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The phytopathogenic fungus *Alternaria brassicicola*: Phytotoxin production and phytoalexin elicitation

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ABSTRACT

The metabolites and phytotoxins produced by the phytopathogenic fungus *Alternaria brassicicola* (Schwein.) Wiltshire, as well as the phytoalexins induced in host plants, were investigated. Brassicicolin A emerged as the most selective phytotoxic metabolite produced in liquid cultures of *A. brassicicola* and spirobrassinin as the major phytoalexin produced in infected leaves of *Brassica juncea* (whole plants). In detached infected leaves of *B. juncea*, the main component was *N*′-acetyl-3-indolylmethanamine, the product of detoxification of the phytoalexin brassinin by *A. brassicicola*. In addition, the structure elucidation of three hitherto unknown metabolites having a fusicoccane skeleton was carried out and the antifungal activity of several plant defenses against *A. brassicicola* was determined.

1. Introduction

Alternaria brassicicola (Schwein.) Wiltshire is a phytopathogenic fungus that, together with *A. brassicae*, causes one of the most economically important diseases of *Brassica* species, Alternaria black spot (also called dark leaf spot). Oilseed brassicas (*Brassica* sp.) are valuable agricultural commodities as they contribute substantially to the global production of food, oil, and fodder, as well as biofuel. The yield losses due to black spot can be catastrophic, particularly because *Brassica juncea* L. (subspecies *oleifera*, brown mustard), a species widely cultivated in Asian countries, is quite susceptible to this disease (Vishwanath et al., 1999; Rimmer and Buchwaldt, 1995). Although the family Brassicaceae (common name crucifer) contains species reported to be resistant to *A. brassicicola*, commercially resistant cultivars of *B. juncea* or other *Brassica* sp. are not available. Therefore, the need to find resistance to black spot disease has encouraged a wealth of research dealing with *A. brassicae*, *A. brassicicola* and their host plants. Nonetheless, the understanding of the molecular interaction between *A. brassicicola* and its hosts is still rather incomplete. For example, brassicicolin A (**1**) was the first metabolite reported and isolated as an "antibiotic complex" from cultures of *A. brassicicola* (Ciegler and Lindenfelser, 1969), but its activity towards brassicas has not been determined. The unique structure of brassicicolin A (**1**) was established as a mixture of epimers only several years later (Gloer et al., 1988). As well, *A. brassicicola* was reported to produce a host-specific toxin protein called AB-toxin (Otani et al., 1998), from spores germinating on host leaves. AB-toxin was induced by a host-derived oligosaccharide of 1.3 kDa, but the encoding gene and its function remain unknown. (Oka et al., 2005). In addition, several fusicoccane-like diterpenes were isolated from phytotoxic extracts of cultures of *A. brassicicola*, but their phytotoxicity was not determined due to the small quantities obtained (MacKinnon et al., 1999).

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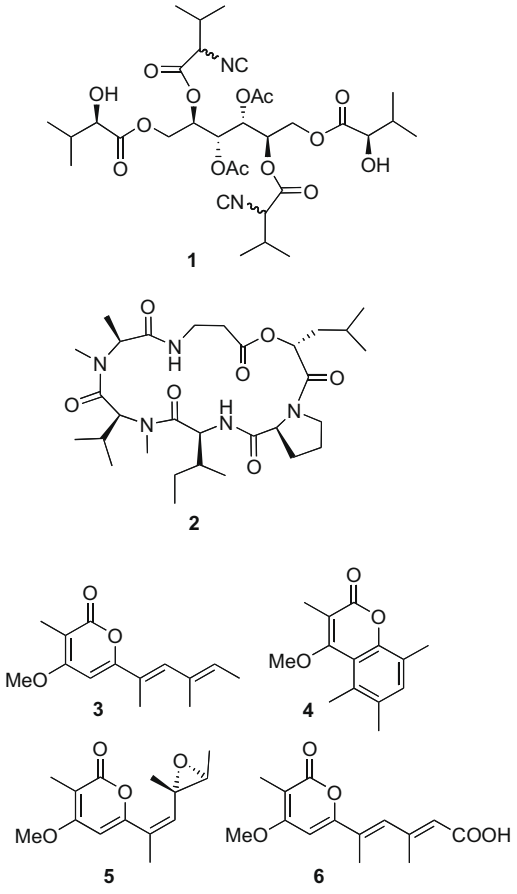
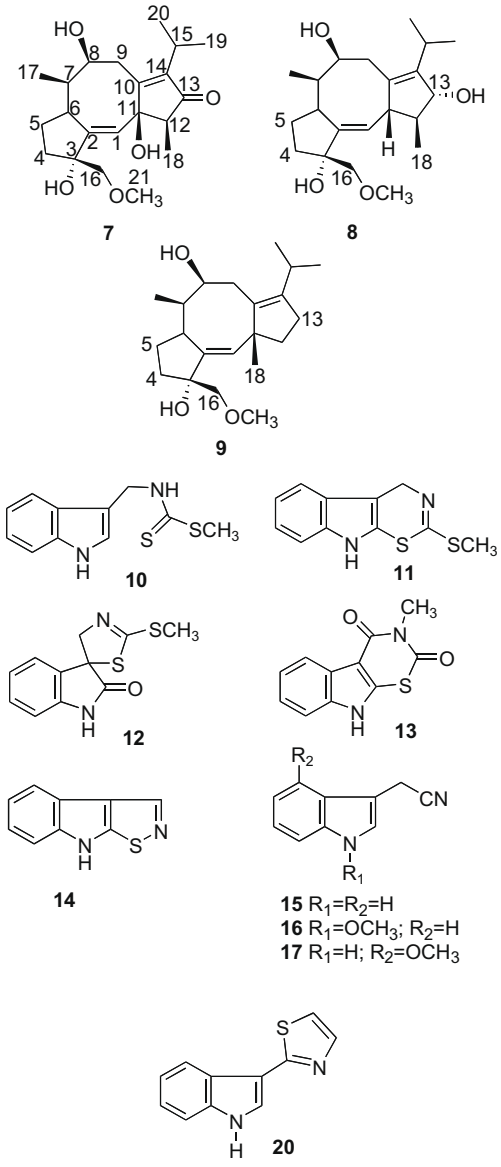


In principle, by analogy to other *Alternaria* species (Thomma, 2003), *A. brassicicola* would be expected to produce non-proteinic host-selective toxins. In fact, a recent study of the interaction between *A. brassicicola* and *B. oleracea* utilizing ESTs (expressed sequence tags) sequencing from infected plant tissue identified several candidate genes potentially involved in the production of phytotoxins (Cramer et al., 2006). Although no reports of such toxins have been published to date, siderophores were reported from *A. brassicicola* (Oide et al., 2006). The gene *NPS6* appears to encode the non-ribosomal peptide synthase (NPS) involved in the biosynthesis of these siderophores (Oide et al., 2006). Lawrence and co-workers (Kim et al., 2007) investigated the function of the *AbNPS2* gene of *A. brassicicola* and determined that it plays an important role in fungal development and in virulence, yet its end product remains undetermined. Some fungal secondary metabolites derived from NPSs were found to be host-selective phytotoxins involved in the virulence of pathogens (e.g. HC-toxin, Walton, 2006). Our work on the interaction of crucifers with *A. brassicae* has shown the significance of an NPS fungal metabolite, the host-selective toxin destruxin B (**2**). Detoxification of destruxin B (**2**) was shown to occur in crucifers resistant to black spot much faster than in susceptible species (e.g. <24 h vs. >96 h) (Pedras et al., 2001, 2003).

