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Detoxification of the phytoalexin brassinin by isolates of *Leptosphaeria maculans* pathogenic on brown mustard involves an inducible hydrolase

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Abstract

Brassinin is a phytoalexin produced by plants from the family Brassicaceae that displays antifungal activity against a number of pathogens of *Brassica* species, including *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.] and *L. biglobosa*. The interaction of a group of isolates of *L. maculans* virulent on brown mustard (*Brassica juncea*) with brassinin was investigated. The metabolic pathway for degradation of brassinin, the substrate selectivity of the putative detoxifying hydrolase, as well as the antifungal activity of metabolites and analogs of brassinin are reported. Brassinin hydrolase activity was detectable only in cell-free homogenates resulting from cultures induced with brassinin, *N*′-methylbrassinin, or camalexin. The phytoalexin camalexin was a substantially stronger inhibitor of these isolates than brassinin, causing complete growth inhibition at 0.5 mM.
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Keywords: Brassinin hydrolase; Camalexin; Brassicaceae; *Leptosphaeria maculans*; Paldoxin; *Phoma lingam*; Phytoalexin

1. Introduction

Brassinin (**1**) is a phytoalexin produced by plants from the family Brassicaceae (syn. Cruciferae) that displays antifungal activity against a number of crucifer pathogens (Pedras et al., 2003a,b, 2007). Phytoalexins are inducible secondary metabolites with antimicrobial activity produced de novo by plants to deter pathogens (Bailey and Mansfield, 1982; Essenberg, 2001). The antifungal activity of brassinin (**1**) is attributed to its dithiocarbamate group. Dithiocarbamates are known to be potent toxophores used in synthetic agrochemicals to control fungi and weeds (Leroux, 2003; Caldas et al., 2001). We have shown that a few economically important fungal plant pathogens can detoxify brassinin (**1**), a process that can cause plants to be more vulnerable to microbial colonization, specially considering that brassinin (**1**) is a biosynthetic precursor of several other phytoalexins (Pedras and Ahiahonu, 2005). Virulent isolates (virulent to canola, *Brassica napus* L. and *B. rapa* L.) of the phytopathogenic fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.] detoxified brassinin (**1**) to 3-indolecarboxaldehyde (**4**) and 3-indolecarboxylic acid (**5**) (Pedras and Ahiahonu, 2005). Avirulent isolates (avirulent to canola, *B. napus* and *B. rapa*; these isolates are now considered a new species named *L. biglobosa* (Shoemaker and Brun, 2001)) converted brassinin (**1**) to 3-indolylmethanamine (**2**), *N*′-acetyl-3-indolylmethanamine (**3**, Fig. 1) and then to 3-indolecarboxaldehyde (**4**) and 3-indolecarboxylic acid (**5**) (Pedras and Taylor, 1993; Pedras and Ahiahonu, 2005). The detoxification of brassinin (**1**) by virulent isolates was recently suggested to involve a putative brassinin oxidase (BO) that could convert directly brassinin (**1**) to 3-indolecarboxaldehyde (**4**) (Pedras et al., 2006). Furthermore, another group of isolates of *L. maculans* (Laird 2 and Mayfair 2, hereon called L2/M2) was discovered in the Canadian prairies with a virulence range which included brown mustard (*Brassica juncea*) (Taylor et al., 1995; Pedras et al., 1998), a usually blackleg resistant species (Keri et al., 1997). Considering that brown mustard is becoming a crop more widely cultivated in North America (Burton

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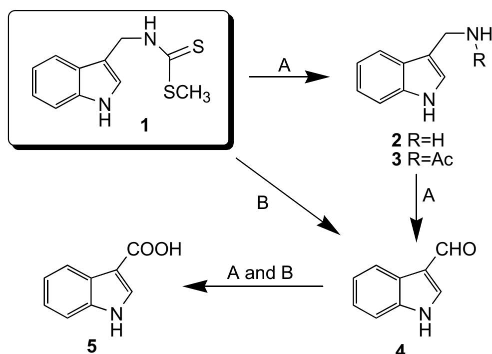


Fig. 1. Detoxification pathway of brassinin (**1**) by *Leptosphaeria maculans*: A – avirulent isolates (avirulent on canola, now considered a new species named *L. biglobosa*), B – virulent isolates (virulent on canola).

