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

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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.,

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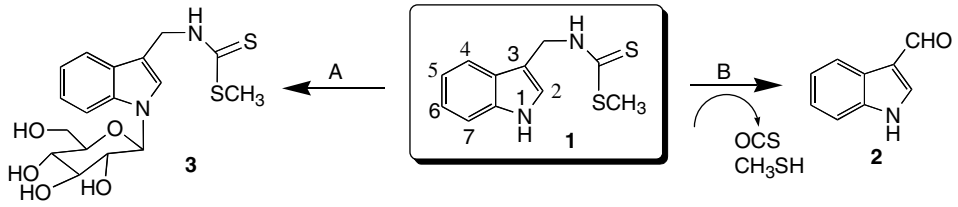
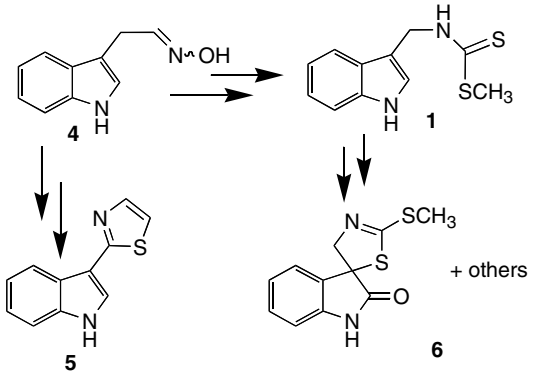


Scheme 1. Detoxification of brassinin (**1**) by plant pathogenic fungi: (A) *Sclerotinia sclerotiorum*; (B) virulent *Leptosphaeria maculans* (*Phoma lingam*) (Pedras and Ahiahonu, 2005).

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**,