Necrotrophic phytopathogens such as *A. alternata* and *A. brassicae* are known to synthesize phytotoxins that damage plant tissues and facilitate colonization (Wolpert et al., 2002; Thomma, 2003), while in response to pathogen attack crucifers biosynthesize phytoanticipins and phytoalexins (Pedras et al., 2007; Pedras, 2008). Phytoalexins are secondary metabolites produced de novo by plants in response to diverse forms of stress including microbial infection, UV irradiation, and heavy metal salts (Pedras et al., 2007), whereas phytoanticipins are constitutive defenses whose concentrations can increase upon stress (VanEtten et al., 1994). To the detriment of cruciferous crops, some of their pathogenic fungi can overcome phytoanticipins and phytoalexins by producing detoxifying enzymes (Pedras and Ahiahonu, 2005).



Our approach to design strategies for controlling plant fungal diseases requires a chemical–ecological understanding of the plant and the pathogen cultured in isolation and in direct contact. Toward this end, we examined the phytotoxic metabolites produced by *A. brassicicola* in liquid culture and the metabolites produced in leaves of whole plants and in detached leaves of *B. juncea* inoculated with the pathogen and report here the results of this systematic investigation.

2. Results and discussion

2.1. Metabolites from cultures of Alternaria brassicicola

Bioassay-directed isolation of metabolites produced by three different isolates of *A. brassicicola* in a chemically defined liquid

medium was carried out. From the organic extracts of the culture broths several metabolites were isolated and their structures were determined using spectroscopic methods (^{1}H, ^{13}C NMR, 2D NMR, HRMS and FT-IR). Multiple chromatographic separations allowed the isolation of the following metabolites, according to their elution order (silica gel column chromatography): phomapyrone F (**4**, 1 mg), phomapyrone A (**3**, 30 mg), phomapyrone G (**5**, 1 mg), brassicicolin A (**1**, 30 mg) brassicicene H (**8**, <1 mg), brassicicene G (**7**, 4 mg), brassicicene I (**9**, 1 mg) and infectopyrone (**6**, 2 mg). The three new fusicoccane diterpenes, brassicicenes G (**7**), H (**8**), and I (**9**), were named by analogy to previous designations (MacKinnon et al., 1999).

From the least polar and non-phytotoxic fractions, phomapyrones A (**3**), F (**4**) and G (**5**) and infectopyrone (**6**) were isolated and their structures were determined by comparison of their chromatographic and spectroscopic data with those of authentic samples isolated from *Leptosphaeria maculans* and from *Leptosphaeria biglobosa* (Pedras et al., 1994; Pedras and Biesenthal, 2001; Pedras and Chumala, 2005).

From the most phytotoxic fractions F4 and F5, brassicicolin A (**1**) was obtained as a colorless and chromatographically homogeneous oil (HPLC R_t = 27.3 min). HR-ESI-MS analysis indicated a molecular formula of $C_{32}H_{48}N_2O_{14}$ and the FT-IR spectrum contained strong bands at 3400, 2145, and 1760 cm^{-1}, suggesting the presence of hydroxyl, isonitrile, and carbonyl groups, respectively. The ^{1}H NMR spectrum was complex, suggesting that the sample was actually a mixture of closely related compounds. Likewise, the ^{13}C NMR spectral data showed that each cluster of signals contained four resonances, indicating the presence of four isomers. Comparison of these spectroscopic data and specific optical activity ($[\alpha]_D$ = +16 (*c* 1.0, CH_2Cl_2); lit. $[\alpha]_D$ = 16.1 (*c* 1.86, CH_2Cl_2) with those previously reported (Gloer et al., 1988) along with chemical hydrolysis results indicated that this metabolite was brassicicolin A (**1**), a derivative of mannitol containing two acetyl, two 2-isocyano-3-methylbutanoyl and two 2-hydroxy-3-methylbutanoyl substituents. Fraction F5 was further separated to yield a chromatographically homogeneous material ($C_{26}H_{41}NO_{13}$) shown to contain a mixture of non-symmetrical mannitol derivatives in which one of the 2-isocyano-3-methylbutanoyl groups was missing. The ^{13}C NMR spectrum displayed clusters of four to six peaks, suggesting that the fraction contained a mixture of at least four compounds. Given the complexity of this fraction and the presence of inseparable epimers, no structures were assigned to each specific component.

Three related metabolites were isolated from fractions displaying low phytotoxicity. The ^{1}H NMR spectrum of each of these metabolites (Table 1) showed the presence of an isopropyl, a methoxymethylene, and two methyl substituents suggesting that these metabolites had fusicoccane structures, similar to those previously reported for brassicicenes A–F (MacKinnon et al., 1999). The most oxidized metabolite (**7**) was obtained as a white solid whose molecular formula was determined to be $C_{21}H_{32}O_5$ (six double bond equivalents) based on NMR and HREI-MS data. The ^{1}H NMR spectrum displayed four methyl doublets, a methoxyl group and one olefinic proton. The proton-decoupled ^{13}C NMR spectrum (Table 2) displayed signals for four olefinic carbons, one carbonyl carbon, and five oxygen-bonded sp^3 hybridized carbons. The presence of the isopropyl moiety was evident from couplings shown for a

Table 2
^{13}C NMR (125 MHz, $CDCl_3$) spectroscopic data (δ ppm) of brassicicenes G (**7**), H (**8**) and I (**9**).

C #	Brassicicene G (**7**)	Brassicicene H (**8**)	Brassicicene I (**9**)
1	135.2	126.4	135.5
2	147.2	153.1	139.3
3	83.0	83.0	82.0
4	36.3	37.0	35.5
5	37.1	36.9	32.0
6	45.9	48.5	40.4
7	49.7	50.0	44.5
8	74.5	75.4	76.3
9	30.0	28.3	28.5
10	168.0	136.5	132.5
11	87.9	52.5	52.8
12	50.9	42.6	42.0
13	203.4	80.3	26.5
14	145.1	144.2	145.9
15	28.1	29.9	27.4
16	78.6	79.1	77.8
17	11.7	12.2	7.9
18	10.6	13.8	27.1
19	18.4	22.0	20.9
20	22.3	21.7	20.8
21	59.7	59.7	59.4

Table 1
^{1}H NMR (500 MHz, $CDCl_3$) spectroscopic data (δ ppm) of brassicicenes G (**7**), H (**8**) and I (**9**) (coupling constants in Hz).

