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Structure and biological activity of maculansin A, a phytotoxin from the phytopathogenic fungus *Leptosphaeria maculans*

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ABSTRACT

During a search for elicitors and phytotoxins produced by virulent isolates of the phytopathogenic fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.], the selective phytotoxin maculansin A was isolated and its structure determined by analysis of spectroscopic data and chemical degradation. Maculansin A, a unique derivative of mannitol containing the unusual chromophore 2-isocyano-3-methyl-2-butenoyl, was isolated from potato dextrose cultures of *L. maculans* virulent on canola (*Brassica napus* L. cv. Westar). Surprisingly, maculansin A was more toxic to resistant plants (*B. juncea* L. cv. Cutlass, brown mustard) than to susceptible plants (canola). Maculansin A, however, did not elicit the production of phytoalexins either in resistant or susceptible plants. In addition, other maculansin type structures and the metabolite 2,4-dihydroxy-3,6-dimethylbenzaldehyde were isolated and the latter was found to be a strong inhibitor of root growth of both brown mustard and canola. Considering that *L. maculans* seems to be expanding its host range to infect brown mustard as well, maculansins could assist in chemotaxonomic studies to group the diverse isolates.

1. Introduction

The plant pathogenic fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.] causes blackleg disease in rapeseed (*Brassica napus* L. and *B. rapa* L.) and canola (*B. napus* L. and *B. rapa* L.) in Canada and worldwide (Chen and Fernando, 2006; Fitt et al., 2006; Howlett et al., 2001). Recent canola field surveys showed the prevalence of *L. maculans* and an increase in more aggressive isolates in both Canada and USA (Chen and Fernando, 2006; Kutcher et al., 2007). Consistent with these field surveys, metabolite profiles of isolates of *L. maculans* collected in different countries suggested that this species comprises distinct groups (Pedras and Biesenthal, 2000; Pedras, unpublished), one of which is highly virulent on canola. In a chemically defined liquid medium (minimal medium, MM), these virulent isolates produce mainly sirodesmin PL (**1**) (Pedras, 2001), a non-host-selective phytotoxin recently detected in infected canola (Elliott et al., 2007). Other sirodesmins, including deacetylsirodesmin PL (**2**), and sirodesmins H (**3**), J (**4**), and K (**5**) and phomamide (**6**) are minor metabolites co-produced with **1** in liquid MM (Pedras, 2001). In addition, the host-selective toxin phomalide (**7**) is produced during early growth stages in MM culture as well as in infected susceptible plants (Pedras and Biesenthal, 1998; Pedras et al., 1993). A rather different metabolite profile was obtained in cultures grown in potato dextrose broth (PDB) where no phytotoxins appeared to be produced, but from which 2,4-dihydroxy-3,6-dimethylbenzaldehyde (**8**) was isolated (Pedras et al., 2007). Previous to our work, **8** had been isolated from *Valsa ambiens* and a few other fungi (Mitova et al., 2006; Ayer et al., 1993) and shown to have root and hypocotyl growth inhibition effect on lettuce seedlings (Jiao et al., 1994) (see Fig. 1).

Using an elicitor-toxin bioassay to detect both elicitor and phytotoxic activity, we searched for bioactive metabolites in cultures of virulent *L. maculans* grown in PDB, where no sirodesmins were produced. We discovered a unique selective phytotoxin derived from D-mannitol and containing two 2-isocyano-3-methyl-2-butenoyl substituents, which was named maculansin A (**9**); as well, maculansin B (**10**), a related metabolite was isolated. The structure of maculansin A (**9**) was determined using NMR and MS spectroscopic data and chemical degradation. Surprisingly, this metabolite caused larger lesions on leaves of resistant brown mustard (*B. juncea* L.) than on susceptible canola, although it was produced by *L. maculans* isolates virulent on canola. In addition, metabolite **8** was isolated and shown to display significant growth inhibitory activity on roots of canola and mustard.

