Benzyl and phenyl glucosinolates are metabolized by the specific plant pathogen *Alternaria brassicicola* but not by the generalist fungal pathogens *Rhizoctonia solani* or *Sclerotinia sclerotiorum*

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Graphical Abstract

Benzyl and phenyl glucosinolates are metabolized by the specific plant pathogen *Alternaria brassicicola* but not by the generalist fungal pathogens *Rhizoctonia solani* or *Sclerotinia sclerotiorum*

Research highlights

- Benzyl and phenyl glucosinolates are metabolized by *Alternaria brassicicola*.
- *Rhizoctonia solani* and *Sclerotinia sclerotiorum* do not metabolize benzyl or phenyl glucosinolates.
- Benzyl isothiocyanate is metabolized by *A. brassicicola*.
- Benzyl isothiocyanate causes complete inhibition of mycelial growth of *R. solani* and *S. sclerotiorum*.
- Myrosinase-type activity is present in mycelia of *A. brassicicola*.

Abstract

The metabolism of benzyl and phenyl glucosinolates by three phytopathogenic fungal species, i.e., the specific *Alternaria brassicicola* that infects stems, leaves and pods of crucifer crops and the generalists *Rhizoctonia solani* that infects mainly below ground plant organs, and *Sclerotinia sclerotiorum* that infects above ground organs are reported. It is established that *A. brassicicola* metabolizes intact benzyl and phenyl glucosinolates and the corresponding desulfo derivatives, whereas the generalists *R. solani* and *S. sclerotiorum* do not. Phenylacetonitrile and benzylisothiocyanate are the first metabolic products of benzyl glucosinolate. The myrosinase-like activity detected in mycelia of *A. brassicicola*, but not in mycelia of *R. solani* or *S. sclerotiorum* is consistent with these metabolic results. Furthermore, benzyl isothiocyanate is metabolized by *A. brassicicola* to 3-benzyl-2-thioxo-2,3-dihydrothiazolidine-4-carboxylic acid. Bioassays showed that benzyl and phenyl isothiocyanates and nitriles are strong growth inhibitors of both *R. solani* and *S. sclerotiorum* but not of *A. brassicicola*. These findings emphasize that the metabolism of aromatic glucosinolates and desulfo congeners by phytopathogenic fungi is dependent on each fungal species.

Keywords: *Alternaria brassicicola*; Brassicaceae; crucifer; benzyl glucosinolate; benzylisothiocyanate; phenyl glucosinolate; myrosinase activity; *Rhizoctonia solani*; *Sclerotinia sclerotiorum*

1. Introduction

Benzyl (**1**) and phenyl (**2**) glucosinolates belong to a class of compounds known as glucosinolates (GLs), which carry a unique functional group composed of an O-sulfated thiohydroximate connected to a β -glucopyranosyl residue through a sulfur atom. Recently, it was estimated that about 140 reliably characterized GLs (Agerbirk and Olsen, 2012; Blazevic et al., 2020) have been reported from plant species belonging to the order Brassicales. Within Brassicales, the family Brassicaceae (common name crucifer) is the most economically relevant. Indeed, Brassicaceae comprises numerous *Brassica* species that yield edible and industrial oils, vegetables, ornamentals and condiments. Because GLs and their metabolic products are involved in plant innate immunity and defense pathways, some of which include phytoalexins (Pedras et al., 2011; Pedras and Abdoli, 2017), these areas are under intensive investigation (Pastorczyk and Bednarek, 2016). Projects investigating GL metabolic processes occurring in plants, insects and microbial organisms are advancing greatly our understanding of the ecological functions of GLs.

Fig. 1. Metabolic transformation of benzyl glucosinolate (**1**) in crucifer species (order Brassicales) and potential transformation of phenyl glucosinolate (**2**).

Namely, *in planta*, myrosinases (thioglucoside glucohydrolases, EC 3.2.1.147) catalyze the hydrolyses of GLs to unstable thiohydroximate intermediates that rearrange spontaneously to isothiocyanates. Additional enzymatic and chemical transformations of thiohydroximates to thiocyanates and nitriles are known to occur in Brassicales species (Fig. 1) (Bones and Rossiter, 2006; Halkier and Gershenzon, 2006; Sønderby et al., 2010). While benzyl GL (**1**) and benzylisothiocyanate (**5**) are produced in various species, as for example *Arabidopsis thaliana* L. (Geu-Flores et al., 2009), production of phenyl GL (**2**) and phenyl isothiocyanate (**6**)

in crucifers is not established. Indeed, to date, reports of detection of **2**, and **6-8** appear unconvincing (Blazevic et al., 2020).

Previously, we investigated the transformation of indole GLs and the corresponding desulfo-GLs by the phytopathogenic fungi *Alternaria brassicicola* (Schwein.) Wiltshire, *Rhizoctonia solani* Kuhn and *Sclerotinia sclerotiorum* (Lib.) de Bary (Pedras and Hossain, 2011). It was established that indole GLs were not transformed by any of the fungal species, although low myrosinase activity was detected in mycelia of *A. brassicicola*. However, indole desulfo-GLs were metabolized to the corresponding indolyl-3-acetonitriles by the generalist pathogens *R. solani* and *S. sclerotiorum*, but not by the specific pathogen *A. brassicicola*. In continuation of that work, to evaluate the metabolic interactions of aromatic glucosinolates with phytopathogenic fungi, we have analyzed the effects of benzyl GL (**1**) and phenyl GL (**2**), the corresponding nitriles **3** and **8**, and benzylisothiocyanate (**5**) on pathogenic fungi. In contrast with previous results (Pedras and Hossain, 2011), it was discovered that the specific pathogen *A. brassicicola* metabolized both GLs **1** and **2**, as well as benzylisothiocyanate (**5**), but the generalists *R. solani* and *S. sclerotiorum* did not (Hossain, 2012). These results were consistent with the enzymatic activity detected in mycelia of *A. brassicicola*, but not in *R. solani* and *S. sclerotiorum*. Here we describe the results of this investigation, including the antifungal activities of various metabolites.