H #	Brassicicene G (**7**)	Brassicicene H (**8**)	Brassicicene I (**9**)
1	5.99, br s	5.85 dd (1.2, 9.5)	5.60, d (2.2)
4	1.64, m	1.68, m	1.44, m
	1.90, ddd (6.2, 13.0, 13.0)	1.89, ddd (6.5, 12.5, 12.5)	1.95, m
5	1.48, m	1.49, m	1.25, m
	2.02, m	2.07, m	2.02, m
6	2.82, d (7.7)	3.35, m	2.89, dd (7.7, 7.7)
7	2.06, m	2.02, m	1.79, m
8	4.42, m	4.29, m	3.89, ddd (3.8, 3.8, 11.5)
9	2.41, dd (11.5, 14.0)	2.09, m	2.12, m
	2.92 ddd (1.4, 5.5, 14.0)	2.56, m	2.32, br d (13.2)
11	–	3.28, m	–
12	2.99, m	2.56, m	1.66, m
			1.79, m
13	–	4.82, br s	2.07, m
			2.10, m
15	2.96, m	2.90, hept (6.8)	2.79, hept (6.8)
16	3.41, d (9.4)	3.42, d (9.3)	3.15, d (9.5)
	3.52, d (9.4)	3.53, d (9.3)	3.39, d (9.5)
17	1.02, d (7.5)	0.99, d (7.5)	0.81, d (7.0)
18	1.34, d (7.5)	1.14d (7.0)	1.13, s
19	1.33, d (7.0)	1.38, d (7.5)	0.99, d (7.0)
20	1.22, d (7.0)	1.13, d (7.5)	0.98 d (7.0)
21	3.45, s	3.45, s	3.42, s

methine proton at δ_H 2.96 (H-15) with methyl doublets at δ_H 1.33 (H_3-19) and δ_H 1.22 (H_3-20) in the ^{1}H COSY spectrum. Furthermore, HMBC correlations shown by the isopropyl methine H-15 (δ_H 2.96) to two olefinic carbons at δ_C 168.8 (C-10) and 145.1 (C-14), and a carbonyl carbon at δ_C 203.4, together with the chemical shifts of olefinic carbons C-10 (δ_C 168.8) and C-14 (δ_C 145.1), established the presence of an α,β-unsaturated carbonyl moiety with an isopropyl group attached at the α-position, i.e. structural subunit **A** (Fig. 1). Additional couplings of H-7 (δ_H 2.06) to the methyl doublet H_3-17 (δ_H 1.02), H-8 (δ_H 4.42) to H_2-9 (δ_H 2.92 and 2.41), and H-6 (δ_H 2.82) to H_2-5 (δ_H 1.48 and 2.02), which in turn coupled to H_2-4 (δ_H 1.90 and 1.64), and allylic coupling between H-1 (δ_H 5.99) and H-6 (δ_H 2.82) suggested structural subunit **B**. Further analysis of HMBC correlations was consistent with this assignment.

The presence of a methoxymethylene moiety and its connectivity with a quaternary carbon C-3 (δ_C 83.0) was established from correlations shown in the HMBC spectrum. Furthermore, the ^{1}H COSY spectrum displayed an isolated spin system where a methyl doublet at δ_H 1.34 (H_3-18) coupled to methine H-12 (δ_H 2.99), which exhibited HMBC correlations to C-1 (δ_C 135.2), C-10 (δ_C 168.0), C-11 (δ_C 87.9), and C-13 (δ_C 203.4). Based on this spectroscopic data structure **7** was assigned to this metabolite. A structure search indicated that compound **7** was not known, although similar diterpenoid metabolites were reported previously from *A. brassicicola* (Mackinnon et al., 1999). Finally, the stereochemistry of **7**, including absolute configuration, was assigned based on NOESY spectral data (C-16, C-17, and C-18 had *trans*-orientation relative to H-6, H-7 and H-8) and by analogy to previous assignments (Cerrini et al., 1979; Muromtsev et al., 1994; Mackinnon et al., 1999). Compound **7** was named as brassicicene G by analogy to previous names (Mackinnon et al., 1999).

Compound **8** was obtained as a white solid, whose molecular formula was established as $C_{21}H_{34}O_4$ based on HREI-MS [*m/z* (M^+-2H_2O) 314.2284, calc. 314.2245] and NMR (^{13}C NMR and HMQC) data (Tables 1 and 2), indicating five double bond equivalents. The ^{1}H NMR spectrum displayed four methyl doublets, a methoxy group and one olefinic proton. The proton-decoupled ^{13}C NMR spectrum displayed 21 resonances, of which four carbon signals at δ_C 126.4, 153.1, 136.5 and 144.2 were assigned to four sp^2 carbons, C-1, C-2, C-10 and C-14, respectively. Additionally, five signals for sp^3 carbons attached to oxygen at δ_C 83.0, 75.4, 79.1, 80.3 and 59.7 were assigned as C-3, C-8, C-16, C-13 and C-21, respectively. The ^{1}H COSY spectrum showed two methyl groups at δ_H 1.13 (H-19) and δ_H 1.38 (H-20) coupled to a methine H-15 (δ_H 2.90), suggesting the presence of an isopropyl group. An isolated spin system showed the methyl protons at δ_H 1.14 (J = 7 Hz, H-18) coupled to a proton at δ_H 2.56 (H-12), which in turn coupled to protons at δ_H 4.82 (H-13) and 3.28 (H-11), and the latter (H-11) further coupled to the olefinic proton at 5.85 (H-1). Comparison of the ^{1}H NMR and ^{13}C NMR spectroscopic data of this compound (**8**) with that of compound **7** indicated that the OH at C-11 and the C-13 carbonyl of **7** were reduced in **8**. Further structural similarity between compounds **7** and **8** was verified by comparison of their ^{1}H COSY and HMBC correlation data. The stereochemistry of this metabolite was established based on spatial correlations displayed in the NOESY spectrum; protons H-6, H-7 and H-8 had *cis* orientation with respect to one another, but *trans* to H-11, H-13, C-16, C-17 and C-18. Consequently, structure **8** was deduced and named brassicicene H.

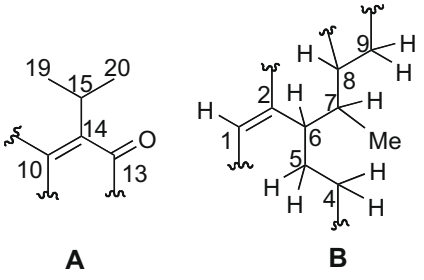


Fig. 1. Partial structures of brassicicene G (**7**).

