Phytochemistry 72 (2011) 2308–2316

Contents lists available at SciVerse ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Interaction of cruciferous phytoanticipins with plant fungal pathogens: Indole glucosinolates are not metabolized but the corresponding desulfo-derivatives and nitriles are

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ARTICLE INFO

Article history:
Received 27 April 2011
Received in revised form 21 July 2011
Available online 13 September 2011

Keywords:
Alternaria brassicicola
Brassicaceae
Crucifer
Indole glucosinolate
Secondary metabolism
Myrosinase activity
Rhizoctonia solani
Sclerotinia sclerotiorum

ABSTRACT

Glucosinolates represent a large group of plant natural products long known for diverse and fascinating physiological functions and activities. Despite the relevance and huge interest on the roles of indole glucosinolates in plant defense, little is known about their direct interaction with microbial plant pathogens. Toward this end, the metabolism of indolyl glucosinolates, their corresponding desulfo-derivatives, and derived metabolites, by three fungal species pathogenic on crucifers was investigated. While glucobrassicin, 1-methoxyglucobrassicin, 4-methoxyglucobrassicin were not metabolized by the pathogenic fungi *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*, the corresponding desulfo-derivatives were metabolized to indolyl-3-acetonitrile, caulilexin C (1-methoxyindolyl-3-acetonitrile) and arvelexin (4-methoxyindolyl-3-acetonitrile) by *R. solani* and *S. sclerotiorum*, but not by *A. brassicicola*. That is, desulfo-glucosinolates were metabolized by two non-host-selective pathogens, but not by a host-selective. Indolyl-3-acetonitrile, caulilexin C and arvelexin were metabolized to the corresponding indole-3-carboxylic acids. Indolyl-3-acetonitriles displayed higher inhibitory activity than indole desulfo-glucosinolates. Indolyl-3-methanol displayed antifungal activity and was metabolized by *A. brassicicola* and *R. solani* to the less antifungal compounds indole-3-carboxaldehyde and indole-3-carboxylic acid. Diindolyl-3-methane was strongly antifungal and stable in fungal cultures, but ascorbigen was not stable in solution and displayed low antifungal activity; neither compound appeared to be metabolized by any of the three fungal species. The cell-free extracts of mycelia of *A. brassicicola* displayed low myrosinase activity using glucobrassicin as substrate, but myrosinase activity was not detectable in mycelia of either *R. solani* or *S. sclerotiorum*.

1. Introduction

Glucosinolates (GLs) represent a large group of plant natural products long known for diverse and fascinating physiological functions and activities (Fenwick and Heaney, 1983; Fahey et al., 2001; Traka and Mithen, 2009; Vig et al., 2009; Hopkins et al., 2009). The functional group glucosinolate contains an *O*-sulfated thiohydroximate connected with a β-glucopyranosyl residue through the sulfur atom (Fig. 1). The R moiety of the molecule is derived from protein amino acids and their homologues (Sønderby et al., 2010; Halkier and Gershenzon, 2006). Many glucosinolate producing plant species have been credited with both beneficial and detrimental health effects. For example, metabolic products of GLs have been shown to induce detoxifying enzymes in humans and suggested to protect against cancer (Traka and Mithen, 2009; Vig et al., 2009). On the other hand, harmful goitrogenic effects have also been attributed to GLs (Fenwick and Heaney, 1983; Traka and Mithen, 2009). Glucosinolate producing plant species have enormous economic importance (family Brassicaceae, common name crucifer) as sources of healthy oils (canola and rapeseed), vegetables (cabbage, cauliflower, broccoli, Brussels sprouts, turnip, rutabaga, and other *Brassica* spp.) and condiments (mustard an wasabi). Central to the huge interest generated by GLs, and their metabolic products, is their involvement in plant defense pathways against very destructive organisms, including a broad range of insects, nematodes, and microbes (Fenwick and Heaney, 1983; Traka and Mithen, 2009; Vig et al., 2009; Hopkins et al., 2009; Ahuja et al., 2010).

In general, the defensive layer of plant metabolites is composed of both phytoanticipins (constitutive) and phytoalexins (inducible) (Smith, 1996). GLs are phytoanticipins of crucifers as metabolic products of their enzymatic degradation are effective in protecting against pests and microbes. A correlation between defense signalling pathways and GL profiles observed in *Arabidopsis thaliana* L. indicated that indole GLs (IGLs) were involved in defense, although the function of each particular compound was not examined (Mikkelsen et al., 2003). Furthermore, IGLs (R = indolyl-3-methyl, **1**–**3**) are potential intermediates in the biosynthesis of cruciferous

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doi:10.1016/j.phytochem.2011.08.018

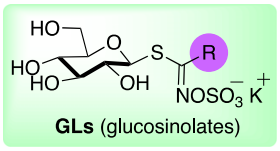


Fig. 1. General chemical structure of glucosinolates.

phytoalexins, indicating that their production is crucial to plant fitness (Pedras et al., 2010, 2011). Therefore, IGLs are among the most important components of the cruciferous defense arsenal.

The metabolic products of GLs result from hydrolyses catalyzed by enzymes known as myrosinases (thioglucoside glucohydrolases, EC 3.2.1.147), which are brought together with their substrates only upon tissue damage. Enzyme catalyzed hydrolysis is followed by additional enzymatic and chemical transformations of the thiohydroximate intermediates to yield volatile isothiocyanates as major products (Bones and Rossiter, 2006); however, the chemical structures of these products depend on various factors, including the structures of the R groups. For example, in the case of IGLs **1**–**3** (e.g. R = indolyl-3-methyl, known as glucobrassicin (**1**)), the isothiocyanates **7**–**9** are neither volatile nor stable, decomposing to indolyl-3-methylene carbocations **13**–**15**. In the plant, these carbocations react with nucleophiles such as water, ascorbate, glutathione, etc., to yield indolyl-3-methanols **22**–**24** and ascorbigens **16**–**18** (Fig. 2) (Agerbirk et al., 2009). In addition, from the thiohydroximate intermediates **4**–**6**, indolyl-3-acetonitriles **10**–**12** are formed by enzymatic catalyses that require particular proteins (e.g. ESP = epithiospecifier protein plus Fe^{2+}; NSP = nitrile specifier protein).