2. Results

2.1 Chemical synthesis and antifungal activity of benzyl GL (**1**), phenyl GL (**2**), benzyl desulfo-GL (**13**) and phenyl desulfo-GL (**14**).

Benzyl GL (**1**) and phenyl GL (**2**) were synthesized as summarized in Fig. 2 (Mays et al., 2008) and detailed in the Supplementary Material. The antifungal activities of benzyl GL (**1**), phenyl GL (**2**), benzyl desulfo-GL (**13**) and phenyl desulfo-GL (**14**) were determined using the mycelial radial bioassay described previously (Pedras and Hossain, 2011). None of these compounds displayed antifungal activity against the fungal species *A. brassicicola*, *R. solani* or *S. sclerotiorum*. These results are consistent with the lack of antifungal activities of indole GLs.

Fig. 2. Synthesis of benzyl GL (**1**), phenyl GL (**2**), benzyl desulfo-GL (**13**) and phenyl desulfo-GL (**14**). Reagents: (i) NCS, Py, DMF; (ii) thioglucose, Et_3N ; (iii) MeOK, MeOH; (iv) HSO_3Cl , Py, KHCO_3 .

2.2 Metabolism of benzyl GL (**1**), phenyl GL (**2**), benzyl desulfo-GL (**13**) and phenyl desulfo-GL (**14**)

Metabolic transformations of GLs and desulfo-GLs were carried out using mycelial cultures in water (Pedras and Hossain, 2011). To obtain sufficient mycelial mass, cultures of *A. brassicicola*, *R. solani*, or *S. sclerotiorum* were first incubated in a chemically defined medium for 2-3 days, as detailed in the experimental section. Next, the fungal mycelia were aseptically filtered, washed, and finally transferred into sterile water (under these conditions the cultures remained viable for at least seven days). Mycelial cultures of each species were then incubated aseptically with compounds for various time periods, samples were withdrawn from cultures immediately after addition of each compound and at different times up to five days, extracted with EtOAc and the resulting aqueous layer immediately frozen and lyophilized (Pedras and Hossain, 2011). For comparison, mycelial cultures of each species, in water containing no amendments, were treated similarly. As well, control solutions of each compound in water were treated similarly, to determine the chemical stability and recovery of compounds in aqueous solutions.

Mycelia cultures of *A. brassicicola*, *R. solani*, or *S. sclerotiorum* in water were incubated with benzyl GL (**1**). HPLC analysis of EtOAc extracts and culture samples indicated that benzyl GL (**1**) was completely transformed by *A. brassicicola* in 24 h (Fig. 3A). The chemical structures of metabolites of benzyl GL (**1**) were determined by comparison of UV and MS-ESI spectra (available in our virtual libraries) and HPLC retention times with those of authentic samples

(commercially available) to be phenylacetonitrile (**3**), phenylacetic acid (**15**) and traces of benzylisothiocyanate (**5**), all detected in cultures after 6 h. The trace amounts of **5** detected in extracts of cultures are likely due to losses through evaporation (rather volatile, see Section 2.3). However, no metabolites were detected in cultures of *R. solani* or *S. sclerotiorum* (up to 120 h). Similarly, mycelial cultures of *A. brassicicola*, *R. solani*, or *S. sclerotiorum* were incubated with phenyl GL (**2**). HPLC analysis of culture samples (Fig. 3B) indicated transformation of phenyl GL (**2**) by *A. brassicicola* to benzonitrile (**8**), but no transformations were detected in cultures of *R. solani* or *S. sclerotiorum* (up to 120 h). The identity of benzonitrile (**8**) was confirmed by comparison with a commercially available sample (HPLC retention time and UV and MS-ESI spectra). The transformation of benzyl GL (**1**) was substantially faster than that of phenyl GL (**2**) (24 h vs. 72 h).

Fig. 3. Kinetics of transformation of (HPLC-method B): **A**, benzyl glucosinolate (**1**), and **B**, phenyl glucosinolate (**2**) in cultures of *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments conducted in triplicate $\pm$ standard deviations.

As above, mycelial cultures of *A. brassicicola*, *R. solani*, and *S. sclerotiorum* in water were incubated separately with desulfo-GLs **13** and **14**. HPLC analysis of samples of cultures of *A. brassicicola* indicated a very slow transformation of benzyl desulfo-GL (**13**) (ca. 20% in 120 h) to phenylacetoneitrile (**3**) whereas HPLC analysis of samples of cultures of *A. brassicicola* indicated complete transformation of phenyl desulfo-GL (**14**) to benzonitrile (**8**) in 120 h (Fig. 4B). No transformation of either **13** or **14** was detected in cultures of *R. solani* or *S. sclerotiorum* (Figs. 4A, 4B). Overall, our results show that transformation of benzyl desulfo-GL (**13**) was substantially slower than that of phenyl desulfo-GL (**14**), contrary to the rates of transformation of benzyl and phenyl GLs **1** and **2**.

Fig. 4. Kinetics of transformation of (HPLC-method B): **A**, benzyl desulfo-GL (**13**), and **B**, phenyl desulfo-GL (**14**) in cultures of *Alternaria brassicicola* (*A.b*), *Rhizoctonia solani* (*R.s*) and *Sclerotinia sclerotiorum* (*S.s*). Concentrations were determined using calibration curves; each point is the average of at least three independent experiments conducted in triplicate $\pm$ standard deviations.