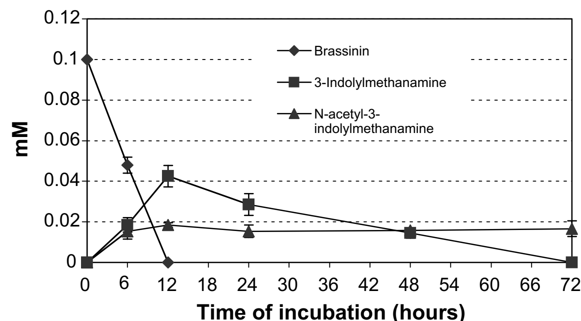


Fig. 2. Progress curve for the transformation of brassinin (**1**, 0.10 mM) and formation of products 3-indolylmethanamine (**2**) and N'-acetyl-3-indolylmethanamine (**3**) in cultures of *Leptosphaeria maculans* isolates L2/M2.

et al., 2004), it is probable that these new isolates L2/M2 of *L. maculans* will spread and become a serious problem.

The potential negative impact of fungicides continues to provide incentives to devise alternative methods to control fungal diseases. Towards this end, it is of great importance to discover new metabolic targets in fungal pathogens that will facilitate development of environmentally sustainable plant treatments and disease control strategies. Potential strategies to control the various groups of *L. maculans* could include treatments with designer compounds coined paldoxins, i.e. phytoalexin detoxification inhibitors (Pedras et al., 2003a,b; Pedras and Jha, 2006). To be able to design and produce paldoxins to inhibit a particular fungal pathogen, it is essential to: (i) determine the detoxification reaction(s) of each crucifer phytoalexin by the particular pathogen, and (ii) understand the substrate selectivity of the enzyme(s) involved in the process. To learn if this strategy is applicable to isolates L2/M2, the metabolism of brassinin (**1**) and analogs **6**, **7** and **11**–**13** was investigated. Furthermore, the substrate selectivity of the putative detoxifying hydrolase as well as the antifungal activity of metabolites and analogs of brassinin (**1**) were determined.

2. Results and discussion

2.1. Kinetics of brassinin transformation in cultures of *L. maculans* isolates L2/M2

The phytoalexin brassinin (**1**) was synthesized as previously reported (Pedras et al., 2003a,b) and its bioactivity was established using mycelial cultures of *L. maculans* isolates L2/M2. Subsequently, 48-h-old cultures of isolates L2/M2 were incubated with brassinin (**1**, 0.1 mM) and transformation was monitored by HPLC (photodiode array detection at 220 nm, brassinin (**1**), $t_R = 18.8 \pm 0.5$ min). Samples were withdrawn from cultures immediately after addition of brassinin (**1**) and then at 6, 12, 24, 48 and 72 h, extracted with the neutral and basic extracts were analyzed by HPLC. Analyses of HPLC chromatograms of neutral extracts showed that brassinin (**1**) was almost completely metabolized within 12 h (cultures of *L. maculans* isolates L2/M2, Fig. 2). Furthermore, the chromatograms of neutral extracts showed the presence of additional peaks that were established to be due to N'-acetyl-3-indolylmethanamine (**3**, $t_R = 5.2 \pm 0.2$ min) and 3-indolecarboxaldehyde (**4**, $t_R = 7.2 \pm 0.2$ min) by comparison with authentic samples. The basic extracts indicated the presence of 3-indolylmethanamine (**2**) (TLC detection) which, due to its alkalinity, was not eluted under our analytical HPLC conditions. HPLC detection and quantification of amine **2** present in basic extracts was carried out after acetylation (acetic anhydride in pyridine) to N'-acetyl-3-indolylmethanamine (**3**). Data analysis for transformation of brassinin (**1**) into amine **2** and acetyl amine **3** showed that amine **2** was transformed in 72 h, whereas acetyl amine **3** remained in culture for more than five days (Table 1).