While altering GL profiles has been shown to affect plant disease resistance to microbial pathogens (Brader et al., 2006; Agerbirk et al., 2009), despite speculation, very little is known about the direct interaction of plant fungal pathogens with GLs. By contrast, there is substantial evidence suggesting that the degradation products of GLs, namely isothiocyanates and nitriles, are strongly antifungal and antibacterial (Manici et al., 1997; Góralska et al., 2009; Aires et al., 2009). Interestingly, transformed *A. thaliana* (expressing CYP79D2) accumulating isopropyl and methylpropyl GLs showed enhanced resistance against the bacterial soft-rot pathogen *Erwinia carotovora*, whereas transformed *A. thaliana* (expressing CYP79A1 or over-expressing the endogenous CYP79A2) accumulating *p*-hydroxybenzyl or benzyl GLs, respectively, showed enhanced susceptibility to the plant fungal pathogen *Alternaria brassicicola* and enhanced resistance to the bacterial pathogen *Pseudomonas syringae*

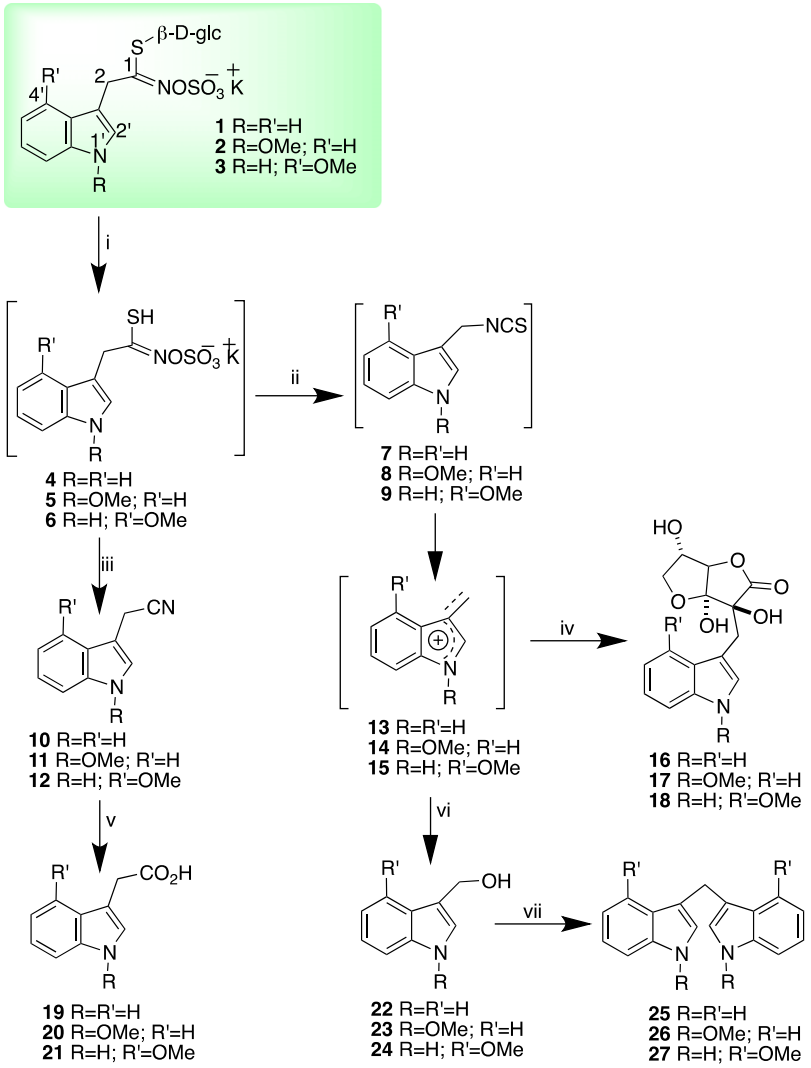


Fig. 2. Metabolism of indole glucosinolates **1**–**3** in plants: (i) hydrolyses catalyzed by myrosinases; (ii) chemical rearrangement; (iii) enzymatic reactions catalyzed by Fe^{2+}/ESP (epithiospecifier protein)/NSP (nitrile specifier protein); (iv) reaction with ascorbic acid; (v) hydrolyses catalyzed by NIT (nitrilases); (vi) reaction with water; (vii) chemical rearrangement.

(Brader et al., 2006). Because fungal pathogens of *Brassica* spp., and crucifers in general, overcome the protection provided by phytoalexins and related metabolites through detoxification reactions (Pedras and Ahiahonu, 2005; Pedras, 2008; Pedras and Yaya, 2010; Pedras et al., 2011), it is of great importance to establish if such reactions occur with IGLs and their metabolites. The paucity of studies dealing with metabolism of IGLs may be due to the commercial unavailability of these compounds as their chemical syntheses are not trivial (Rollin and Tatibouet, 2011) and the isolation and purification of intact products require extensive chromatography, affording insufficient amounts for such studies (e.g. ca. 5 mg from 1.3 kg of leaf tissue, Pedras et al., 2008).

To better understand the roles of IGLs in the interaction of crucifers with their fungal pathogens, their potential metabolism, and that of the corresponding desulfo-derivatives, by the phytopathogenic fungi *A. brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* was investigated. In addition, the products of enzymatic and chemical reactions of IGLs by the three fungal species was determined. It was established that antifungal plant metabolites derived from IGLs such as indolyl-3-acetonitriles and indolyl-3-methanol are metabolized to products with lower antifungal activity. In addition, indole glucosinolates and desulfo-derivatives showed no inhibitory activity against any of the three plant pathogens. Interestingly, myrosinase activity was detected in mycelia of

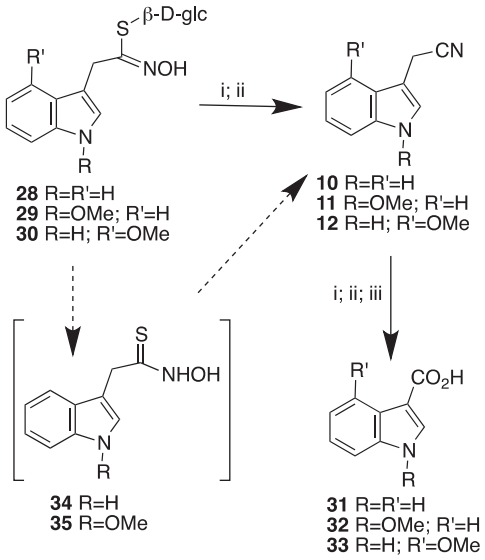


Fig. 3. Metabolism of indole desulfo-glucosinolates **28** and **29** in cultures of *Rhizoctonia solani* (i) and *Sclerotinia sclerotiorum* (ii) and nitriles **10**–**12** in cultures of *R. solani* (i), *S. sclerotiorum* (ii) and *Alternaria brassicicola* (iii). Dashed arrows represent hypothetical steps.