Similarly, to determine the fates of phenylacetoneitrile (**3**) and benzonitrile (**8**) in mycelial cultures of *A. brassicicola*, *R. solani*, and *S. sclerotiorum*, their transformations were investigated. Phenylacetoneitrile (**3**) was administered to cultures of *A. brassicicola*, *R. solani*,

and *S. sclerotiorum*, samples were collected at different times up to 120 h, extracted, lyophilized and the extracts and residues were analyzed by HPLC. HPLC chromatograms showed a decrease of the phenylacetonitrile (**3**) peak and appearance of new peaks corresponding to phenylacetic acid (**15**) and benzoic acid (**16**) in samples of cultures of *A. brassicicola* and *S. sclerotiorum* (both showed 100 % transformation in 12 h), but no transformation was detected in cultures of *R. solani* (Fig. 5A). Similar experiments using benzonitrile (**8**) and HPLC analyses indicated that it was metabolized to benzoic acid (**16**) only by *S. sclerotiorum* (100% in 96 h), but not by *A. brassicicola* or *R. solani* (Fig. 5B). The summary and sequence of these transformations by both *A. brassicicola* and *S. sclerotiorum* are summarized in Fig. 6.

Fig. 5. Kinetics of transformation of (HPLC-method A): **A**, phenyl acetonitrile (**3**) and **B**, benzonitrile (**8**) in cultures of *Alternaria brassicicola* (*A.b*), *Rhizoctonia solani* (*R.s*) and *Sclerotinia sclerotiorum* (*S.s*). Concentrations of phenyl acetonitrile (**3**) and benzonitrile (**8**) were determined using calibration curves; each point is the average of at least three independent experiments conducted in triplicate $\pm$ standard deviations.

Fig. 6. Transformation of benzyl GL (**1**), phenyl GL (**2**), benzyl desulfo-GL (**13**) and phenyl desulfo-GL (**14**) by *Alternaria brassicicola*, and metabolites **3** and **15** by *A. brassicicola* and *Sclerotinia sclerotiorum*.

2.3 Metabolism of benzyliothiocyanate (**5**) by *Alternaria brassicicola*

The volatility of benzylisothiocyanate (**5**) and phenylisothiocyanate (**6**) was a hindrance to investigate their potential metabolism in fungal cultures. Hence, preliminary experiments to test their detection, stability and recovery in aqueous solutions were carried out. When solutions of each compound (**5** and **6** in CH₃CN) were added to water (final concentration ca. 0.1 mM) and the aqueous solutions were directly analyzed by HPLC, ca. 30% of benzylisothiocyanate (**5**) was detected after 72 h of incubation, whereas only ca. 10% of phenylisothiocyanate (**6**) was detected after incubation for 6 h (Fig. S1A). These differences were attributed to the faster rate of evaporation of phenylisothiocyanate (**6**) than that of benzylisothiocyanate (**5**). In addition, incubation of benzylisothiocyanate (**5**) with mycelial cultures of *A. brassicicola* (0.10 mM), followed by direct HPLC analysis of cultures (no extractions carried out) indicated that only traces of **5** were detected after 24 h of incubation. Importantly, benzylisothiocyanate (**5**) was found to be stable in fungal cultures of *R. solani* and *S. sclerotiorum* since its concentrations in cultures were similar to those of control solutions even after 72 h of incubation (Fig. 7).

Fig. 7. Kinetics of transformation of (HPLC-method A) benzylisothiocyanate (**5**) in cultures of *Alternaria brassicicola* (A.b) and cultures of *Rhizoctonia solani* (R.s) and *Sclerotinia sclerotiorum* (S.s) and control solutions. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments conducted in triplicate $\pm$ standard deviations.

Next, to identify the products of transformation of benzylisothiocyanate (**5**) by *A. brassicicola*, mycelial cultures were incubated in water with **5** and culture samples were collected immediately and then every 12 h up to four days. The culture samples were lyophilized and the residues (dissolved in aqueous CH₃CN) were analyzed by HPLC-DAD and HPLC-DAD-ESI-MS. Mycelial cultures of *A. brassicicola* were prepared and analyzed similarly. The HPLC chromatograms of cultures of *A. brassicicola* incubated with benzylisothiocyanate (**5**) showed the presence of a broad but prominent peak with retention time of ca. 4.0 min, not present in control cultures (Fig. S2A). The UV spectrum of this metabolite (t_R = 4.0 min) was similar to that of a synthetic compound available in our HPLC-UV-MS libraries, i.e., 4-hydroxy-3-benzyl-2-thioxothiazolidine-4-carboxylic acid (**17**). Furthermore, the structure of metabolite **17** was confirmed by comparison of its HPLC-DAD retention time, HRMS (ESI-negative mode, m/z 268.0104 consistent with the molecular formula C₁₁H₁₁NO₃S₂) and UV spectra with those of a synthetic sample (Pedras and Thapa, 2020). That is, the identity of the metabolite with t_R = 4.0 min was confirmed to be 3-benzyl-2-thioxo-2,3-dihydrothiazolidine-4-carboxylic acid (**17**). Compound **17** was synthesized by reacting near stoichiometric amounts of benzylamine (**19**), CS₂ and bromopyruvic acid; during chromatographic separation compound **17** underwent dehydration to yield the more stable compound **20** (Fig. 8) (Pedras and Thapa, 2020).

Fig. 8. Synthesis and dehydration of 3-benzyl-2-thioxo-2,3-dihydrothiazolidine-4-carboxylic acid (**17**) to **20** (Pedras and Thapa, 2020).

Importantly, when mycelial cultures of *A. brassicicola* were incubated with benzylisothiocyanate (**5**) and L-cysteine, the formation of **17** increased substantially, as summarized in Fig. 9. To confirm that **17** was formed in cultures of *A. brassicicola* incubated with benzyl GL (**1**), additional incubation experiments were carried out. Namely, culture samples were collected and immediately lyophilized without any preliminary extraction. Under these conditions, HPLC analysis of culture samples (using elution method C, Fig. S2C) confirmed that 4-hydroxy-3-benzyl-2-thioxothiazolidine-4-carboxylic acid (**17**) was produced in cultures of *A. brassicicola* incubated with **1** (Fig. S3).