To establish the sequence of steps of brassinin (**1**) transformation, amine **2**, acetyl amine **3**, and aldehyde **4** were administered separately to cultures of isolates L2/M2, which the cultures then incubated and analyzed as described for brassinin (**1**). The HPLC chromatograms indicated that amine **2** was completely metabolized in 48 h to yield acetyl amine **3** and aldehyde **4**, whereas conversion of acetyl amine **3** to aldehyde **4** and amine **2** occurred at slower rates (Table 1). Aldehyde **4** was transformed to 3-indolecarboxylic acid (**5**) in 72 h. That is, the transformation pathway of brassinin (**1**) by isolates L2/M2 was similar to the pathway previously observed for the avirulent isolates of *L. maculans* shown in Fig. 1 (Pedras and Taylor, 1993). It appeared that the acetylation reaction was reversible whereas oxidation of the amine **2** to aldehyde **4** was not.

To probe the potential substrate selectivity of the enzyme(s) of isolates L2/M2 involved in the transformation of brassinin, the structurally related compounds **6** and **7** (Fig. 3) were administered to cultures. First, methyl tryptamine dithiocarbamate (**6**) was selected as it contained the same dithiocarbamate group as brassinin (**1**) but had an additional CH_2 group on the side-chain of the indole nucleus. HPLC analysis of the cultures incubated with **6**

Table 1
Transformation of brassinin (**1**), metabolites **2**–**4** and analogs **6**, **7** and **11**–**13** in mycelial cultures of *Leptosphaeria maculans* isolates L2/M2

Compounds/percentage of transformation[a]	Metabolic products
Brassinin (**1**)/100% in 12 h	3-Indolylmethanamine (**2**), *N*′-acetyl-3-indolylmethanamine (**3**), indole-3-carboxaldehyde (**4**)
3-Indolylmethanamine (**2**)/100% in 48 h	*N*′-Acetyl-3-indolylmethanamine (**3**)
N′-Acetyl-3-indolylmethanamine (**3**)/ca. 20% recovered after 72 h	3-Indolylmethanamine (**2**), 3-indolecarboxaldehyde (**4**)
3-Indolecarboxaldehyde (**4**)/100% in 72 h	3-Indolecarboxylic acid (**5**)
Methyl tryptamine dithiocarbamate (**6**)/100% in 48 h	*N*′-Acetyltryptamine (**9**)
Methyl tryptophol dithiocarbonate (**7**)/100% in 72 h	Tryptophol (**10**)
N′-Methylbrassinin (**11**)/ca. 75% recovery after 72 h	No metabolism
Methyl *N*′-(3-indolylmethyl)carbamate (**12**)/ca. 75% recovery after 72 h	No metabolism
Methyl 3-indolylpropanoate (**13**)/complete in 8 h	Indolyl-3-propanoic acid (**14**)

[a] Percentages were determined using calibration curves and are averages of at least two independent experiments conducted in triplicate.

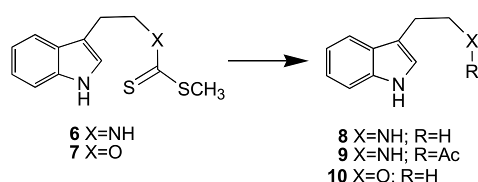


Fig. 3. Transformation of methyl tryptamine dithiocarbamate (**6**) and methyl tryptophol dithiocarbonate (**7**) by *Leptosphaeria maculans* isolates L2/M2.

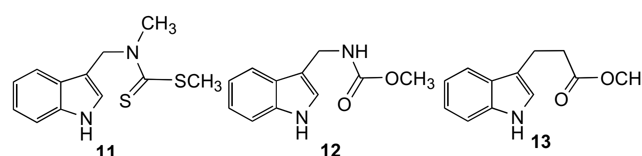


Fig. 5. Chemical structures of brassinin analogs: *N*′-methylbrassinin (**11**), methyl *N*′-(3-indolylmethyl)carbamate (**12**), and methyl 3-indolylpropanoate (**13**).

indicated it to be metabolized to acetyl tryptamine **9**, the expected tryptamine (**8**) was not detected in either neutral or basic extracts (Figs. 3 and 4, Table 1). On the other hand, methyl tryptophol dithiocarbonate (**7**) was hydrolyzed to tryptophol (**10**, Fig. 3) and no acetyl derivative was detected in the cultures (**10**, Fig. 3, Table 1). Furthermore, the brassinin analog **11**, containing an additional methyl group at the nitrogen atom of the side chain, and analog **12**, in which the sulfur atoms of brassinin (**1**) were replaced with oxygen, were not transformed and remained in culture for the duration of the experiments (five days, Table 1). Carboxylic ester **13**, resulting formally from replacement of the NH of brassinin (**1**) side-chain with CH_2 and both sulfur atoms with oxygen, was hydrolyzed to the corresponding carboxylic acid **14** (Fig. 5, Table 1).