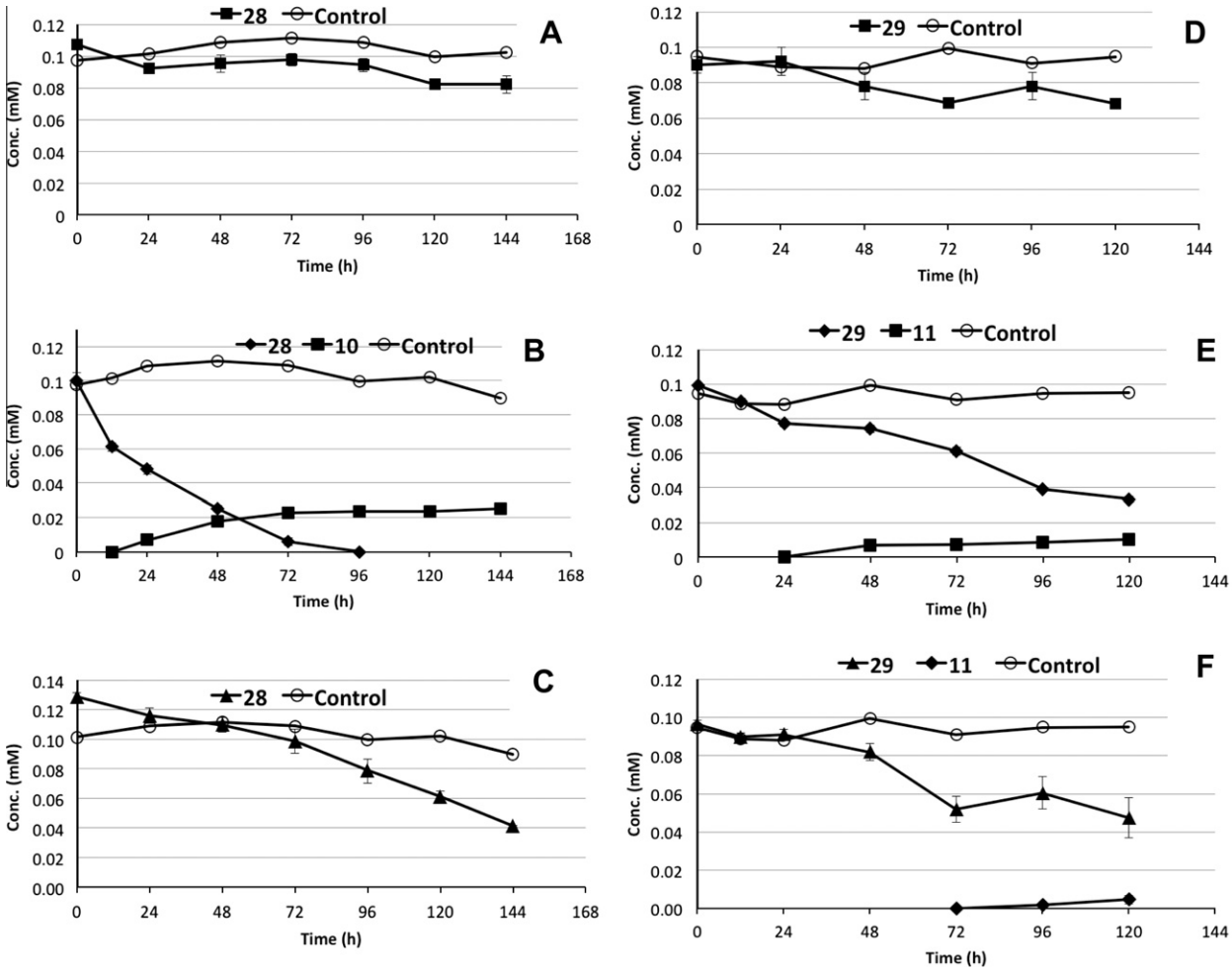


Fig. 4. Kinetics of transformation of indole desulfo-glucosinolates in cultures of: (A) *Alternaria brassicicola* incubated with **28**; (B) *Rhizoctonia solani* incubated with **28** and formation of nitrile **10**; (C) *Sclerotinia sclerotiorum* incubated with **28**; (D) *A. brassicicola* incubated with **29**; (E) *R. solani* incubated with **29** and formation of nitrile **11**; (F) *S. sclerotiorum* incubated with **29** and formation of nitrile **11**; control curves indicate the stability of compounds **28** and **29** incubated in water under similar conditions but without the fungus.

A. brassicicola, but no detectable activity was found in mycelia of *R. solani* or *S. sclerotiorum*.

2. Results and discussion

Three fungal species pathogenic to crucifers were chosen to carry out metabolic studies of IGLs and derivatives, based on their host-range and amenability to culture under aseptic conditions. *A. brassicicola* (Schwein.) Wiltshire, together with *A. brassicae*, causes one of the most economically important diseases of *Brassica* species, the so-called Alternaria black spot (also called dark leaf spot) (Cooke et al., 1997). *A. brassicicola* is quite selective in its host-range, infecting mainly cultivars of *B. juncea* L. or other *Brassica* spp. (Rimmer and Buchwaldt, 1995; Vishwanath et al., 1999). By contrast, both *R. solani* Kuhn, and *S. sclerotiorum* (Lib.) de Bary infect crucifers and a wider range of plant families, but while *S. sclerotiorum* infects mainly above ground plant organs (Bolton et al., 2006), *R. solani* infects mainly underground organs (Luebeck, 2004).

2.1. Chemical synthesis and antifungal activity of compounds

The chemical synthesis of compounds was carried out as described in the experimental and those commercially available were used as such, after confirming their purity by HPLC analysis. IGLs **1**–**3**, desulfo-IGLs **28** and **29** and known derivatives of indole glucosinolates (compounds **10**–**12**, **16**, **22** and **25**, Fig. 2) were assayed for antifungal activity. *A. brassicicola*, *R. solani* and *S. sclerotiorum* were used in standard mycelial growth assays described in the experimental. As summarized in Table 1, neither IGLs **1**–**3** nor their desulfo-derivatives **28** and **29** displayed detectable antifungal activity. While diindolyl-3-methane (**25**) was the most active of all compounds, inhibiting growth of the three fungal species completely at 0.50 mM, ascorbigen (**16**) displayed the lowest activity. Among the nitriles, caulilexin C (1-methoxyindolyl-3-acetonitrile) (**11**) was the most active, inhibiting completely the growth of *R. solani* at 0.50 mM.