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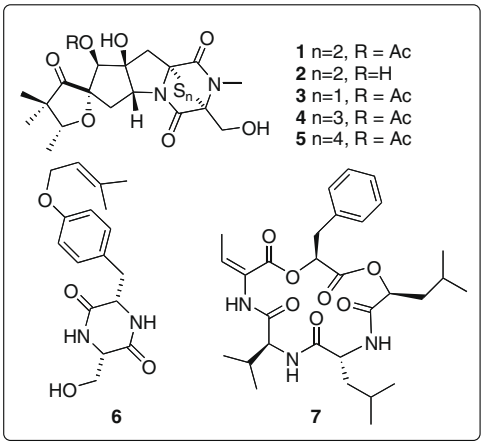
2. Results and discussion

2.1. Structure determination of maculansins A (**9**) and B (**10**)

We have reported that cultures of *L. maculans* appeared to produce mainly benzaldehyde **8** in PDB (Pedras et al., 2007). However, an accidental power failure that caused an increase in the room temperature from 23 to 28 °C appeared to induce the production of additional metabolites not available in our fungal metabolite libraries (Fig. 2). To isolate these potentially new metabolites produced in PDB cultures, their production was optimized by analyzing the relative concentration of each metabolite (determined by peak areas of HPLC chromatograms) in function of the incubation time (four and seven days) and temperature (23–29 °C). It was established that the production of compound **8** (R_t = 11.4 min) was maximized at 25 °C after seven days of incubation, whereas the production of the new metabolites with R_t = 16.4 and 18.3 min (later determined to be compound **9** and **10**) was maximized around four days of incubation at 27 °C (Fig. 2).

Next, cultures of *L. maculans* virulent isolate BJ 125 were grown in PDB medium and extracted as reported in the Experimental. The EtOAc extract of the broth showed phytotoxic activity using the elicitor-toxin bioassay. As previously reported, no sirodesmins (**1**–**5**) were detected in the extract obtained from cultures grown in PDB medium, as determined by HPLC analysis. Metabolite **8** (ca. 1.7 mg/l, R_t = 11.4 min) together with maculansin A (**9**) (ca. 1.6 mg/l, R_t = 16.4 min) were the main components isolated from the culture extract, as described in the Experimental.



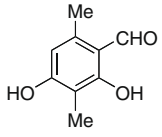
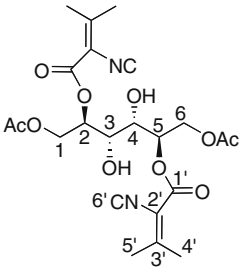
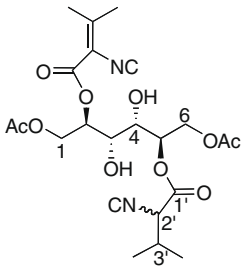
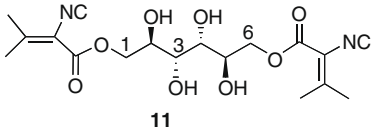
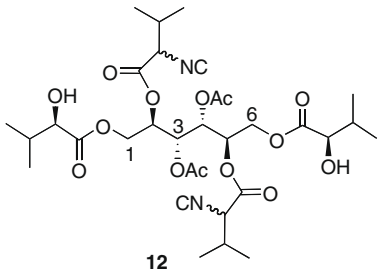


Fig. 1. Metabolites produced by *Leptosphaeria maculans* in liquid minimal medium (**1**–**7**) and in potato dextrose medium (**8**).