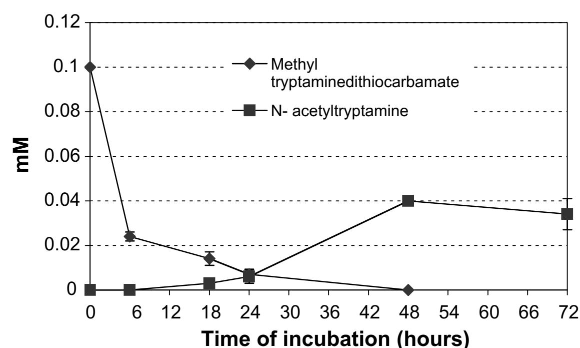


Fig. 4. Progress curve for transformation of methyl tryptamine dithiocarbamate (**6**, 0.10 mM) and formation of *N*′-acetyltryptamine (**9**) in cultures of *Leptosphaeria maculans* isolates L2/M2.

Considering the structural similarity between brassinin (**1**) and methyl tryptamine dithiocarbamate (**6**), it was rather surprising to find that tryptamine (**8**) did not appear to be formed. Absence of this transformation in mycelial cultures was even more puzzling considering that methyl tryptophol dithiocarbonate (**7**) was hydrolyzed to tryptophol. This result suggested that either acetylation of tryptamine (**8**) occurred immediately after hydrolysis (a slower reaction) or the metabolism involved different enzyme(s). Overall, the biotransformation results using brassinin analogs suggested that perhaps the enzyme(s) involved in the metabolism of brassinin (**1**) can discriminate among various substrates, indicating some degree of specificity. None-

Table 2
Antifungal activity[a] of brassinin (**1**), metabolites and analogs against *Leptosphaeria maculans* isolates L2/M2 (5 days incubation)

Compounds	Activity[a] (%) at concentrations		
	0.50 mM	0.20 mM	0.10 mM
Brassinin (**1**)	38 ± 3	23 ± 5	0
3-Indolylmethanamine (**2**)	24 ± 6	0	0
N′-Acetyl-3-indolylmethanamine (**3**)	0	0	0
3-Indolecarboxaldehyde (**4**)	0	0	0
3-Indolecarboxylic acid (**5**)	0	0	0
Methyl tryptamine dithiocarbamate (**6**)	95 ± 5	62 ± 4	15 ± 5
Methyl tryptophol dithiocarbonate (**7**)	41 ± 7	30 ± 5	27 ± 7
N′-Methylbrassinin (**11**)	71 ± 5	60 ± 7	39 ± 5
Methyl *N*′-(3-indolylmethyl)carbamate (**12**)	21 ± 4	0	0
Methyl indolyl-3-propanoate (**13**)	0	0	0
Camalexin (**15**)	100 ± 0	65 ± 5	35 ± 5

[a] Percentage of inhibition = 100 − [(growth on medium containing compound/growth on control medium) × 100] ± standard deviation.

theless, the possibility that other factors might affect the metabolism of these brassinin analogs cannot be ruled out at this point.

The antifungal activity of brassinin (**1**), its metabolites **2**–**5**, and compounds **6**, **7**, **11**–**13** were evaluated against isolates L2/M2 using a standard mycelial radial growth assay. Metabolites **2**–**5** were less inhibitory to isolates L2/M2 than brassinin (**1**), and neither acetylamine **3** nor aldehyde **4** displayed inhibitory activity on mycelial growth (Table 2). Overall, the bioassays indicate that brassinin metabolism is a detoxification process. It is of interest to note that camalexin (**15**, Fig. 5) was the most inhibitory of the compounds tested followed by methyl tryptamine dithiocarbamate (**6**) and N'-methylbrassinin (**11**).