2.2. Metabolism of indole glucosinolates *1*–*3*, desulfo-glucosinolates *28* and *29* and myrosinase activity

Liquid cultures of *A. brassicicola*, *R. solani* and *S. sclerotiorum* grown for 72 h were incubated with IGLs **1**–**3** for various time periods, samples were withdrawn from cultures immediately after addition of each compound and up to 5 days and immediately frozen and then lyophilized (Pedras and Suchy, 2005). Media incubated with each compound (control solutions) were treated similarly to determine the chemical stability of compounds during the incubation experiments. HPLC analysis of the concentrated culture samples indicated no transformation of any of IGLs **1**–**3**. Although a slight decrease in the concentration of **2** was detected, this decrease was similar to that observed in control solutions and attributed to its slow decomposition. The final results indicated that none of the IGLs were metabolized by any of the fungal species.

Similar experiments conducted with desulfo-IGLs **28** and **29** indicated that these compounds were transformed by *R. solani* and *S. sclerotiorum* to the corresponding nitriles (Fig. 3) at slower rate in *S. sclerotiorum* ($t_{1/2}$ 120 h for both **28** and **29**) than in *R. solani* ($t_{1/2}$ 24 h for **28**, $t_{1/2}$ 90 h for **29**) (Fig. 4B, C, E and F). By contrast, *A. brassicicola* did not metabolize any of the desulfo-IGLs **28** and **29**, which remained in culture for the duration of the experiments (5 and 6 days, respectively, Fig. 4A and D). Nitriles **10** and **11** accumulated in cultures of *R. solani* and could be quantified after 12-h incubations (Fig. 4B and E), but in cultures of *S. sclerotiorum* **10** was detected only in trace amounts (Fig. 4C) and nitrile **11** was quantifiable only after 72-h incubations (Fig. 4F). These nitriles were further metabolized to the corresponding carboxylic acids, as described below in Section 2.3. Because no potential intermediates of the transformations of **28** and **29** to **10** and **11** were detected in any of the cultures, we hypothesize that a glucosyl hydrolase(s) catalyzes these transformations to yield thiohydroxamic acids **34** and **35**, which are further transformed on standing (enzymatically or spontaneously) to the corresponding nitriles **10** and **11**. Similar transformation of isotopically labeled **34**–**10** were observed *in planta* (Pedras and Okinyo, 2008).

To the best of our knowledge, this is the first report of the transformation of desulfo-IGLs by fungi. Previously, a soil isolate of the fungus *Aspergillus flavus* was shown to convert enzymatically 2-propenyl and 2-phenylethyl glucosinolates and their desulfo-derivatives to nitriles, that is sulfatase and myrosinase activities were reported in *A. flavus* (Galletti et al., 2008). The metabolism of desulfo-IGLs **28** and **29** suggests that both *R. solani* and *S. sclerotiorum* produce glucosyl hydrolases that do not have myrosinase activity, and do not produce sulfatases, as intact IGLs are not metabolized. However, these transformations were not detoxification processes, since the nitriles were more inhibitory to each fungal species than the corresponding desulfo-IGLs (Tables 1 and 2).

Although some *Aspergillus* spp. have been reported to produce myrosinases (Ohtsuru et al., 1973; Smits and Knol, 1993; Rakariyatham et al., 2006), this enzymatic activity does not appear to be a known trait of plant fungal pathogens. Hence, the myrosinase activity of cell-free extracts of mycelia of *A. brassicicola*, *R. solani* and *S. sclerotiorum* grown under various conditions was determined using glucobrassicin (**1**) as substrate. Under the conditions tested (Chevolleau et al., 1997), low but consistent myrosinase activity was detected in cell-free extracts of mycelia of *A. brassicicola* (0.4 nmoles/mg/min of indolyl-3-methanol (**22**) and dindolylmethane (**25**) formed), but not in mycelia of *R. solani* or *S. sclerotiorum*. However, since glucobrassicin (**1**) was not transformed in cultures of *A. brassicicola*, the myrosinase activity detected in cell-free extracts is likely not secreted into the culture media, as previously observed for *Aspergillus* spp. (Ohtsuru et al., 1973). Although ascorbate is a known stabilizer of myrosinase activity (Shikita et al., 1999), we found that when ascorbate was used in enzyme assays using glucobrassicin (**1**) as substrate, it reacted with the reaction product indolyl-3-methanol (**22**), thus ascorbate was not used in subsequent assays. Myrosinase activity was not induced by 1-methoxyglucobrassicin (**2**). Considering that myrosinase activity has not been reported for any other plant fungal pathogens, it is difficult to draw conclusions before further work with other fungal species under various conditions is carried out.

2.3. Metabolism of indolyl-3-acetonitriles *10*–*12*

Cultures of *A. brassicicola* were incubated with nitriles **10**–**12** and cultures of *R. solani* and *S. sclerotiorum* were incubated with nitriles **11** and **12** (because studies with **10** were previously reported, Pedras and Montaut, 2003) and their transformations were monitored by HPLC (photodiode array and ESI detection). Samples were withdrawn from cultures immediately after addition of each compound, were lyophilized, and analyzed by HPLC, as reported in the experimental. Media samples incubated with nitriles **10**–**12** (control solutions) were analyzed similarly, to determine the chemical stability of each compound during the incubation experiments. Nitriles **10**–**12** were metabolized by each species to the corresponding indole-3-carboxylic acids **31**–**33** (Fig. 3). As shown in Fig. 5 and summarized in Table 3, the rates of transformation of nitriles **10** and **11** were faster in *A. brassicicola* than in *R. solani* or *S. sclerotiorum*, whereas transformations of 4-methoxy nitrile **12** were similar in the three species. The identity of each acid was confirmed by direct comparison with authentic samples synthesized as reported in the experimental. Interestingly, the inhibitory activity of nitriles **10**

Table 1
Antifungal activity of compounds **1**–**3**, **10**–**12**, **16**, **22**, **25** and **28** and **29** against the plant fungal pathogens *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (as described in Section 4.3) under continuous light.