Compound **9** was obtained as a white solid with the molecular formula $C_{21}H_{34}O_3$ (HREI-MS), indicating five double bond equivalents. The ^{1}H NMR spectrum displayed signals for three methyl doublets, a methyl singlet, a methoxy group and an olefinic proton (Table 1). The proton-decoupled ^{13}C NMR spectrum (Table 2) displayed 21 resonances, of which four signals at δ_C 145.8, 139.3, 135.5, and 132.5 were attributed to olefinic carbons and another set of four signals at δ_C 82.0, 77.8, 76.7 and 59.3 were attributed to carbons attached to oxygen. The ^{1}H COSY spectrum displayed two methyl doublets H_3-19 (δ_H 0.99, J = 7 Hz) and H_3-20 (δ_H 0.98, J = 7 Hz) coupled to a methine at δ_H 2.79 (H-15, J = 7 Hz), suggesting presence of an isopropyl group. In addition, allylic coupling was observed between H-1 (δ_H 5.60, J = 2 Hz) and proton H-6 (δ_H 2.89). An isolated spin system displayed showed couplings of methylene H-12 (δ_H 1.66 and 1.79) with methylene H-13 (δ_H 2.07 and 2.10). Moreover, the existence of a methoxymethylene moiety was evident from correlations of H_2-16 (δ_H 3.39 and 3.15) with C-21 (δ_C 59.4), C-4 (δ_C 35.5) and C-2 (δ_C 139.3) displayed in the HMBC spectrum, and also from the presence of methylene H_2-16 at δ_H 3.39 (J = 9.5 Hz) and 3.15 (J = 9.5 Hz) and the methoxy group at δ_H 3.42. Further, comparison of NMR (^{13}C NMR, HMBC, COSY) data of compound **9** with those of compounds **7** and **8** indicated that all had similar structures. The relative configurations at C-3, C-6, C-7, C-8 and C-11 were determined based on NOESY data, as well as by comparison with brassicicenes reported from *A. brassicicola* (Mackinnon et al., 1999). Hence, the structure **9** was assigned and the metabolite was named brassicicene I.

Additional brassicicenes were isolated but the very small amounts recovered precluded structure elucidation. It is possible that some of these minor metabolites are identical to those reported previously (Mackinnon et al., 1999); however, considering that brassicicolin A (**1**) or related structures were not reported by Mackinnon et al. (1999), the isolates of *A. brassicicola* reported here are from a likely different pathogenicity group, and perhaps a rather more virulent one.

2.2. Metabolites from leaves of Brassica juncea inoculated with Alternaria brassicicola

2.2.1. Metabolites from leaves of whole plants

To determine phytoalexin accumulation in leaves of *B. juncea*, 2-week-old plants were inoculated with spores of *A. brassicicola*, and were incubated up to 120 h; leaves were excised every 24 h, extracted and analyzed by HPLC–DAD. No phytoalexins were detected 24 h post inoculation; four phytoalexins were detected 48 h post inoculation and were quantified using calibration curves constructed with synthetic samples (Pedras et al., 2007). The phytoalexin spirobrassinin (**12**) was produced in larger amounts than brassinin (**10**), cyclobrassinin (**11**), or rutalexin (**13**), as shown in Fig. 2. As well, indolyl-3 acetonitrile (**15**) was detected in higher concentration in leaves (0.8–4.0 nmol/g fresh weight) of infected plants than in control leaves (<0.1 nmol/g fresh weight). Furthermore, HPLC–MS analysis of leaf extracts did not detect any of the metabolites produced by *A. brassicicola* in liquid cultures.

2.2.2. Metabolites from detached leaves

To determine phytoalexin accumulation in detached leaves of *B. juncea* excised leaves of 2-week-old plants were inoculated with spores of *A. brassicicola*, and were incubated up to 120 h, extracted

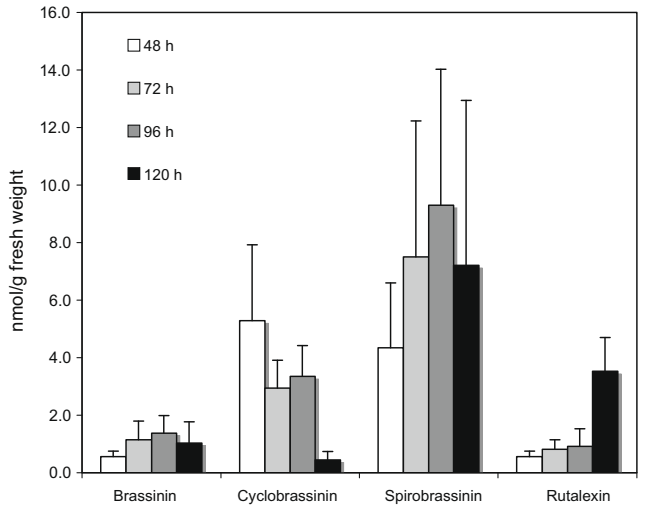


Fig. 2. Accumulation of the phytoalexins brassinin (**10**), cyclobrassinin (**11**), spirobrassinin (**12**) and rutalexin (**13**) in leaves of *Brassica juncea* cv. Cutlass (whole plants) inoculated with *Alternaria brassicicola*.

and analyzed by HPLC–DAD. Phytoalexins and metabolites were detected and quantified in crude extracts (Table 3) using calibration curves constructed with synthetic samples (Pedras et al., 2007).

Because no fungal metabolites were detected in leaves of *B. juncea*, larger scale experiments were carried out using detached leaves inoculated with *A. brassicicola* to establish whether fungal metabolites are produced in infected leaves. Control detached leaves were inoculated with water and incubated under similar conditions. Leaves were extracted after 72 h and 120 h of incubation and the extracts were fractionated as described in Section 4. Each fraction was analyzed by HPLC–DAD–MS and spectroscopic data of each peak were compared with those obtained for the metabolites of *A. brassicicola*. As summarized in Table 4, several phytoalexins were detected in amounts different from those detected in leaves of whole plants (Fig. 2); while the major phytoalexin was cyclobrassinin (**11**), brassinin (**10**) was not detected. Instead, *N*′-acetyl-3-indolylmethanamine **(19)** was detected. The structure of *N*′-acetyl-3-indolylmethanamine was confirmed upon isolation and analysis of its spectroscopic data. Since *N*′-acetyl-3-indolylmethanamine **(19)** was not present in control leaves, it was suspected to be a detoxification product of brassinin (**10**) in a transformation mediated by *A. brassicicola.* Hence, biotransformation of brassinin in cultures of *A. brassicicola* was investigated, as described below.

Table 3
Metabolites produced in detached leaves of *Brassica juncea* cv. Cutlass inoculated with *Alternaria brassicicola* (after 120 h of incubation, quantification of metabolites carried out in extracts).