Fig. 9. Progress curve for the formation of 4-hydroxy-3-benzyl-2-thioxothiazolidine-4-carboxylic acid (**17**) (HPLC-method C) in cultures of *Alternaria brassicicola* incubated with benzylisothiocyanate (**5**) and benzylisothiocyanate (**5**) plus L-cysteine (L-Cys). Concentrations were determined using calibration curves; each point is the average of at least two independent experiments conducted in triplicate $\pm$ standard deviations.

2.4 Antifungal activity of metabolites

The antifungal activities of nitriles **3** and **8**, isothiocyanates **5** and **6**, and carboxylic acids **15** and **16** were determined against *A. brassicicola*, *R. solani* and *S. sclerotiorum*, as summarized in Table 1. Among all compounds, benzylisothiocyanate (**5**) was the most inhibitory, causing complete inhibition of mycelial growth of *R. solani* and *S. sclerotiorum* at 0.50 mM, although it was only slightly inhibitory to *A. brassicicola* at the same concentration. These results are consistent with previous work reporting inhibitory activity of benzylisothiocyanate (**5**) against *A. brassicicola* (EC₅₀ 2.4 – 6.2 mM; Sellam et al., 2007) and *S. sclerotiorum* (complete inhibition at 0.47 mM; Kurt et al., 2011). In addition, benzylisothiocyanate (**5**) was more inhibitory to *S. sclerotiorum* than phenylisothiocyanate (**6**), which is also consistent with recent work (74% inhibition at 0.47 mM; Kurt et al., 2011).

Table 1. Antifungal activity of isothiocyanates **5** and **6**, nitriles **3** and **8** and carboxylic acids **15** and **16** against the plant fungal pathogens *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* under continuous light (**17** was not tested as it is not sufficiently stable).

Compound (#)	Conc. (mM)	% Inhibition (± S. E.)*		
		<i>A. brassicicola</i>	<i>R. solani</i>	<i>S. sclerotiorum</i>
Benzylisothiocyanate (5)	0.50	11 ± 1	100	100
	0.20	NI	59 ± 1	50 ± 1
	0.10	NI	20 ± 1	18 ± 1
Phenylisothiocyanate (6)	0.50	NI	44 ± 4	63 ± 1
	0.20	NI	16 ± 1	48 ± 1
	0.10	NI	9 ± 1	10 ± 1
Phenylacetonitrile (3)	0.50	NI	51 ± 1	59 ± 1
	0.20	NI	29 ± 1	44 ± 1
	0.10	NI	9 ± 1	17 ± 1
Benzonitrile (8)	0.50	NI	51 ± 1	59 ± 1
	0.20	NI	29 ± 1	44 ± 1
	0.10	NI	9 ± 1	17 ± 1
Phenyl acetic acid (15)	0.50	14 ± 1	18 ± 1	8 ± 1
	0.20	9 ± 1	10 ± 1	NI
	0.10	NI	NI	NI
Benzoic acid (16)	0.50	17 ± 1	NI	11 ± 1
	0.20	NI	NI	5 ± 1
	0.10	NI	NI	NI

*Percentage of growth inhibition was calculated using the formula: $100 - [(\text{diameter of mycelia on amended medium} / \text{diameter of mycelia in control medium}) \times 100]$. Data are means ± S. E. two independent experiments conducted in triplicate; NI= no inhibition.

2.5 Myrosinase activity

Myrosinase activity in cruciferous fungal pathogens has been reported only for *A. brassicicola* (Pedras and Hossain, 2011) and was determined using glucobrassicin (**21**) as substrate; the products of this enzymatic reaction were indolyl-3-methanol and diindolyl-3-methane. To determine myrosinase activity, the amounts of each product were quantified by HPLC using calibration curves built with synthetic samples. For this reason, ascorbate was not included in the assay mixture to avoid its reaction with indolyl-3-methanol, although it has been reported to increase myrosinase activity (Shikita et al., 1999). Using this assay, crude cell-free extracts of mycelia of *A. brassicicola* showed low but consistent myrosinase activity (0.4 nmoles/mg/min) (Pedras and Hossain, 2011). In this work, to confirm myrosinase activity in *A. brassicicola* and also screen for myrosinase activity in *R. solani* and *S. sclerotiorum*, a more sensitive enzymatic assay was used. Myrosinase activity was determined by calculating the concentration of glucose formed due to myrosinase activity on any glucosinolate (glucose assay Sigma kit). In brief, the assay involves phosphorylation of glucose released by myrosinase (using ATP and hexokinase), followed by product oxidation to 6-phosphogluconate in a reaction catalyzed by glucose-6-phosphate dehydrogenase in the presence of NAD. During this oxidation step, an equimolar amount of NAD is reduced to NADH, causing an increase in absorbance at 340 nm, which is directly proportional to glucose concentration. The experimental procedure is described in Section 4.3 and the results are summarized in Table 2, using benzyl glucosinolate (**1**), phenyl glucosinolate (**2**), glucobrassicin (**21**), 1-methoxyglucobrassicin (**22**) and sinigrin (**23**) as substrates. Myrosinase activity of *R. solani* and *S. sclerotiorum* was tested similarly in the crude cell-free exact of mycelia, but no activity was detected with any of the glucosinolates tested. The determination of myrosinase activity by measuring the amount of glucose released seems to be more sensitive than the direct measurement of indolyl-3-methanol and diindolyl-3-methane from glucobrassicin (**21**) (Pedras and Hossain, 2011). Using this glucose assay kit, the specific activity of the myrosinase present in crude cell-free extracts of *A. brassicicola* was higher with benzyl and phenyl GLs than with glucobrassicins as substrates. These results are consistent with the metabolism results reported above and those previously reported (Pedras and Hossain, 2011), which demonstrated that *A. brassicicola* metabolized both benzyl and phenyl GLs, but not glucobrassicins. A commercially available plant myrosinase (from *Sinapis alba* L., ca. 100 units/g solid; one unit produces 1.0 μ mole of glucose per min from sinigrin at pH 6.0) was tested

with the same substrates to compare its specific activities with those of fungal myrosinase from *A. brassicicola*. Interestingly, as shown in Table 2, commercial myrosinase from *S. alba* displayed similar activities for benzyl GL (**1**), glucobrassicin (**21**), 1-methoxyglucobrassicin (**22**) and sinigrin (**23**), but much lower activity with phenyl GL (**2**). Furthermore, considering that the commercial myrosinase was purified, its substantially higher specific activity than that present in crude cell-free extracts of *A. brassicicola* was expected; the specific activity of commercial myrosinase was about eightfold higher with benzyl glucosinolate (**1**) than with phenyl glucosinolate (**2**) (Table 2).