Compound name (#)	Concentration (mM)	% Inhibition[a] (standard error)		
		A. brassicicola	*R. solani*	*S. sclerotiorum*
Glucobrassicin (**1**)	0.50	NI	NI	NI
1-Methoxyglucobrassicin (**2**)	0.50	NI	NI	NI
4-Methoxyglucobrassicin (**3**)	0.50	NI	NI	NI
Indolyl-3-acetonitrile (**10**)	0.50	27 (2) a, x	41 (2) a, y	27 (0) a, x
	0.20	13(1)	15 (2)	16 (0.3)
	0.10	3 (0.3)	3 (2)	4 (0.3)
1-Methoxyindolyl-3-acetonitrile (**11**)	0.50	43 (1) b, x	100 (0) b, y	54 (0) b, z
	0.20	29 (0)	45 (1)	13 (1)
	0.10	18 (0)	29 (1)	0 (0)
4-Methoxyindolyl-3-acetonitrile (**12**)	0.50	59 (1) c, x	70 (1) c, y	77 (0) c, y
	0.20	15 (1)	46 (0)	46 (0)
	0.10	0 (0)	26 (1)	24 (1)
Ascorbigen (**16**)	0.50	36 (1) d, x	12 (0.3) d, y	10 (0) d, y
	0.20	12 (0)	6 (0)	7 (0)
	0.10	0 (0)	0 (0)	3 (0)
Indolyl-3-methanol (**22**)	0.50	43 (1) b, x	44 (0) a, x	73 (0.3) c, y
	0.20	29 (0)	27 (0.3)	60 (0)
	0.10	18 (0)	11 (0)	41 (0.3)
Diindolyl-3-methane (**25**)	0.50	100 (0) e, x	100 (0) b, x	100 (0) e, x
	0.20	77 (1)	61 (1)	81 (2)
	0.10	60 (1)	32 (1)	72 (2)
Desulfoglucobrassicin (**28**)	0.50	NI	NI	NI
1-Methoxydesulfoglucobrassicin (**29**)	0.50	NI	NI	NI

[a] Percentage of growth inhibition calculated using the formula:% inhibition = 100 − [(growth on amended medium/growth in control medium) × 100]. Data are the mean ± SE; for statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; $n = 3$; different letters in the same column (a–e) indicate significant differences ($P < 0.05$); different letters in the same row (x–z) indicate significant differences ($P < 0.05$).

Table 2
Antifungal activity of compounds **19**, **31**–**33** and **36** against the plant fungal pathogens *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (as described in Section 4.3) under continuous light.

Compound name (#)	Concentration (mM)	% Inhibition[a] (standard error)		
		A. brassicicola	*R. solani*	*S. sclerotiorum*
Indolyl-3-acetic acid (**19**)	0.50	28 (2) a, x	19 (1) a, y	18 (1) a, y
	0.20	13 (2)	12 (0.3)	6 (0)
	0.10	NI	7 (0)	NI
Indole-3-carboxylic acid (**31**)	0.50	22 (0) b, x	9 (0.3) b, y	34 (2) b, z
	0.20	11 (0)	5 (0.3)	9 (0)
	0.10	6 (0)	NI	4 (0)
1-Methoxyindole-3-carboxylic acid (**32**)	0.50	48 (1) c, x	14 (0) c, y	67 (0) c, z
	0.20	24 (0)	7 (0)	43 (0.3)
	0.10	12 (0)	3 (0.3)	27 (0)
4-Methoxyindole-3- carboxylic acid (**33**)	0.50	18 (0) b, x	14 (0) c, y	11 (0.3) d, z
	0.20	11 (1)	6 (0.3)	3 (0)
	0.10	7 (1)	NI	NI
Indole-3-carboxaldehyde (**36**)	0.50	29 (1) a, x	32 (1) d, x	16 (0.3) a, y
	0.20	21 (1)	17 (1)	6 (0)
	0.10	6 (1)	7 (1)	NI

[a] Percentage of growth inhibition calculated using the formula: % inhibition = 100 − [(growth on amended medium/growth in control medium) × 100]. Data are the mean ± SE; for statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; $n = 3$; different letters in the same column (a–d) indicate significant differences ($P < 0.05$); different letters in the same row (x–z) indicate significant differences ($P < 0.05$).

and **11** was stronger against *R. solani*, than that of acids **31** (41% vs. 9% at 0.50 mM) and **32** (100% vs. 14% at 0.50 mM), respectively, whereas acid **32** was more inhibitory to *S. sclerotiorum* than the corresponding nitrile **11** (54% vs. 67% at 0.50 mM) (Tables 1 and 2). By contrast, the 4-methoxy nitrile **12** was more inhibitory to the three species than the corresponding acid (59% vs. 18%, 70% vs. 14%, 77% vs. 11% at 0.50 mM) (Tables 1 and 2).

*2.4. Metabolism of ascorbigen (**16**), indolyl-3-methanol (**22**) and diindolyl-3-methane (**25**)*

Initially, the stability of ascorbigen (**16**), indolyl-3-methanol (**22**) and diindolyl-3-methane (**25**) in water was evaluated by HPLC analyses of lyophilized samples. The compounds were considered sufficiently stable to incubate with cultures of *A. brassicicola*, *R. solani* and *S. sclerotiorum*, although ascorbigen (**16**) showed ca. 40% decomposition in 4 days and indolyl-3-methanol (**22**) showed <10% decomposition in 2 days. The potential transformations of each compound were monitored by HPLC (photodiode array and ESI detection) analyses of concentrated cultures. Ascorbigen (**16**) was not metabolized by any of the pathogens and was found to decompose slowly in cultures or in water to indolyl-3-methanol (**22**). Indolyl-3-methanol (**22**) was metabolized in ca. 12 h to indole-3-carboxaldehyde (**36**) and then to indole-3-carboxylic acid (**31**) by both *A. brassicicola* and *R. solani* (Figs. 6 and 7). We suspect that *S. sclerotiorum* metabolizes **22** similarly; however, because **22**