Metabolite	Fresh weight 120 h post inoculation 2(nmol/g)
Cyclobrassinin (**11**)	5.5 ± 2.3
Spirobrassinin (**12**)	4.6 ± 1.9
Rutalexin (**13**)	1.6 ± 0.7
Brassilexin (**14**)	1.2 ± 0.4
Indolyl-3-acetonitrile (**15**)[a]	2.0 ± 0.5
1-Methoxyindolyl-3-acetonitrile (caulilexin) (**16**)[a]	0.51 ± 0.02
4-Methoxyindolyl-3-acetonitrile (arvelexin) (**17**)[a]	n. d.[b]
N′-acetyl-3-indolylmethanamine (**19**)	10.3 ± 3.6
Indole-3-carboxylic acid	0.01 ± 0.01

[a] Traces (<0.1 nmol/g fresh weight) detected in some extracts of control leaves.
[b] n.d. = Not detected.

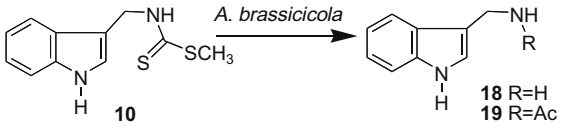


Fig. 3. Detoxification pathway of the phytoalexin brassinin (**10**) by *Alternaria brassicicola*.

Table 4
Metabolites produced in detached leaves of *Brassica juncea* cv. Cutlass inoculated with *Alternaria brassicicola* (larger scale experiment, quantification of metabolites carried out in fractions.)

Metabolite	Fresh weight 72 h post inoculation (nmol/g)	Fresh weight 120 h post inoculation (nmol/g)
Cyclobrassinin (**11**)	16.7	7.5
Spirobrassinin (**12**)	2.5	6.2
Rutalexin (**13**)	3.0	3.8
Brassilexin (**14**)	1.3	0.5
Indolyl-3-acetonitrile (**15**)[a]	25.6	0.6
1-Methoxyindolyl-3-acetonitrile (caulilexin) (**16**)[a]	2.3	0.3
4-Methoxyindolyl-3-acetonitrile (arvelexin) (**17**)[a]	3.8	n.d.[b]
N′-acetyl-3-indolylmethanamine (**19**)	11.4	26.1
Indole-3-carboxylic acid	<0.01	<0.01

[a] Traces (<0.1 nmol/g fresh weight) detected in some extracts of control leaves.
[b] n.d. = Not detected.

2.3. Biotransformation of brassinin (*10*)

The phytoalexin brassinin (**10**) was synthesized as previously reported (Pedras et al., 2003) and its inhibitory activity against *A. brassicicola* was determined using mycelial cultures. Subsequently, 48 h-old cultures were incubated with brassinin (**10**, 0.1 and 0.2 mM) and its transformation was monitored by HPLC–DAD (**10**, t_R = 18.8 ± 0.5 min). Samples were withdrawn from cultures immediately after addition of brassinin (**10**) and then at 6, 12, 24, 48, 72, and 96 h, extracted and the neutral and basic extracts were analyzed by HPLC–DAD. Analyses of HPLC chromatograms of neutral extracts showed that brassinin (**10**) was almost completely metabolized within 12 h (**10**, 0.1 mM) or 24 h (**10**, 0.2 mM) depending on its initial concentration (Table 5). Furthermore, the chromatograms of neutral extracts of cultures of *A. brassicicola* showed the presence of additional peaks due to *N*′-acetyl-3-indolylmethanamine (**19**, t_R = 5.2 ± 0.2 min). The identity of this compound was established after isolation by comparison of its spectroscopic data with those of an authentic sample. The basic extracts indicated the presence of 3-indolylmethanamine (**18**) (TLC detection), which, due to its alkalinity was not eluted under our analytical HPLC reverse-phase conditions but detected under normal phase conditions using a CN column. Data analysis for transformation of brassinin (**10**) into amine **18** and acetyl amine **19** showed that amine **18** was transformed in 96 h, whereas acetyl amine **19** remained in culture for more than seven days (Table 5).

To establish the sequence of steps of brassinin (**10**) biotransformation, amine **18** and acetyl amine **19** were administered separately to cultures of *A. brassicicola*, and cultures were incubated and analyzed as described above for brassinin (**10**). The HPLC chromatograms indicated that amine **18** was completely metabolized in 48 h to yield acetyl amine **19**, which was slowly transformed

Table 5
Transformation of brassinin (**10**) and metabolites **18** and **19** in mycelial cultures of *Alternaria brassicicola*.

Compounds (#, concentration)/percentage of transformation[a]	Metabolic products
Brassinin (**10**, 0.1 mM)/ca. 100% in 12 h	3-indolylmethanamine (**18**); *N*′-acetyl-3-indolylmethanamine (**19**)
Brassinin (**10**, 0.2 mM)/ca. 100% in 24 h	3-indolylmethanamine (**18**); *N*′-acetyl-3-indolylmethanamine (**19**)
3-Indolylmethanamine (**18**, 0.1 mM)/ca. 40% in 120 h	*N*′-acetyl-3-indolylmethanamine (**19**)
N′-Acetyl-3-indolylmethanamine (**19**, 0.1 mM)/ca. 40% after 168 h	Undetermined

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

to undetermined product(s). That is, the first two steps of the transformation pathway of brassinin (**10**) by *A. brassicicola* are similar to the pathway previously observed for avirulent (now reclassified as *L. biglobosa*) and Laird 2 isolates of *L. maculans* (Pedras and Taylor, 1993; Pedras et al., 2007). The detoxification of brassinin (**10**) by isolates of *L. maculans* pathogenic on brown mustard appeared to be carried out by an inducible hydrolase (Fig. 3).

2.4. Phytotoxicity and antifungal assays

Phytotoxicity assays carried out on leaves of *B. juncea* cv. Cutlass (susceptible), *Brassica napus* cv. Westar (tolerant), and *Sinapis alba* cv. Ochre (resistant), as described in Section 4, indicated that brassicicolin A (**1**) is a host-selective phytotoxin, causing chlorosis and necrosis on leaves of the susceptible species (*B. juncea*) at 0.5 mM, but no detectable damage on leaves of the resistant species (*S. alba*) (Table 6). In addition, the mixture of mannitol derivatives of fraction F5 showed similar phytotoxic effects.

Leaves of *B. juncea*, *B. napus*, and *S. alba* treated with brassicicolin A (**1**, dissolved in MeOH–H_2O) were harvested after incubation for seven days, then extracted with the extracts analyzed by HPLC–DAD. The extracts of control leaves (treated with MeOH–H_2O) were analyzed similarly. The chromatograms of treated and non-treated leaves were similar, that is brassicicolin A (**1**) did not elicit phytoalexins in either susceptible or resistant plants and was not detected in extracts of treated leaves. It is possible that the concentration of brassicicolin A (**1**) in the plant tissue was too low to be detected or it may have been transformed or decomposed.

Table 6
Phytotoxic activity[a] of brassicicolin A (**1**) on leaves of *Brassica napus* cv. Westar (susceptible), *B. juncea* cv. Cutlass (susceptible), and *S. alba* cv. Ochre (resistant).

