin-Swartz, IBCN curator, Agriculture and Agri-Food Canada Research Station, Saskatoon SK, for kindly providing fungal isolates.

References

Bailey, J.A., Mansfield, J.W., 1982. Phytoalexins. Blackie & Son, Glasgow, UK, pp. 1–334.

Caldas, E.D., Conceição, M.H., Miranda, M.C.C., de Souza, L.C.K.R., Lima, J.F., 2001. Determination of dithiocarbamate fungicide residues in food by a spectrophotometric method using a vertical disulfide reaction system. J. Agric. Food Chem. 49, 4521–4525.

Essenberg, M., 2001. Prospects for strengthening plant defenses through phytoalexin engineering. Physiol. Mol. Plant Pathol. 59, 71–81.

Frebort, I., Matsushita, K., Adachi, O., 1997. Involvement of multiple copper/topa quinone-containing and flavin-containing amine oxidases and NAD(P) + aldehyde dehydrogenases in amine degradation by filamentous fungi. J. Ferment. Bioeng. 84, 200–212.

Glawischnig, E., Hansen, B.G., Olsen, C.E., Halkier, B.A., 2004. Camalexin is synthesized from indole-3-acetaldoxime, a key branching point between primary and secondary metabolism in Arabidopsis. Proc. Natl. Acad. Sci. USA 101, 8245–8250.

Leroux, P., 2003. Mode of action of agrochemicals towards plant pathogens. C. R. Biol. 326, 9–21.

Pedras, M.S.C., Ahiahonu, P.W.K., 2002. Probing the phytopathogenic stem rot fungus with phytoalexins and analogues: unprecedented glucosylation of camalexin and 6-methoxycamalexin. Bioorg. Med. Chem. 10, 3307–3312.

Pedras, M.S.C., Ahiahonu, P.W.K., Hossain, M., 2004. Detoxification of the phytoalexin brassinin in *Sclerotinia sclerotiorum* requires an inducible glucosyltransferase. Phytochemistry 65, 2685–2694.

Pedras, M.S.C., Ahiahonu, P.W.K., 2005. Metabolism and detoxification of phytoalexins and analogs by phytopathogenic fungi. Phytochemistry 66, 391–411.

Pedras, M.S.C., Biesenthal, C.J., 1998. Production of the host-selective phytotoxin phomalide by isolates of *Leptosphaeria maculans* and its correlation with sirodesmin PL production. Can. J. Microbiol. 44, 547–553.

Pedras, M.S.C., Jha, M., Ahiahonu, P.W.K., 2003. The synthesis and biosynthesis of phytoalexins produced by cruciferous plants. Curr. Org. Chem. 7, 1635–1647.

Pedras, M.S.C., Khan, A.Q., 1996. Biotransformation of the Brassica phytoalexin brassicanal A by the blackleg fungus. J. Agric. Food Chem. 44, 3403–3407.

Pedras, M.S.C., Okanga, F.I., 1999. Strategies of cruciferous pathogenic fungi: detoxification of the phytoalexin cyclobrassinin by mimicry. J. Agric. Food Chem. 47, 1196–1202.

Pedras, M.S.C., Okanga, F.I., Zaharia, I.L., Khan, A.Q., 2000. Phytoalexins from crucifers: synthesis, biosynthesis, and biotransformation. Phytochemistry 53, 161–176.

Rodriguez, J.G., Lafuente, A., Garcia-Almaraz, P., 2000. Synthesis and structural analysis of (*E*)-2-(2′-nitrovinyl)indoles from the corresponding 2-formylindole derivatives. J. Heterocycl. Chem. 37, 1281–1288.

VanEtten, H., Temporini, E., Wasmann, C., 2001. Phytoalexin (and phytoanticipin) tolerance as a virulence trait: why is it not required by all pathogens? Physiol. Mol. Plant Pathol. 59, 83–93.