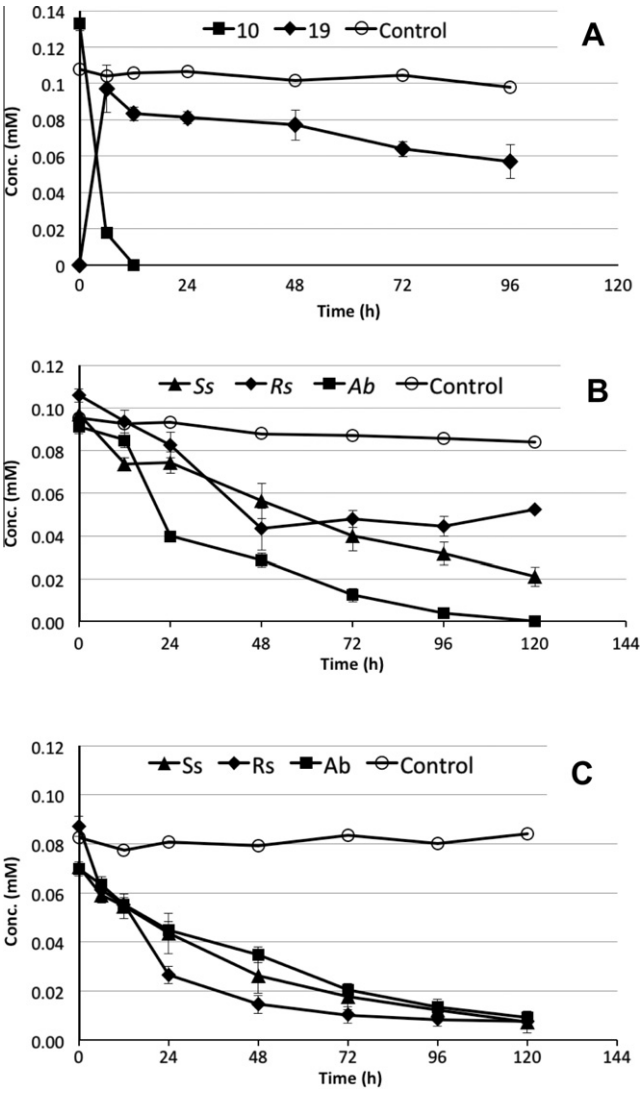


Fig. 5. Kinetics of transformation of nitriles **10**–**12** in cultures of: A, *Alternaria brassicicola* incubated with **10** and formation of **19**; B, *A. brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* incubated with **11**; C, *A. brassicicola*, *R. solani* and *S. sclerotiorum* incubated with **12**; control curves indicate the stability of nitriles **10**–**12** incubated in water under similar conditions but without the fungus.

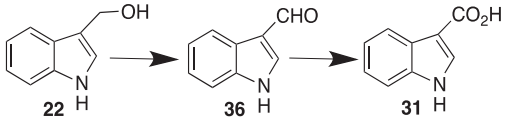
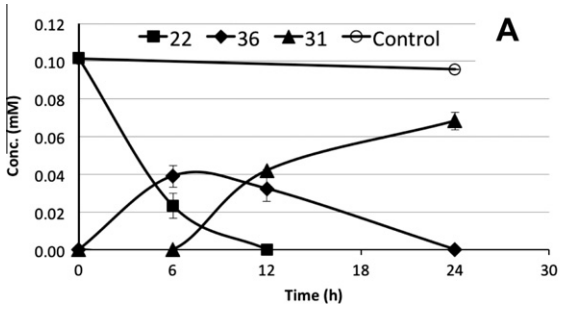


Fig. 6. Metabolism of indolyl-3-methanol (**22**) by *Alternaria brassicicola* and *Rhizoctonia solani*.



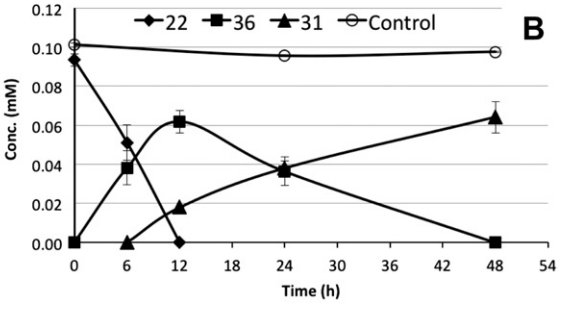


Fig. 7. Kinetics of transformation of indolyl-3-methanol (**22**) and product formation in cultures of: A, *Alternaria brassicicola*; B, *Rhizoctonia solani*; control curves indicate the stability of **22** incubated in water under similar conditions but without the fungus.

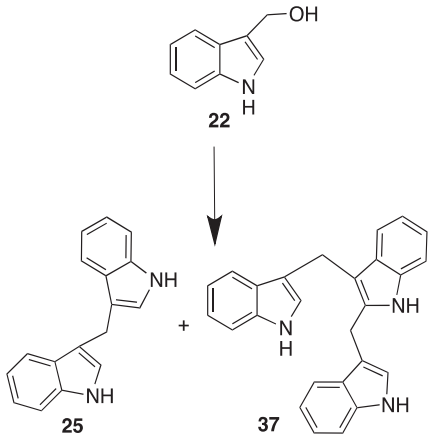


Fig. 8. Non-enzymatic transformation of indolyl-3-methanol (**22**) in cultures of *Sclerotinia sclerotiorum*.

decomposed quickly (ca. 90% in 2 h) in cultures of *S. sclerotiorum*, no evidence could be obtained. Isolation of the products of decomposition of **22** and NMR spectroscopic data showed that the main products resulted from dimerization and trimerization of **22** to yield **25** and **37**, respectively, as shown in Fig. 8; transformations are likely to occur via the indolyl-3-methylene cation (**13**) (Grose and Bjeldanes, 1993). This decomposition was eventually found to be due to the acidic pH of the culture solution; it was determined that in water, mycelia of *S. sclerotiorum* excreted sufficient amounts of acids to decrease the pH of the culture solution to 3.8. In the case of *A. brassicicola* and *R. solani* the pH of the culture solution remained around 6.5. Production of oxalic acid by *S. sclerotiorum* has been documented under various conditions (Bolton et al., 2006). In control solutions, indolyl-3-methanol (**22**) decomposed mainly to **25** and **37**, albeit at much slower rates (Fig. 7, <10% in 48 h) than in acidic medium. Indolyl-3-methanol (**22**) displayed stronger antifungal activity against each of the three fungal species than indole-3-carboxylic acid (**31**). Oxidation of indolyl-3-methanol (**22**) to **36** and **31** has been reported in mammalian systems and in plants, but to the best of our knowledge, has not been reported in fungi (Agerbirk et al., 2009).

In similar time-course experiments, incubation of diindolyl-3-methane (**25**) with each pathogen and analyses of concentrated cultures by HPLC indicated no additional compounds present in the culture. The recovery of diindolyl-3-methane (**25**) from cultures was ca. 90%, 50% was recovered from the broth and 40% from the fungal mycelia (mycelial adsorption likely due to its lipophilicity).

3. Conclusion

The first investigation of the fate of three IGLs (**1**–**3**) in cultures of three plant fungal pathogens, differing on their host-range and preferred plant tissues (*A. brassicicola*, *R. solani* and *S. sclerotiorum*),

Table 3
Metabolism of compounds **10**–**12**, **22**, **28** and **29** by the plant fungal pathogens *Alternaria brassicicola*, *Sclerotinia sclerotiorum*, and *Rhizoctonia solani*.