Brassicicolin A (**1**) (mM)	*B. napus*	*B. juncea*	*S. alba*
0.5	1	5	0
0.1	0	2	0
0.05	0	1	0

[a] Lesion size scale: 0 = 1.0–1.5 mm; 1 = 1.5–2.5 mm; 2 = 2.5–3.0 mm; 3 = 3.0–4.0 mm; 4 = 4.0–5.0 mm; 5 = 5.0–5.5 mm; 6 = 5.5–6.5 mm.

Table 7
Antifungal activity[a] of brassinin (**10**) and its metabolites **18** and **19**, cyclobrassinin (**11**), spirobrassinin (**12**) and indolyl-3-acetonitrile (**15**) against *Alternaria brassicicola* (72 h of incubation).

Compounds	Activity[a] (%) at concentrations of		
	0.50 mM	0.20 mM	0.10 mM
Brassinin (**10**)	39 ± 2	31 ± 2	22 ± 1
Cyclobrassinin (**11**)[b]	61 ± 8	34 ± 4	9 ± 4
Spirobrassinin (**12**)	54 ± 4	32 ± 5	11 ± 7
Brassilexin (**14**)	85 ± 2	59 ± 2	50 ± 3
Indolyl-3-acetonitrile (**15**)	32 ± 0	0	0
3-Indolylmethanamine (**18**)	16 ± 1	0	0
N′-Acetyl-3-indolylmethanamine (**19**)	0	0	0
Camalexin[c] (**20**)	100 ± 0	85 ± 1	53 ± 1

[a] Percentage of inhibition = 100−[(growth on medium containing compound/growth on control medium) × 100)] ± standard deviation.
[b] Not completely soluble at this concentration.
[c] Reference phytoalexin; it is not produced by *Brassica* species.

The antifungal activity of the phytoalexins brassinin (**10**), cyclobrassinin (**11**) and spirobrassinin (**12**), which were detected in plant leaves inoculated with *A. brassicicola,* was determined as summarized in Table 7 (Pedras et al., 2007). *A. brassicicola* appeared to be equally sensitive to these phytoalexins but much more sensitive to camalexin (**20**), a phytoalexin produced by wild species, including *Arabidopsis thaliana* (Pedras et al., 2007). To the best of our knowledge, this is the first time that phytoalexins other than camalexin are detected in plant tissues inoculated with *A. brassicicola*. These results are consistent with data published previously in which brassinin and camalexin were against tested 13 different isolates of *A. brassicicola* (Sellam et al., 2007).

3. Summary and conclusions

We have established that *A. brassicicola* produces brassicicolin A (**1**, a mixture of epimers, Gloer et al., 1988) as the major host-selective phytotoxin. Brassicicolin A (**1**) appeared to be more phytotoxic to the susceptible cruciferous species *B. juncea* than to the tolerant *B. napus* (Table 6). Previous claims that *A. brassicicola* produced destruxins (Cooke et al., 1997; Evans et al., 1996) could not be confirmed; in spite of the availability of several destruxins in our metabolite library (Pedras et al., 2000), none of these phytotoxins could be detected in either extracts of liquid cultures or in infected leaf tissues. In addition, the four polyketides phomapyrones A (**3**), F (**4**) and G (**5**) and infectopyrone (**6**), previously isolated from *L. maculans* and *L. biglobosa* (Pedras et al., 1994; Pedras and Biesenthal, 2001; Pedras and Chumala, 2005), were also isolated from *A. brassicicola*. Clones of *A. brassicicola* (altr203xn03 and altr014xe01) were recently reported (Cramer et al., 2006) to encode enzymes involved in the biosynthesis of undetermined polyketides, which could be the phomapyrones **3**–**5** reported here. In this context, it is pertinent to point out that genome sequences currently available in public databases have indicated the existence of numerous "orphan pathways", i.e. gene clusters for which the encoded natural product is unknown (Gross, 2007). Therefore, it is expected that further new metabolites and/or phytotoxins could potentially be isolated from fungal species previously investigated, such as *A. brassicicola*.

Phytoalexin production in leaves of *B. juncea* infected with *A. brassicicola* led to the detection and isolation of two known phytoalexins and a phytoanticipin: cyclobrassinin (**11**) and spirobrassinin (**12**) and indolyl-3-acetonitrile (**15**), respectively. Indolyl-3-acetonitrile (**15**), was occasionally detected in control leaves, therefore it is more appropriate to consider it a phytoanticipin (Table 3). In addition, other minor phytoalexins (**13** and **14**) and phytoanticipins (**16** and **17**) were detected in fractions of leaf extracts. Importantly, the product of detoxification of brassinin (**10**), *N*′-acetyl-3-indolylmethanamine (**19**), was isolated from infected leaves, but was never detected in control leaves. Hence, we suspected that brassinin (**10**) did not accumulate in infected tissue because it was quickly detoxified to **19**, similar to the detoxification that occurs in *A. brassicicola* cultures. To support this hypothesis, we determined that *A. brassicicola* detoxified brassinin (**10**) to

indolyl-3-methanamine (**18**) and *N*′-acetyl-3-indolylmethanamine (**19**) in fungal cultures (Table 5). Although brassinin (**10**) did not appear to be strongly antifungal against *A. brassicicola*, considering that this phytoalexin is a precursor of brassilexin (**14**) and other phytoalexins of *B. juncea* (Pedras et al., 2007), it is likely that its detoxification makes the plant more vulnerable to fungal invasion. That is, brassinin detoxifying enzyme(s) produced by *A. brassicicola* might contribute to its virulence. Finally, comparison of the in vitro antifungal activity of the phytoalexins (Table 7) present in infected tissues of *B. juncea* and that of camalexin (**20**, not produced in *Brassica* species) suggested that camalexin might be a more effective plant defense against *A. brassicicola*.

4. Experimental

4.1. General

All chemicals were purchased from Sigma–Aldrich Canada Ltd., Oakville, Ont. All solvents were analytical grade except for high-performance liquid chromatography (HPLC) grade solvents used for HPLC analysis. HPLC analysis was carried out with an HPLC system equipped with quaternary pump, automatic injector and diode array detector (DAD, wavelength range 190-600 nm), degasser and a Hypersil ODS column (5 mm particle size silica, 200 × 4.6 internal diameter mm) equipped with an in-line filter. HPLC method A was used for metabolite analysis except where stated otherwise: linear gradient of H_2O–CH_3CN (75:25) to H_2O–CH_3CN (0:100) for 35 min, with flow rate 1.0 ml/min. For HPLC analysis of amine **18**, a Zorbax XDB-CN (5 mm particle size silica, 150 × 4.6 internal diameter mm) column equipped with an in-line filter was used with elution method B: linear gradient of 2-PrOH-hexane (85:15) to 2-PrOH-hexane (100:0) for 15 min, with a flow rate 1.0 ml/min.

Liquid Chromatography–Mass Spectrometry (LC–MS) data were obtained using an Agilent 1100 series HPLC system (Agilent Technologies USA) equipped with an autosampler, binary pump, degasser, and a DAD connected directly to a mass detector (MSD, 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 XSB C-18 column (5 mm particle size silica, 150 mm × 4.6 mm internal diameter). The mobile phase consisted of a gradient of 0.2% formic acid in H_2O (A) and 0.2% HCOOH in CH_3CN (B) (75% A to 75% B in 35 min, to 100% B in 5 min) and a flow rate of 1.0 ml/min. Additional conditions for the MSD were as previously reported (Pedras et al., 2006).