2.2. Detection of brassinin hydrolase (BH) activity and transformation of brassinin (1) in crude cell-free homogenates (CFH)

To further understand the potential specificity of the enzyme(s) involved in brassinin hydrolysis, crude CFH of mycelial cultures of isolates L2/M2 were studied. Previously, we showed that conversion of brassinin (**1**) to 3-indolecarboxaldehyde (**4**) was carried out by constitutive BO produced in cultures of virulent isolates (Pedras et al., 2005). BO activity could be increased several fold by incubating cultures with compounds such as the phytoalexin camalexin (**15**) (Pedras et al., 2005). Since the transformation of brassinin (**1**) in cultures of isolates L2/M2 appeared to involve first its hydrolysis to 3-indolylmethanamine (**2**), it was important to find conditions to obtain this enzymatic activity in CFH. In preliminary experiments, cultures of isolates of L2/M2 were incubated with and without camalexin (**15**) (at three concentrations) for a 24-h period, with the mycelia used to prepare CFH. CFH obtained in Tris/HCl buffer were used for enzymatic assays with brassinin (**1**) as a substrate. These assays showed that the CFH obtained from the mycelia of isolates L2/M2 incubated with camalexin (**15**) transformed brassinin (**1**) to amine **2**, whereas CFH obtained from cultures containing no camalexin (control) did not transform brassinin (**1**). The CFH resulting from mycelial cultures incubated with camalexin (**15**) at 0.1 mM and extracted with buffer containing Triton X-100 showed a sevenfold increase in brassinin hydrolase (BH) activity relative to cultures incubated with camalexin (**15**) at 0.01 mM (Fig. 6). BH activity increased substantially when the extraction buffer contained Triton X-100 (0.057 ± 0.001 versus 0.010 ± 0.002 nmol/min/mg of protein). Brassinin (**1**), N'-methylbrassinin (**11**), and camalexin (**15**) (at 0.05 mM) induced similar BH activity (Fig. 7). Furthermore, the substrate specificity of BH and/or related hydrolase(s) produced by isolates L2/M2 was examined in CFH using the compounds shown in Table 3. These assays revealed that, besides brassinin (**1**), only methyl tryptophol dithiocarbonate (**7**) was transformed. Based on the metabolic results obtained with mycelial cultures, the methyl ester **13** was expected to be hydrolyzed to propanoic acid

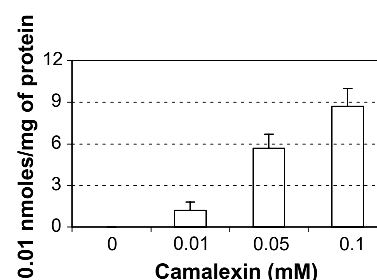


Fig. 6. Brassinin (**1**) hydrolase activity in cell-free homogenates of mycelia of *Leptosphaeria maculans* isolates L2/M2: control culture (incubated without camalexin (**15**)) and cultures incubated with camalexin at different concentrations for 24 h.

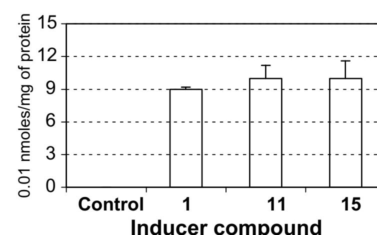


Fig. 7. Brassinin hydrolase activity in cell-free homogenates of mycelia of *Leptosphaeria maculans* isolates L2/M2 incubated in medium containing no additives (control), and in medium containing inducer compounds brassinin (**1**), N'-methylbrassinin (**11**), or camalexin (**15**) (0.05 mM) for 6 h.

derivative **14**. This difference between metabolism in the mycelial cells and in CFH suggests that BH is different from the hydrolase involved in the conversion of methyl ester **13**. The hydrolase involved in the metabolism of methyl ester **13** could require extraction and/or reaction conditions different from BH (e.g. pH, co-factor). Similarly, tryptamine dithiocarbamate did not appear to be transformed in CFH; neither tryptamine (**8**) nor acetyl tryptamine (**9**) were detected in the reaction assay mixture, even when acetyl

Table 3
Substrate affinity of brassinin hydrolase (BH) in cell-free homogenates (CFH) of mycelia obtained from induced cultures of *Leptosphaeria maculans* isolates L2/M2

Substrate (0.5 mM)	Specific activity[a] (nmol/min/mg) ± SD	Relative activity % (brassinin = 100)
Brassinin (**1**)	0.075 ± 0.005	100
Methyl tryptamine dithiocarbamate (**6**)	No detectable conversion	0
Methyl tryptophol dithiocarbonate (**7**)	0.043 ± 0.002	62
N'-Methylbrassinin (**11**)	No detectable conversion	0
Methyl N'-(3-indolylmethyl)-carbamate (**12**)	No detectable conversion	0
Methyl 3-indolyl-propanoate (**13**)	No detectable conversion	0
Camalexin (**15**)	No detectable conversion	0

[a] The specific activity of CFH was defined as the amount (nmol) of product (3-indolylmethanamine or tryptophol) formed per min per mg of protein; results are from three independent experiments carried out in triplicate.