Table 2. Myrosinase activity of crude cell-free extracts of *Alternaria brassicicola* and commercial myrosinase^a (thioglucosidase from *Sinapis alba* seed).

Compound	Specific activity (nmol mg ⁻¹ min ⁻¹) ± standard deviation	
	Cell-free extracts	Commercial myrosinase
Benzyl glucosinolate (1)	2.29 ± 0.28	45.2 ± 7.4
Phenyl glucosinolate (2)	2.14 ± 0.42	5.8 ± 3.0
Glucobrassicin (21)	1.19 ± 0.33	38.5 ± 3.4
1-Methoxyglucobrassicin (22)	1.29 ± 0.35	46.7 ± 5.0
Sinigrin (23)	2.32 ± 0.11	54.1 ± 0.8

^a Purchased from Sigma-Aldrich Canada.

3. Discussion and conclusion

To the best of our knowledge, this is the first report demonstrating that a specific fungal plant pathogen, *A. brassicicola*, is able to metabolize benzyl GL (**1**), phenyl GL (**2**), desulfo-GLs **13** and **14**, and benzylisothiocyanate (**5**), whereas the generalist pathogens *R. solani* and *S. sclerotiorum* are not (Table 3). These results indicate that *A. brassicicola* produces myrosinase(s)-type activity that catalyzes the transformations of GLs **1** and **2** to the thiohydroximate sulfates that spontaneously rearrange to the corresponding isothiocyanates **5**

and **6** (Table 3); this conclusion is consistent with the enzymatic activity detected in mycelia of *A. brassicicola*.

Table 3. Summary of metabolism of compounds **1–3**, **5**, **8**, **13** and **14** by *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* under continuous light.

Compound (#)	Metabolic products; % transformation		
	<i>A. brassicicola</i>	<i>R. solani</i>	<i>S. sclerotiorum</i>
Benzyl glucosinolate (1) (HPLC-methods B and C)	Phenylacetonitrile (3), benzyl isothiocyanate (5); phenyl acetic acid (15), benzoic acid (16); 3-benzyl-2-thioxo-2,3-dihydrothiazole-4-carboxylic acid (17); 100% in 24 h	No transformation	No transformation
Phenyl glucosinolate (2) (HPLC-method B)	Benzonitrile (8); 100% in 72 h	No transformation	No transformation
Benzyl desulfoglucosinolate (13) (HPLC-method B)	Phenylacetonitrile (3); (very slow transformation) 20% in 120 h	No transformation	No transformation
Phenyl desulfoglucosinolate (14) (HPLC-method B)	Benzonitrile (8); 50% in 60 h	No transformation	No transformation
Phenylacetonitrile (3) (HPLC-method A)	Benzoic acid (16); 100% in 12 h	No transformation	Benzoic acid (16); 100% in 12 h
Benzonitrile (8) (HPLC-method A)	No transformation	No transformation	Benzoic acid (16); 50% in 24 h
Benzylisothiocyanate (5) (HPLC-method C)	Transformed to 3-benzyl-2-thioxo-2,3-dihydrothiazole-4-carboxylic acid (17); 100% in 24 h	No transformation	No transformation

In addition, the formation of nitrile **3** from GL **1**, similar to metabolic transformations occurring in crucifers, suggests the presence of a nitrile specifier protein(s) (Kissen and Bones, 2009). As well, *A. brassicicola* is likely to produce glucosyl hydrolase(s) that catalyze the transformations of desulfo-GLs **13** and **14** to nitriles **3** and **8**, respectively; however, since these transformations were much slower than those of GLs, we suggest that these hydrolases are not specific for desulfo-GLs. These discoveries are in contrast with our previous work showing

that indole GLs were not metabolized by any of these fungal species (Pedras and Hossain, 2011).

The transformation of benzyliothiocyanate (**5**) to benzyl-2-thioxothiazolidine **17** (4-hydroxy-3-benzyl-2-thioxothiazolidine-4-carboxylic acid) in cultures of *A. brassicicola* is likely to result from conjugation with L-Cys, similar to the metabolism of **5** in mammalian systems (Görler et al., 1982). Furthermore, it is noteworthy that the formation of a metabolite related to **17**, 4-hydroxy-3-(4-methoxy-1*H*-indol-3-yl)-2-thioxothiazolidine-4-carboxylic acid (**25**), was recently reported from cultures of *Colletotrichum* species incubated with the cruciferous phytoalexin rapalexin A (**24**) (Pedras and Thapa, 2020). That is, the phytopathogenic fungal species *Colletotrichum higginsianum* and *C. dematium* were able to metabolize 4-methoxyindolyl-3-isothiocyanate (**24**) to carboxylic acid **25** (Fig. 10). This transformation was proposed to result from electrophilic addition of the thiol group of glutathione (GSH) to the isothiocyanate carbon of rapalexin A (**24**) to yield an L-Cys-conjugate that undergoes transamination and cyclization to **25** (on standing in solution, **25** decomposes slowly to yield back intact rapalexin A (**24**)).