Compound name	Metabolic products		
	A. brassicicola	*R. solani*	*S. sclerotiorum*
Indolyl-3-acetonitrile (**10**)	Indole-3-carboxylic acid (**31**); 100% transformation in 12 h	Indole-3-carboxylic acid (**31**); 75% transformation in 5 days[a]	Indole-3-carboxylic acid (**31**); 100% transformation in 24 h[a]
1-Methoxyindolyl-3-acetonitrile (**11**)	1-Methoxyindole-3-carboxylic acid (**32**); 50% transformation in 20 h	1-Methoxyindole-3-carboxylic acid (**32**); 50% transformation in 42 h	1-Methoxyindole-3-carboxylic acid (**32**); 50% transformation in 54 h
4-Methoxyindolyl-3-acetonitrile (**12**)	4-Methoxyindole-3-carboxylic acid (**33**); 50% transformation in 48 h	4-Methoxyindole-3-carboxylic acid (**33**); 50% transformation in 14 h	4-Methoxyindole-3-carboxylic acid (**33**); 50% transformation in 36 h
Indolyl-3-methanol (**22**)	Indolyl-3-carboxylic acid (**31**); 100% transformation in 12 h	Indolyl-3-carboxylic acid (**31**); 100% transformation in 12 h	Unstable in acidic medium, not determined
Desulfoglucobrassicin (**28**)	No transformation	Indolyl-3-acetonitrile (**10**); 50% transformation in 24 h	Indolyl-3-acetonitrile (**10**); 50% transformation in 108 h
1-Methoxydesulfoglucobrassicin (**29**)	No transformation	1-Methoxyindolyl-3-acetonitrile (**11**); 50% transformation in 84 h	1-Methoxyindolyl-3-acetonitrile (**11**); 50% transformation in 72 h

[a] Results from previous work Pedras and Montaut (2003).

indicated that none of the three species metabolized these compounds. By contrast, the corresponding indole desulfo-glucosinolates **28** and **29** were metabolized by the non-host-selective pathogens *R. solani* and *S. sclerotiorum* to indolyl-3-acetonitriles **10** and **11**, but not by the host-selective *A. brassicicola* (Table 3). These results suggest that both *R. solani* and *S. sclerotiorum* produce glucosyl hydrolases that do not have myrosinase activity, since intact IGLs are not metabolized. This conclusion is consistent with the lack of myrosinase activity in mycelia of these pathogens. To date, there is no evidence of metabolism of IGLs **1**–**3** by plant fungal pathogens; however, considering that IGLs do not appear to have detrimental effects on the fungal species investigated, the lack of metabolizing enzymes should not be surprising. Consistent with previous work, our overall results indicate that indolyl-3-acetonitriles **10**–**12** and indolyl-3-methanol (**22**) are antifungal and thus potentially able to provide additional protection against some fungal species. In general, the methoxy substituted nitriles **11** and **12** were more inhibitory than nitrile **10**; however while the 4-methoxy nitrile **12** was more inhibitory to both *A. brassicicola* and *S. sclerotiorum*, the 1-methoxy nitrile **11** was more inhibitory to *R. solani*. That is, cruciferous species that contain larger amounts of **11** in the roots are likely to show lower susceptibility to *R. solani*, a root pathogen. Nonetheless, since these pathogens are able to detoxify **10**–**12** and **22**, such protection can only be temporary. Of all metabolites derived from IGLs, diindolyl-3-methane (**25**) appears the most useful to the plant, since it is not metabolized and is a good growth inhibitor of fungal growth. Nonetheless, because **25** arises from non-enzymatic transformation of **22**, it is difficult to predict which metabolic modifications (e.g. increase the levels of IGL **1**, decrease formation of nitrile **10** by removing any nitrile specifier activity, or both) are necessary to increase its concentration. Hence, to improve the natural resistance traits of *Brassica* spp. to microbial pathogens, pathways regulating both IGL and phytoalexin biosynthesis ought to be carefully examined.

4. Experimental

4.1. Chemicals and instrumentation

All solvents were HPLC grade and used as such, except for any solvents used in synthetic procedures (dried as necessary for each procedure). Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Flash column chromatography (FCC): silica gel, grade 60, 230–400 μm. Organic extracts were dried over Na_2SO_4 and the solvents were removed under vacuum using a rotary evaporator. HPLC analysis was carried out with Agilent high performance liquid chromatographs equipped with quaternary pump, automatic injector, and diode array detector (DAD, wavelength range 190–600 nm), degasser, and a column, having an in-line filter. Elution method A: column Eclipse XDB-C18 (5 μm particle size silica, 4.6 i.d. × 150 mm), mobile phase 50% H_2O – 50% CH_3OH to 100% CH_3OH, for 25.0 min, linear gradient, and at a flow rate 0.75 ml/min; elution method B (indolyl glucosinolates and other polar metabolites): column Zorbax SB-C18 (3.5 μm particle size silica, 100 × 3.0 mm i.d.), equipped with an in-line filter, with the mobile phase H_2O (with 0.1% TFA) – CH_3OH (with 0.1% TFA) from 85:15 to 70:30 in 25 min, to 50:50 in 5 min, to 40:60 in 5 min and a flow rate of 0.40 ml/min. HPLC–DAD–ESI-MS analysis was carried out with an Agilent 1100 series HPLC system equipped with an autosampler, binary pump, degasser, and a diode array detector connected directly to a mass detector (Agilent G2440A MSD-Trap-XCT ion trap mass spectrometer) with an electrospray ionization (ESI) source. Chromatographic separation was carried out at room temperature using an Eclipse XDB C-18 column (5 μm particle size silica, 4.6 i.d. × 150 mm). The mobile phase consisted of a linear gradient of 0.2% formic acid in water and 0.2% formic acid in CH_3CN (75:25 to 25:75 in 35 min, to 0:100 in 5 min) and a flow rate of 1 ml/min. Data acquisition was carried out in positive and negative polarity modes in a single LC run. All other experimental conditions as reported previously (Pedras et al., 2010).

4.2. Synthesis and characterization of new compounds

All compounds gave satisfactory spectroscopic data. The commercially available compounds **10**, **19**, **22**, **31** and **36** were used as such; compounds **1**–**3** (Rollin and Tatibouet, 2011), **11** and **12** (Pedras et al., 2007), **16** (Piironen and Virtanen, 1962), **25** (Nagarajan and Perumal, 2004), **28** and **29** (Robertson and Botting, 1999), and **32** and **33** (Somei et al., 2001) were synthesized according to literature procedures and their purity was established by ^1H NMR spectroscopy and HPLC analysis. The first synthesis and spectroscopic characterization of compound **33** is reported below and NMR spectra are provided in Supplementary data.