Crucifer phytoalexins were synthesized as previously reported (Pedras et al., 2007) with purities above 98% (determined by HPLC–DAD and ^{1}H NMR). Calibration curves were prepared for each phytoalexin using synthetic samples dissolved in CH_3CN to prepare stock solutions, followed by serial dilutions. Calibration curves were prepared using peak areas of the HPLC chromatograms obtained using method A (λ 220 nm). The correlation coefficients of phytoalexin calibration curves were $\geqslant$0.9998.

High resolution (HR) electron impact (EI) mass spectra (MS), were obtained on a VG 70 SE mass spectrometer employing a solids probe.

NMR spectra were recorded on 500 MHz spectrometers. For ^{1}H NMR spectra (500 MHz) the chemical shifts (δ) are reported in parts per million (ppm) relative to TMS. The δ values were referenced to $CDCl_3$ ($CHCl_3$ at 7.28 ppm), CD_3CN (CD_2HCN at 1.94 ppm), CD_3OD (CD_2HOD at 3.31 ppm), D_2O (HDO at 4.79 ppm). For ^{13}C NMR (125.8 MHz) the chemical shifts (δ) were referenced to $CDCl_3$ (77.4 ppm), CD_3OD (49.0 ppm). The multiplicities of the ^{13}C signals refer to the number of attached protons: s = C, d = CH, t = CH_2, q = CH_3. Fourier transform infrared (FT-IR) spectral data were recorded on a Bio-Rad FTS-40 spectrometer and spectra were measured by the diffuse reflectance method on samples dispersed in KBr. Ultraviolet (UV) spectra were recorded on a spectrophotometer using a 1 cm path length quartz cell.

4.2. Fungal cultures of Alternaria brassicicola

A. brassicicola isolates Ab-C1, Ab-C2, and ATCC 96866 were subcultured in V8-agar plates under constant light at room temperature for 10 days; the spores were collected by adding autoclaved H_2O (10 ml) to each agar plate followed by gentle scraping of agar to dislodge spores, and centrifugation of suspension; the pellet was washed by resuspending in sterile H_2O (10 ml) followed by centrifugation. Finally, the spores were counted and stored at −20 °C. Cultures of *A. brassicicola* were initiated by inoculating spores (10^6 spores per flask) in 250 ml Erlenmeyer flasks containing 100 ml of minimal medium plus thiamine (Pedras et al., 1997) and incubated on a shaker at 120 rpm, at 23 ± 1 °C for 7 days. HPLC analysis of the EtOAc extracts of 7-day-old cultures indicated that the three isolates produced identical metabolites.

4.3. Isolation and characterization of metabolites from cultures of Alternaria brassicicola

Cultures of *A. brassicicola* were grown in minimal medium (Pedras et al., 1997) for seven days. The cultures were filtered and the broths (total ca. 50 L) were combined and extracted with EtOAc. The combined EtOAc extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield an oily residue (1.5 g). The EtOAc extract was subjected to flash silica gel CC and eluted with CH_2Cl_2–MeOH (99:1, 150 ml; 95:5, 150 ml; 90:10, 150 ml; 85: 15, 150 ml; 80: 20, 150 ml; 50:50, 150 ml). All fractions were concentrated to dryness under reduced pressure, analyzed by HPLC and TLC, bioassayed, and further fractionated until pure compounds were obtained. Fraction 1 (20 mg) was subjected to preparative TLC (hexane–EtOAc, 75:25) to yield phomapyrone F (**4**, 1 mg). Fraction 2 (76 mg) was further fractionated to yield phomapyrone A (**3**, 30 mg) and phomapyrone G (**5**, 1 mg). Fractions 3 and 4 were combined (355 mg) and further fractionated to yield brassicicolin A (**1**, 30 mg) and brassicicene I (**9**, 1 mg). Fraction 5 (300 mg) was further fractionated to afford a fraction containing a mixture of mannitol derivatives. Fraction 6 (44 mg) was further fractionated to yield brassicicene G (**7**, 4 mg). Fraction 8 (32 mg) was further fractionated to afford brassicicene H (**8**, <1 mg). Fraction 9 was further fractionated to yield infectopyrone (**6**, 2 mg).

The EtOAc extract of the mycelium of liquid cultures of *A. brassicicola* was fractionated by FCC (CH_2Cl_2–MeOH, 100:0, 300 ml; 99:1, 300 ml; 98:2, 300 ml; 97:3, 300 ml, 95:5, 300 ml; 90:10, 300 ml; 80:20, 300 ml; 50:50, 300 ml) to produce eleven fractions containing different fatty acids and fraction 4 containing ergosterol (20 mg).

4.3.1. Brassicicene G (**7**)

HPLC R_t = 4.8 min. HRMS-EI: *m/z* 319.1901 [M-CH_2OCH_3]$^+$, calcd. 319.1909 for $C_{19}H_{27}O_4$. MS-EI: *m/z* (relative intensity) 319 (9%), 301 (8%), 283 (8%). For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HMBC: H-1 → C-6/C-3/C-11/C-2, H-4 → C-3/C-6/C-16, H-5 → C-2/C-3/C-7/C-6/C-4, H-6 → C-17/C-4/C-7/C-3/C-1/C-2, H-7 → C-17/C-8/C-6/C-9, H-9 → C-7/C-8/C-14/C-10, H-12 → C-18/C-11/C-1, H-15 → C-19/C-20/C-14/C-10/C-13, H-16 → C-4/C-21/C-2/C-3, H-17 → C-8/C-6, H-18 → C-12/C-11/C-10, H-19 → C-14/C-15/C-20, H-20 → C-19/C-14/C-15, H-21 → C-16. NOESY: H-1 ↔ H-18/H-16, H-8 ↔ H-6/H-7, H-16 ↔ H-17/H-1, H-6 ↔ H-7/H-8, H-17 ↔ H-16/H-18. FT-IR (KBr, cm^{-1}): 3397, 2961,

2937, 2876, 1735, 1695, 1460, 1234. UV (CH_3CN-H_2O) λ_{max} 192, 215, 270 nm; $[\alpha]_D = -191$ (*c* 0.12, $CHCl_3$).