Fig. 10. Transformation of rapalexin (**24**) to 4-hydroxy-3-(4-methoxy-1*H*-indol-3-yl)-2-thioxothiazolidine-4-carboxylic acid (**25**) mediated by *Colletotrichum higginsianum* and *C. dematium* (Pedras and Thapa, 2020).

Although benzyl-2-thioxothiazolidine **17** is not stable in solution and was found to dehydrate to the corresponding 3-benzyl-2-thioxo-2,3-dihydrothiazole-4-carboxylic acid (**20**), it was detected and isolated from cultures of *A. brassicicola* in sufficient amounts for spectroscopic characterization. Consequently, based on the above results and by analogy to our previous work (Pedras and Thapa, 2020), we propose that benzyl GL (**2**) is transformed by *A. brassicicola* via two main pathways: pathway A, via conjugation of benzyliothiocyanate (**5**) with GSH and subsequent hydrolyses to yield 4-hydroxy-3-benzyl-2-thioxothiazolidine-4-carboxylic acid (**17**), and pathway B through hydrolysis and oxidation to yield nitrile **3**, phenylacetic acid (**15**), and finally benzoic acid (**16**) (stable in culture, no further transformation detected) (Fig. 11). The formation of **17** is consistent with the production of isothiocyanate-inducible glutathione transferases (GSTs) in *A. brassicicola* (Calmes et al. 2015).

Fig. 11. Proposed pathway for transformation of benzyl glucosinolate (1) and benzyliothiocyanate (5) to 4-hydroxy-3-benzyl-2-thioxothiazolidine-4-carboxylic acid (17) and benzoic acid (16) mediated by *Alternaria brassicicola*; two arrows indicate more than one step.

Related work has shown that a soil isolate of the fungus *Aspergillus flavus* transformed 2-propenyl GL and 2-phenylethyl GL and the corresponding desulfo-GLs to nitriles; these transformations were postulated to involve first a sulfatase followed by a thioglucosidase (Galletti et al., 2008). More recently, it was reported that *S. sclerotiorum* metabolizes aliphatic and phenylethyl isothiocyanates via two independent pathways, conjugation to glutathione and hydrolysis to amines (Chen et al., 2020). Since we did not use 2-phenylethylisothiocyanate and our results indicate that neither *S. sclerotiorum* nor *R. solani* metabolize benzyliothiocyanate (5), it is not sensible to speculate about the reasons for this apparent discrepancy until further work is carried out.

In contrast to the paucity of studies on GL metabolism by fungi, metabolism of GLs in the class Insecta has shown that many species detoxify GLs and their hydrolysis products (Jeschke et al., 2016; Jeschke et al., 2017). For example, larvae of *Pieris rapae* express a nitrile-specifier protein suggested to redirect the hydrolysis of benzyl GL (1) towards the formation of nitrile 3 (instead of isothiocyanate 5), followed by hydrolysis and conjugation of 3 with glycine (Winde and Wittstock, 2011). Because benzyliothiocyanate (5) is detrimental to

P. rapae, redirection of this pathway is favorable the pest. Similarly, metabolism of benzyl GL (**1**) to *N*-(benzoyl) aspartic acid in larvae of *Phaedon cochleariae* was thought to be a detoxification step (Friedrichs et al., 2020).

In this work, considering that neither GLs nor desulfo-GLs are toxic to *A. brassicicola*, and that the final products of their metabolism, acids **15** and **16**, are slightly inhibitory, the benefits of these transformations to the pathogen are unclear. However, a potential explanation is provided by work carried out using transformed *A. thaliana* (expressing CYP79A1 or over-expressing the endogenous CYP79A2 and accumulating *p*-hydroxybenzyl GL or benzyl GL) and *A. brassicicola* (Brader et al., 2006). Because CYP79A1 and CYP79A2 plants showed higher susceptibility to *A. brassicicola* than the wild-type, it was suggested that tissue damage caused by *A. brassicicola* infection releases aromatic breakdown products in the transformed plants, triggering salicylic acid mediated defenses that in turn suppress the jasmonic acid signaling pathway. A plant deprived of the jasmonic acid pathway is more susceptible to colonization by *A. brassicicola*, hence we suggest that transformation of **1** may be advantageous to the pathogen. On the other hand, our results indicate that since isothiocyanates **5** and **6** and nitriles **3** and **8** are inhibitory to both *R. solani* and *S. sclerotiorum*, it would not be advantageous for these pathogens to metabolize GLs **1** or **2** since the metabolic products are toxic, and indeed no metabolism was detected.

In conclusion, taking into consideration results of our work on aromatic and indole GLs, we suggest that before generalizations can be made, pathways regulating the fungal metabolism of GLs and their hydrolysis products ought to be carefully examined using different phytopathogenic fungal species. Ultimately, a better understanding of such pathways will facilitate the design of crucifer crops with increased resistance to fungal pathogens.

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). Flash column chromatography (FCC), HPLC, HPLC-DAD-ESI-MS, NMR and FTIR were carried out as previously reported (Pedras and Hossain, 2011).

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 μ m particle size silica, 4.6 i.d. $\times$ 150 mm), mobile phase 50% H₂O - 50% CH₃OH to 100% CH₃OH, 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 μ m particle size silica, 100 $\times$ 3.0 mm I.D.), equipped with an in-line filter, with the mobile phase H₂O (with 0.1% TFA) - CH₃OH (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; elution method C: column Eclipse XDB C-18 column (5.0 μ m particle size silica, 150 $\times$ 2.1 mm I.D.), mobile phase H₂O-CH₃CN from 100:0 to 10:90, linear gradient for 23 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 μ m particle size silica, 4.6 i.d. $\times$ 150 mm). The mobile phase consisted of a linear gradient of 0.2% formic acid in water and 0.2% formic acid in CH₃CN (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.

4.2 Mycelial radial growth antifungal bioassays, fungal cultures and metabolism studies

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 $\pm$ 1°C were used for mycelial

radial growth assays. Assays and conditions were identical to previously reported work (Pedras and Hossain, 2011).