CoA was added to the assay. These results might reflect the specificity of BH, although only after purification and isolation of BH would such speculation be confirmed.

Our previous work on brassinin (**1**) detoxification by isolates of *L. maculans* virulent on canola indicated that the putative detoxifying enzyme BO was constitutive and that its production could be increased by incubating cultures with camalexin (**15**) (Pedras et al., 2005). On the other hand, the detoxification of brassinin (**1**) in *Sclerotinia sclerotiorum* involved a totally inducible brassinin glucosyl transferase whose activity could be induced by camalexin as well (**15**) (Pedras et al., 2004). These different enzymatic detoxifications appear to reflect differences in the ecological adaptation and evolution of both fungal species. In addition, the brassinin (**1**) detoxifying enzyme BH produced by the *L. maculans* isolates virulent on brown mustard is clearly different from the detoxifying enzyme BO produced by isolates virulent on canola. The metabolic differences in the detoxification of brassinin (**1**) between these two groups might suggest that they belong to different species as well. Nonetheless, because both groups appear to co-occur in canola and brown mustard fields, putative paldoxins mixtures that could inhibit both BO and BH activity may provide a reasonable alternative to fungicides.

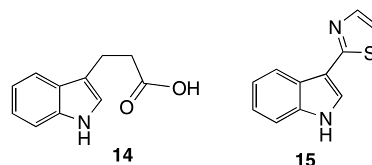
3. Conclusions

L. maculans isolates L2/M2 appeared to detoxify the phytoalexin brassinin (**1**) using a pathway similar to that of *L. biglobosa* (i.e. *L. maculans*, avirulent on canola, Pedras and Taylor, 1993). Assays using CFH incubated with brassinin (**1**) demonstrated that the enzyme involved in its conversion to amine **2**, BH, was strictly inducible. BH activity was induced by brassinin (**1**), *N*′-methylbrassinin (**11**) and camalexin (**15**). A standard mycelial growth assay indicated that brassinin (**1**) was not as toxic to isolates L2/M2 as to virulent isolates of *L. maculans* (caused complete inhibition at 0.5 mM, Pedras and Jha, 2006). Nonetheless, because brassinin (**1**) is a precursor of several antifungal phytoalexins in *Brassica* species (Pedras et al., 2007), it is of interest to inhibit its detoxification by isolates virulent on brown mustard. Therefore, the design and screening of potential inhibitors of BH activity and the concomitant isolation of BH is of great importance. Toward this end, since neither camalexin (**15**) nor *N*′-methylbrassinin (**11**) are metabolized in mycelial cultures, both inducers will be useful for increasing production of BH in mycelial cultures.

4. Experimental

4.1. Chemicals

All solvents were HPLC grade and used as such, except for CH_2Cl_2 that was redistilled. Brassinin (**1**), 3-indolylmethanamine (**2**), *N*′-acetyl-3-indolylmethanamine (**3**), methyl tryptamine dithiocarbamate (**6**), methyl tryptophol dithiocarbonate (**7**), *N*′-acetyltryptamine (**9**), *N*′-methylbrassinin (**11**), methyl *N*′-(3-indolylmethyl)carbamate (**12**), methyl 3-indolylpropanoate (**13**) and camalexin (**15**) were synthesized as previously reported (Pedras and Jha, 2006; Pedras et al., 2003a,b). 3-Indolecarboxaldehyde (**4**), tryptophol (**10**), and indolyl-3-propanoic acid (**14**) were purchased from Sigma–Aldrich Canada Ltd., Oakville, ON.



4.2. Fungal cultures and antifungal assays

L. maculans single-spore isolates Laird 2 and Mayfair 2 (L2/M2) were grown on V8 agar under continuous light at 23 ± 1 °C; after 15 days, 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 10^6 or 10^7 ml^{-1} in 250 ml Erlenmeyer flasks followed by incubation on a shaker under constant light at 23 ± 1 °C.