4.3.2. Brassicicene H (**8**)

HPLC R_t = 9.5 min. HRMS-EI: *m/z* 314.2284 $[M\text{-}2H_2O]^+$, 314.2245 calcd. for $C_{21}H_{30}O_2$, 287.2007 $[M\text{-}CH_2OCH_3\text{–}H_2O]^+$, calcd. 287.2011 for $C_{19}H_{27}O_2$. MS-EI: *m/z* (relative intensity) 314 (14%), 287 (79%). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HMBC: H-1 → C-6/C-3/C-2/C-12, H-4 → C-6/C-3/C-2, H-5 → C-3/C-7, H-6 → C-17/C-5/C-7/C-8/C-3/C-1/C-2, H-7 → C-8, H-8 → C-17, H-9 → C-14/C-7/C-8/C-10, H-11 → C-12/C-13/C-1/C-10/C-14/C-2, H-12 → C-11/C-1/C-10, H-13 → C-10, H-15 → C-20/C-13/C-10, H-16 → C-2, H-17 → C-7/C-8, H-18 → C-12/C-11/C-10, H-19 → C-15/C-20/C-14, H-20 → C-15/C-19/C-14, H-21 → C-16. NOESY: H-1 ↔ H-18/H-11/H-16, H-8 ↔ H-6, H-17 ↔ H-16, H-13 ↔ H-18. FT-IR (KBr, cm^{-1}): 3352, 2951, 2922, 2861, 1455, 1104. UV (CH_3CN–H_2O) λ_{max} 210 nm.

4.3.3. Brassicicene I (**9**)

HPLC R_t = 19.0 min. HRMS-EI: *m/z* 334.2495 $[M]^+$, calcd. 334.2507 for $C_{21}H_{34}O_3$. MS-EI: *m/z* (relative intensity) 334 (14%), 316 (6%), 289 (85%), 271 (75%). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2. HMBC: H-1 → C-6/C-11/C-10/C-2, H-4 → C-3/C-2/C-16/C-6/C-5, H-5 → C-2/C-3/C-7/C-6/C-4, H-6 → C-17/C-5/C-8/C-3/C-1/C-2, H-7 → C-17/C-6/C-5/C-8, H-8 → C-17/C-9/C-6/C-10, H-9 → C-8/C-11/C-7/C-10/C-14, H-12 → C-13/C-18/C-11/C-10/C-1/C-14, H-13 → C-12/C-11/C-10/C-14/C-11, H-15 → C-13/C-20/C-10/C-14, H-16 → C-4/C-21/C-2, H-17 → C-6/C-7/C-8, H-18 → C-1/C-10/C-11/C-12, H-19 → C-20/C-15/C-14, H-20 → C-19/ C-15/C-14, H-21 → C-16. NOESY: H-1 ↔ H-18/H-16, H-8 ↔ H-7/H-6, H-16 ↔ H-17. FT-IR (KBr, cm^{-1}): 3406, 2959, 2932, 2873, 1453, 1097. UV (CH_3CN–H_2O) λ_{max} 206 nm.

4.4. Metabolites from leaves of Brassica juncea inoculated with Alternaria brassicicola and preparation of extracts for time-course analyses

Seeds of brown mustard (*B. juncea*) cv. Cutlass were sown in individual pots in peat-lite in a growth chamber at day/night temperature 20/16 °C, and 16 h photoperiod (250 μmol s^{-1} m^{-2}). Leaves were punctured with a needle and inoculated with spores of *A. brassicicola* by applying 10 μl spore suspensions (3×10^6 spores/ml) in sterile H_2O at punctured spots (8–10 spots per leaf) and were incubated in a growth chamber. Control leaves were treated similarly, but using sterile H_2O. Samples were collected 24 h after elicitation and every 24 h up to five days. Plant tissues were frozen and ground in liq. N_2 and the plant material (500–800 mg) was extracted with MeOH (6–8 ml) on a shaker at 120 rpm for 3 h. Leaf solids were filtered to remove and the solvent was concentrated. The oily residue was rinsed with CH_2Cl_2 (5 ml) and the remaining solid residue (polar extract) was dissolved in MeOH–H_2O (1:1, 200 μl) for HPLC analysis. The CH_2Cl_2 extract (non-polar fraction) was concentrated under reduced pressure and dissolved in CH_3CN (60 μl) for HPLC analysis.

4.5. Metabolites from detached leaves of Brassica juncea inoculated with Alternaria brassicicola

Leaves of two-week-old *B. juncea* cv. Cutlass grown as described above were excised at the base of the petiole, and petioles were wrapped with moist cotton wool and placed in Petri dishes. Leaves were punctured with a needle and inoculated with spores of *A. brassicicola* by applying 10 μL spore suspensions (3×10^6 spores/ml) in sterile H_2O at punctured spots. Control leaves were treated similarly, but using sterile H_2O. Petri dishes with treated and control leaves were sealed with parafilm, and the incubation was carried out at 23 °C under constant light. After 72 h and 120 h, both infected and control leaves (ca. 24 g, fresh weight) were frozen in liq. N_2, crushed and extracted twice with MeOH (30 ml). The MeOH extract was fractionated by FCC (1 × 10 cm, silica gel, 60 ml fractions) with CH_2Cl_2, CH_2Cl_2–MeOH (98:2), and CH_2Cl_2–MeOH (95:5). All fractions of MeOH extracts of infected and control plant leaves were analyzed by HPLC–DAD.

4.6. Biotransformation of brassinin in cultures of Alternaria brassicicola

Erlenmeyer flasks (125 ml, containing 50 ml of liquid minimal media (Pedras and Biesenthal, 1998) were inoculated with spores (10^6 spores/100 ml) of *A. brassicicola* and incubated at 23 ± 1 °C on shaker at 120 rpm under constant light. After 48 h, brassinin (**10**) was added to cultures and samples (10 ml) were withdrawn at different times and either frozen or immediately extracted with EtOAc. The EtOAc extracts were concentrated in a rotary evaporator under vacuum. The remaining aqueous phase was made alkaline by adding NH_4OH (pH >9) and extracted with $CHCl_3$–MeOH (95:5). The concentrated extract was analyzed by HPLC using a CN column, as described above.

4.7. Phytotoxicity and antifungal assays

Phytotoxicity: Brown mustard (*B. juncea* cv. Cutlass, susceptible), canola (*B. napus* cv. Westar, tolerant) and white mustard (*S. alba* cv. Ochre resistant) plants were grown in a growth chamber at 24 ± 2 °C. The phytotoxicity assays of fractions and isolated metabolites from *A. brassicicola* were conducted on leaves of two-week-old plants. Samples were prepared in MeOH–H_2O (1:1) at concentrations of 5×10^{-4} M–5×10^{-6} M (for pure compounds) and 1×10^{-3} M (about 2 mg/ml for fractions). Each leaf was punctured at four places with a needle and inoculated at each puncture with a 10 μL droplet. Control leaves were treated similarly with MeOH–H_2O (1:1). Plants were incubated in a growth chamber and the diameter of the lesions was measured after seven days of incubation. Leaves inoculated with brassicicolin A (**1**) and control leaves were excised and treated with liquid nitrogen before crushing with a glass rod, and the powdered tissue was extracted with MeOH (20 ml × 2). The MeOH extracts of leaves incubated with brassicicolin A and of control leaves (*B. juncea*, *B. napus*, and *S. alba*) were analyzed by HPLC.

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.01.005.

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