For metabolic experiments, liquid cultures of each fungus 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* (Pedras and Hossain, 2011). Cultures were incubated for different periods, the mycelia from each culture were transferred to 250 mL flasks containing sterile water (100 mL), and a solution of each compound 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, except for L-cysteine used at 0.20 mM. Samples (5-10 mL) were withdrawn at various times and immediately extracted with EtOAc, frozen and lyophilized, or immediately frozen and lyophilized. The extracts were concentrated and organic and aqueous residues were dissolved in MeOH, CH_3CN , or MeOH- H_2O and analyzed by HPLC, using method A (extracts, less polar metabolites) or methods B and C for aqueous residues (desulfo-GLs and polar metabolites).

4.3 Myrosinase activity

Cell-free extracts of mycelia of *A. brassicicola*, *R. solani* and *S. sclerotiorum* were prepared as previously established (Pedras and Hossain, 2011). To initiate a reaction, each GL (250 μL , 10 mM) dissolved in buffer solution (KH_2PO_4 , pH 7.0) was added to the cell-free extract (720 μL) containing ascorbic acid (30 μL , 10 mM). After 90 min of incubation, the reaction assay (200 μL) was used to analyze its glucose content using a glucose test kit (Glucose (HK) Assay Kit GAHK20, Sigma-Aldrich). The specific activity was calculated as the number of nmoles of product (glucose) formed per mg of protein per min. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration (Sigma-Aldrich bulletin). The Bradford assay method was used to calculate the protein content of cell-free extracts using a standard BSA calibration curve (wavelength 595 nm).

Commercially available myrosinase from *S. alba* (Sigma-Aldrich, thioglucosidase from *Sinapis alba* (white mustard) seed, ≥ 100 units/g solid, one unit produces 1.0 μmol glucose per min from sinigrin at pH 6.0 at 25 °C) was dissolved in buffer (pH 7.0) in 0.05 mg/mL and each GL (250 μL , 10 mM) dissolved in buffer solution was added to the commercial myrosinase solution (720 μL) containing ascorbic acid (30 μL of 10 mM). After 90 min of incubation, the reaction assay (200 μL) was used to analyze the glucose content as above.

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Supplementary Material

Syntheses and characterization of benzyl desulfo-glucosinolate (13) and phenyl desulfo-glucosinolate (14)

Benzyl desulfo-glucosinolate (13). Phenylacetaldehyde (500 mg, 4.20 mmol) was dissolved in EtOH (15 mL) and a solution of $\text{NH}_2\text{OH}\cdot\text{HCl}$ (580 mg, 8.30 mmol) and Na_2CO_3 (460 mg, 4.60 mmol) in water (8 mL) was added. After stirring at 60 °C for 1 h, the reaction mixture was diluted with water and extracted with Et_2O . The organic phase was dried over Na_2SO_4 and concentrated to dryness to give phenylacetaldehyde oximes (9). The crude reaction mix was separated by FCC (hexane : EtOAc, 4:1, v/v) to give mixture of E and Z oximes 9 in 70% yield. A solution of phenylacetaldehyde oximes (9, 200 mg, 1.50 mmol) in DMF (4.0 mL) was cooled to 0 °C in an ice bath and NCS (198 mg, 1.50 mmol) was added in portions over a period of 45 min. Upon completion, the ice bath was removed and stirring was continued for 2 h. 1-Thio-2,3,4,6-tetra-O-acetyl-β-D-glucopyranose (270 mg, 0.74 mmol) dissolved in DMF (4.0 mL) was added to the reaction mixture followed by triethylamine (200 μl) and the reaction mixtures was stirred for another 18 h at r.t. After 18 h the reaction mixture was diluted with diethyl ether followed by H_2SO_4 (10 mL, 0.5 M) and extracted with EtOAc. The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give the crude reaction mixture, which was separated by FCC (hexane:EtOAc, 1:1, v/v) to give

pure 2,3,4,6-tetra-O-acetyl- β -glucopyranosylbenzylthiohydroximate (**11**) in 78% yield. To a cooled and stirred solution of 2,3,4,6-tetra-O-acetyl- β -glucopyranosylbenzylthiohydroximate (**11**, 460 mg, 0.95 mmol) in anhydrous MeOH (8.5 mL) under Ar, potassium methoxide (1 M) was added to attain pH 11. After stirring at room temperature for 2 h the solution was neutralized with glacial acetic acid. The solvents were then evaporated under reduced pressure, the residue was dissolved in water, filtered and freeze dried to give benzyl desulfo-GL (**13**) in quantitative yield (mp 95-96 °C). HPLC t_R = 16.5 min (Method B). $[\alpha]_D = -52$ (c 0.18, H₂O). UV (HPLC, MeOH-H₂O) λ_{max} (nm): 210. ¹H NMR (500 MHz, D₂O) δ : 7.43-7.40 (2H, m), 7.36-7.33 (3H, m), 4.70-4.68 (1 H, m), 4.03 (2H, brs), 3.66 (1H, dd, J = 13, 2.5 Hz), 3.61 (1H, dd, J = 13, 5 Hz), 3.38-3.36 (1H, m), 3.33-3.30 (2H, m), 3.26-3.23 (1H, m). ¹³C NMR (125.8 MHz, D₂O) δ : 154.6, 136.0, 129.1, 128.0, 127.3, 81.0, 79.7, 77.0, 72.0, 68.9, 60.3, 37.9. HR-ESI-MS m/z calc. for C₁₄H₁₈NO₆S: 328.0849, found 328.0848 [M-1]⁻. FTIR (KBr, cm⁻¹) ν_{max} : 3341, 2883, 1562, 1494, 1412, 1270, 1050, 982, 704.