For bioassays, isolates L2/M2 were grown on potato dextrose agar (PDA) plates at 23 ± 1 °C under constant light for 7 days. The antifungal activity of compounds was determined following a mycelial radial growth bioassay, as described previously (Pedras and Suchy, 2006). All bioassays were carried out in triplicate, at least twice.

4.3. Metabolism and HPLC analysis

Erlenmeyer flasks (125 ml, containing 50 ml of liquid minimal media (Pedras and Biesenthal, 1998)) were inoculated with spores (10^8 spores/100 ml) of *L. maculans* and incubated at 23 ± 1 °C on shaker at 120 rpm under constant light. After 48 h, compounds in Table 1 were added to cultures and samples (2 ml) were withdrawn at different times and either frozen or immediately extracted with EtOAc (2 × 4 ml). The combined EtOAc extracts were concentrated in a rotary evaporator in vacuo. The remaining aqueous phase was made alkaline by adding NH_4OH (pH > 9) and extracted with $CHCl_3$ (4 ml). The concentrated $CHCl_3$ extract was acetylated as described below to yield a basic acetylated residue. The neutral and basic acetylated residues were individually dissolved in a minimum amount of CH_3CN and were analyzed by HPLC.

HPLC analysis was carried out with a high-performance liquid chromatograph equipped with a quaternary pump, automatic injector, and diode array detector (wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), equipped with

an in-line filter. Mobile phase: H_2O–CH_3CN (3:1, v/v) to CH_3CN (100%), for 35 min, linear gradient, and a flow rate 1.0 ml/min.

4.4. Acetylation reaction of basic extracts

The $CHCl_3$ extracts were concentrated to complete dryness using a rotary evaporator, and then dissolved in 100 μl of pyridine–acetic anhydride (4:1) and kept at room temperature for 60 min. The reaction mixture was diluted with toluene (2 ml) and concentrated under reduced pressure (this step was repeated twice). The acetylated extracts were dissolved in CH_3CN and analyzed by HPLC.

4.5. Preparation of crude cell-free homogenates, protein and enzyme assays

L. maculans was grown in liquid cultures as reported above (10^7 spores/ml); after 48 h, camalexin (**15**), dissolved in DMSO (final concentration in culture 0.1, 0.05 and 0.01 mM) or DMSO only (control), was added to cultures and the cultures were incubated for additional 24 h. The fungal mycelia was removed by filtration, washed with sterile distilled H_2O, the remaining H_2O squeezed out between cheese cloth and the mycelial pad (25–30 g mycelia per liter of medium) frozen immediately. Frozen mycelia was mixed with ice-cold extraction buffer (1 g mycelia in 0.6 ml of buffer, 20 mM Tris–HCl, pH 7, 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 22,000 rpm for 40 min to yield the cell-free homogenate and the pellet. In additional experiments, brassinin (**1**), *N*′-methylbrassinin (**11**) and camalexin (**15**) (final concentration in culture 0.05 mM) were added to 48-h old cultures, the cultures were incubated for an additional 6 h and the mycelia used to prepare cell-free homogenates as described above.

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

Enzyme assays were individually carried out at 23 ± 1 °C, using either brassinin (**1**) as substrate or the reported compounds at 0.5 mM in the assay mixture. The specific activity of the cell-free homogenates was defined as the number of moles of 3-indolylmethanamine (detected as the acetyl derivative **3**) formed per min per mg of protein. The standard enzyme assay mixture contained the cell-free homogenate (1000 μl, 8–10 mg protein) in the extraction buffer; the reaction assay was initiated by adding brassinin (**1**) in DMSO followed by incubation for 60 min with constant shaking at 23 ± 1 °C. The reaction assay was stopped by extracting with EtOAc (2 ml), and removal of the solvent in a rotary evaporator was carried to yield a neutral residue. The aqueous phase was made alkaline by adding NH_4OH (pH > 9) with the whole then extracted with $CHCl_3$. The concentrated $CHCl_3$ extract was acetylated using pyridine–acetic anhydride (4:1) as described above. Both neutral and basic acetylated residues were individually dissolved in a minimum amount of CH_3CN and analyzed by HPLC. Quantification of products was carried out by HPLC using standard calibration curves (average $r^2 \geqslant 0.998$).

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