4.2.1. 4-Methoxyindole-3-carboxylic acid (**33**)

Indole-3-carboxaldehyde (100.2 mg; 0.69 mmol, **36**) was added to a solution of thallium trifluoroacetate (562.1 mg, 1.03 mmol) in TFA (1.2 ml), and the mixture was stirred at 30 °C for 3 h. After evaporation of the solvent under reduced pressure, I_2 (525.8 mg, 2.07 mmol), CuI (525.8 mg, 2.76 mmol), and DMF (5.0 ml) were added to the residue. After stirring the reaction mixture at r.t. for 1 h, $NaOCH_3$ (15% w/v, 5 ml) was added and the stirring continued at 100–110 °C for 3 h. The reaction was cooled to room temperature, diluted with CH_2Cl_2–MeOH (95:5) and filtered through Celite. The filtrate was washed with water and brine, dried and concentrated under reduced pressure. Purification of the product by FCC

over silica using EtOAc:hexane (3:1) as the eluent yielded pure 4-methoxyindole-3-carboxaldehyde (62.1 mg, 75%).

A solution of $NaHClO_2$ (870.5 mg, 8.0 mmol) and $NaH_2PO_4{\cdot}2H_2O$ (940.2 mg, 6.0 mmol) in H_2O was added to a solution of 4-methoxyindole-3-carboxaldehyde (70.2 mg, 0.40 mmol) in t-BuOH (4.2 ml) and 2-methoxy-2-butene (4.2 ml) at 0 °C. After stirring at r.t. for 48 h, the reaction mixture was extracted with Et_2O (30 ml × 2), dried and the product purified by FCC over silica using CH_2Cl_2–CH_3OH (99:1), to yield 69.5 mg (91%) of the desired compound **33** (Somei et al., 2001).

HPLC t_R = 5.2 min (method A). ^{1}H NMR δ (500 MHz, CD_3CN): 11.6 (1H, s), 10.2 (1H, s, br), 7.99 (1H, d, J = 3.1 Hz), 7.24–7.20 (2H, m), 6.84–6.80 (1H, m), 4.09 (3H, s). ^{13}C NMR (125.8 MHz, CD_3CN) 165.0, 152.0, 139.7, 135.1, 125.3, 114.9, 109.1, 108.4, 103.8, 57.7. HREI-MS m/z: calc. for $C_{10}H_9NO_3$: 191.0582, found 191.0583; m/z (%): 191.1 [M]+ (100), 172.0 (12), 162.1 (8), 144.0 (33), 132.0 (17), 118.1 (15), 104.1 (64). UV (HPLC, MeOH–H_2O) λ_{max} (nm): 210, 230, 290. FTIR (KBr, cm^{-1}) ν_{max}: 3183, 1696, 1526, 1395, 1245, 1089, 740, 735.

4.3. Mycelial radial growth antifungal bioassays

Three-day-old cultures of *S. sclerotiorum*, 4-day-old cultures of *R. solani* and 7-day-old cultures of *A. brassicicola* grown on PDA under constant light at 23 ± 1 °C were used for mycelial radial growth assays. Plugs (4 mm) were cut from the edges of mycelia and placed inverted onto six-well agar plates (for *S. sclerotiorum* and *R. solani*) or twelve-well agar plates (*A. brassicicola*) amended with test compounds (dissolved in DMSO or H_2O). The final concentrations of each compound in agar varied from 0.10 to 0.50 mM, with a DMSO or H_2O concentration of 1%. The plates were allowed to grow under constant light at 23 ± 1 °C; the diameter of the mycelial mat was measured after 24 h (*S. sclerotiorum*), 48 h (*R. solani*) and 96 h (*A. brassicicola*) and compared to control mycelia grown on plates containing DMSO or H_2O only.

4.4. Liquid fungal cultures and metabolism studies

Liquid cultures of each pathogen were grown in 250 ml Erlenmeyer flasks containing 100 ml of minimal medium inoculated fungal spores for a final concentration of 1×10^6 per 100 ml for *A. brassicicola*, or six mycelial plugs (6 mm diameter) per 100 ml for *R. solani* and *S. sclerotiorum*, and cultures were incubated on a shaker at 120 rpm. After 48–72 h at 23 ± 1 °C, under constant light the cultures were filtered aseptically and the mycelia washed with sterile water. Then the mycelia from each culture were transferred to 250 ml flasks containing sterile water (100 ml), and a solution of each compound (**1**–**3**, **10**–**12**, **16**, **22**, **25**, **28**, and **29**) in sterile water or CH_3CN (100–250 μl depending on solubility) was added to the cultures, for a final concentration of 0.10 mM. Similar control solutions containing compounds only or mycelia were prepared. The flasks were returned to the shaker, and samples (5–10 ml) were withdrawn at various times and immediately frozen and lyophilized. The residue was dissolved in MeOH, CH_3CN, or MeOH–H_2O and analyzed by HPLC, using method A for compounds **10**–**12**, **16**, **22**, and **25**, and method B for compounds **1**–**3**, **28**, and **29**, as described previously (Pedras et al., 2010).

4.5. Myrosinase activity

For induction, 5-day-old fungal cultures were incubated with 1-methoxyglucobrassicin (**2**) to a final concentration of 0.1 mM for 24 h. The mycelia were filtered, washed and dried under vacuum, weighed and frozen immediately. The frozen mycelia (6.5 g) were ground with NaH_2PO_4 buffer (pH 7.0, 13 ml) at 4 °C, centrifuged and the supernatant (cell-free extract) was used for myrosinase activity assays. Glucobrassicin (**1**) in buffer (500 μl, 2.1 mM) was added to the cell-free extract (4.5 ml) and samples (1 ml) were collected after 1, 3 and 6 h, extracted with Et_2O (2 × 2 ml), dried, dissolved in CH_3OH (200 μl) and analyzed by HPLC. The specific activity was calculated as the number of nmoles of product (indolyl-3-methanol (**22**) and diindolyl-3-methane (**25**)) formed per mg of protein per min. The amounts of product formed were quantified using calibration curves built with authentic synthetic samples. The Bradford assay method was used to calculate the protein content of cell-free extracts using a standard BSA calibration curve (wavelength 595 nm). Similar assays were carried out using mycelia grown in the absence of 1-methoxyglucobrassicin (**2**).

Acknowledgements

We thank P.B. Chumala for HPLC–MS data, K. Thoms for HRMS-ESI data and K. Brown for NMR data. Financial support for the authors' work was obtained from the Natural Sciences and Engineering Research Council of Canada (Discovery Grant to M.S.C.P.), the Canada Research Chairs program, Canada Foundation for Innovation, the Saskatchewan Government, and the University of Saskatchewan (teaching assistantship to S.H.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.08.018.

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