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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.

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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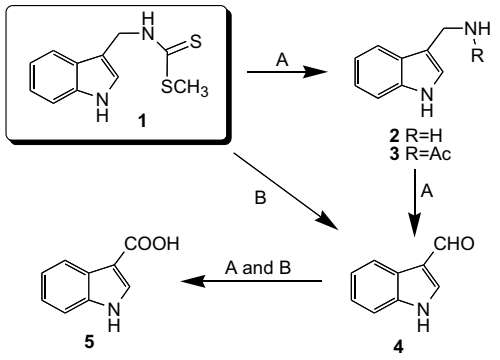

Fig. 1. Detoxification pathway of brassinin (**1**) by *Leptosphaeria maculans*: A – avirulent isolates (avirulent on canola, now considered a new species named *L. biglobosa*), B – virulent isolates (virulent on canola).

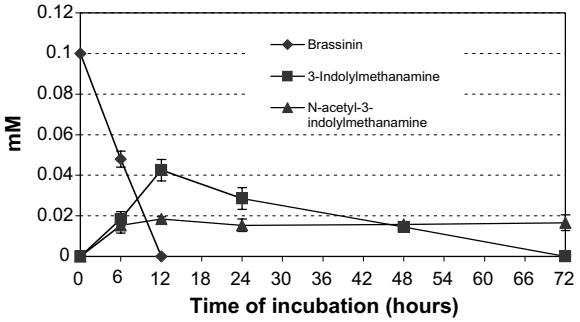

Fig. 2. Progress curve for the transformation of brassinin (**1**, 0.10 mM) and formation of products 3-indolylmethanamine (**2**) and *N*′-acetyl-3-indolylmethanamine (**3**) in cultures of *Leptosphaeria maculans* isolates L2/M2.

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**