Phenyl desulfo-glucosinolate (14). Benzaldehyde (520 μ L, 4.70 mmol) was dissolved in EtOH (5 mL) and a solution of NH₂OH.HCl (340 mg, 4.90 mmol) and Na₂CO₃ (280 mg, 2.60 mmol) in water (5 mL) was added. After stirring at 60 °C for 4 h, the reaction mixture was diluted with water and extracted with CH₂Cl₂. The organic phase was dried over Na₂SO₄ and concentrated to dryness to give benzaldehyde oximes (**12**) (mixture of E and Z isomer) (99%). A solution of benzaldehyde oximes (**12**, 200 mg, 1.60 mmol) in DMF (3.0 mL) and 100 μ L pyridine was cooled to 0° C in an ice bath and NCS (214 mg, 1.60 mmol) was added in portions over a period of 45 min. Upon completion, the ice bath was removed and stirring was continued for 2 h. 1-Thio-2,3,4,6-tetra-O-acetyl- β -D-glucopyranose (270 mg, 0.74 mmol) dissolved in DMF (4.0 mL) was added to the reaction mixture followed by triethylamine (200 μ L) and the reaction mixture was stirred for another 18 h at r.t. After 18 h the reaction mixture was diluted with Et₂O followed by H₂SO₄ (10 mL, 0.5 M) and extracted with EtOAc. The combined organic layer was dried over Na₂SO₄ and concentrated under reduced pressure to give the crude reaction mixture, which after separation by FCC (hexane:EtOAc, 1:1, v/v) yielded pure 2,3,4,6-tetra-O-acetyl- β -glucopyranosylphenylthiohydroximate (**12**) in 60% yield. To a cooled and stirred solution of 2,3,4,6-tetra-O-acetyl- β -glucopyranosylphenylthiohydroximate (**12**, 460 mg, 0.95 mmol) in anhydrous MeOH (8.5 mL) under Ar atmosphere, a few drops of potassium methoxide (ca. 1 M) was added until pH 11 was attained. After stirring at room temperature for 2 h, the solution

was neutralized with glacial acetic acid, the solvents were evaporated under reduced pressure, the residue was dissolved in water, filtered and freeze dried to give phenyl desulfo-GS (**14**) in 85% yield (mp 105-106 °C). HPLC t_R = 11.4 min (Method B). $[\alpha]_D = -22$ (c 0.19, H₂O). ¹H NMR (500 MHz, D₂O) δ 7.55-7.50 (5H, m), 4.25 (1 H, d, J = 10 Hz), 3.65 (1H, dd, J = 13, 2.5 Hz), 3.56 (1H, dd, J = 13, 5 Hz), 3.41 (1H, dd, J = 10, 10 Hz), 3.33 (1H, dd, J = 10, 9.5 Hz), 3.22 (1H, dd, J = 9, 9 Hz), 2.70-2.68 (1H, m). ¹³C NMR (125.8 MHz, D₂O) δ : 156.0, 131.7, 130.4, 129.0, 128.7, 83.1, 79.9, 77.2, 71.9, 68.9, 60.3. UV (HPLC, MeOH-H₂O) λ_{max} (nm): 210. HR-ESI-MS m/z calc. for C₁₃H₁₆NO₆S: 314.0692, found 314.0686 [M-1]⁻. FTIR (KBr, cm⁻¹) λ_{max} : 3360, 2887, 1560, 1494, 1410, 1340, 1232, 1050, 984, 703.

Recoveries of benzylisothiocyanate (5), and phenylisothiocyanate (6) in aqueous solutions

Fig. S1. Recoveries of (HPLC-method A): **A**, benzylisothiocyanate (**5**), and **B**, phenylisothiocyanate (**6**) in aqueous solutions. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments $\pm$ standard deviations.

HPLC-DAD chromatograms (method A, 220 nm) of transformation of benzyl GL and benzylisothiocyanate (5) by Alternaria brassicicola.

Fig. S2. HPLC-DAD chromatograms (method C, 220 nm) of transformation by *Alternaria brassicicola* of: **A**, benzylisothiocyanate (**5**, t_R =18.1 min), mycelial cultures incubated with **5** in water; **B**, mycelial cultures incubated with **5** plus L-cysteine in water; **C**, mycelial cultures incubated with benzyl GL (**1**, t_R =1.8 min) in water. Peak at t_R = 4.0 min is the product of transformation of **5** (3-benzyl-2-thioxo-2,3-dihydrothiazolidine-4-carboxylic acid (**17**)), peaks in 72 h control at t_R = 5.4 min (infectopyrone) and at 14.0 min (phomapyrone G) are due to metabolites produced by *A. brassicicola* (Pedras et al., 2009).

Kinetics of transformation of benzyl glucosinolate (1) and formation of 4-hydroxy-3-benzyl-2-thioxothiazolidine-4-carboxylic acid (17)

Fig. S3. Kinetics of transformation (HPLC-method C) of benzyl glucosinolate (**1**, $\blacksquare$) and formation of 4-hydroxy-3-benzyl-2-thioxothiazolidine-4-carboxylic acid (**17**, $\blacktriangle$) in cultures of *Alternaria brassicicola* and recovery of **1** in control cultures ($\square$). Concentrations were determined using calibration curves; each point is the average of two independent experiments conducted in triplicate $\pm$ standard deviations.

*^1H and ^{13}C NMR spectra of benzyl desulfo-glucosinolate (**13**) and phenyl desulfo-glucosinolate (**14**)*

154.603

135.994

129.077

128.028

127.315

81.035

79.733

77.010

71.979

68.850

60.331

37.927

4.261
4.241

3.661
3.657
3.636
3.632
3.576
3.567
3.551
3.541
3.431
3.414
3.412
3.394
3.350
3.331
3.312
3.238
3.220
3.202

2.701
2.697
2.692
2.687
2.682
2.677
2.672
2.667

181.312

156.011

131.670

130.381

129.043

128.745

83.137

79.875

77.177

71.916

68.924

60.253

23.249