The HRMS–EI of the metabolite with R_t = 16.4 min (**9**) indicated the molecular formula $C_{22}H_{28}N_2O_{10}$ (*m*/*z* 480.1749), however, the ^1H and ^{13}C NMR spectra showed only 14 and 11 signals, respectively, suggesting a symmetry element in the molecule. The ^1H NMR spectrum of **9** displayed signals for two methylene protons at δ_H 4.64 (*dd*, *J* = 12.5, 2.5 Hz, H_a-1) and δ_H 4.41 (*dd*, *J* = 12.5, 4.2 Hz, H_b-1), two methines at δ_H 5.13 (*m*, H-2) and δ_H 3.89 (*t*, *J* = 8.2, 8.2 Hz, H-3), an exchangeable proton at δ_H 3.00 (*d*, *J* = 7.7 Hz, OH), and three methyl singlets at δ_H 2.11, 2.18 and 2.31 (i.e. 14 protons, Table 1). The ^{13}C NMR spectrum showed 11 carbons, six of which were sp^3 hybridized, four carbons were sp^2 hybridized and one was sp hybridized (Table 1). COSY, HMQC and HMBC NMR spectroscopic data showed that the methine proton at δ_H 3.89 was coupled to both H-2 and H(O–) signals, and that the methyl resonances at δ_H 2.31 and 2.18 correlated to sp^2 carbons at δ_C 158.3 and 115.0. In addition, the H-2 signal (δ_H 5.13) showed a correlation with an sp^2 carbon at δ_C 160.5, whereas a carbon at δ_C 168.0 did not show detectable correlations. Altogether, these NMR correlations suggested that the carbonyl at δ_C 160.5 was conjugated with an isopropenyl fragment. Since the IR spectrum contained a characteristic absorption at 2116 cm^{-1} attributable to an isonitrile group and the signal at δ_C 168.0 was consistent with an isonitrile carbon (Karuso et al., 1989), a 2-isocyano-3-methyl-2-butenoyl partial structure was assigned. The overall spectroscopic data suggested that **9** was derived from a polyol containing two acetyl and two 2-isocyano-3-methyl-2-butenoyl substituents and two free

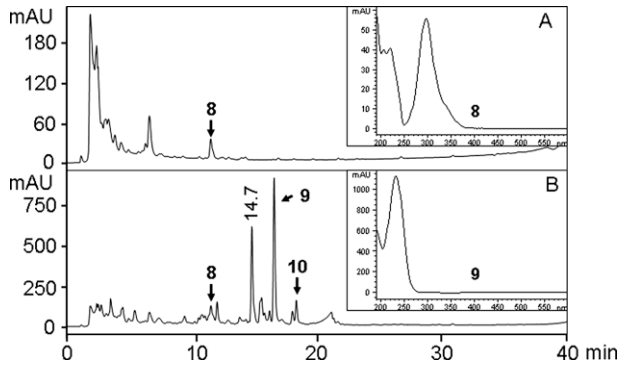


Fig. 2. HPLC chromatograms of extracts of cultures of *Leptosphaeria maculans* grown in potato dextrose medium (PDB); (A) EtOAc extract of culture incubated at 23 °C for four days; (B) EtOAc extract of culture incubated at 27 °C for four days; numbered peaks identified as: 2,4-dihydroxy-3,6-dimethylbenzaldehyde (**8**), maculansin A (**9**), maculansin B (**10**), peak at 14.7 min identified as mixture of metabolites related to **9**. Inserts; UV/Vis spectra of metabolites **8** and **9**.

Table 1
^{13}C NMR (125 MHz) and ^{1}H NMR (500 MHz) chemical shifts (ppm) and multiplicities (*J* in Hz) of maculansin A (**9**) (in $CDCl_3$)

C/H #	Maculansin A (**9**)	
	δ_C	δ_H
1, 6	62.3	4.64, 2H, *dd*, (12.5, 2.5) 4.41, 2H, *dd*, (12.5, 4.2)
2, 5	72.8	5.13, 2H, *m*
3, 4	67.4	3.89, 2H, *t*, (8.2)
1′	160.5	–
2′	115.0	–
3′	158.3	–
4′/5′	21.4	2.31, 6H, *s*
5′/4′	25.1	2.18, 6H, *s*
6′	168.0	–
1″	171.6	–
2″	21.1	2.11, 6H, *s*
(O)H	–	3.00, 2H, *d*, (7.7)

hydroxyl groups. The polyol backbone of metabolite **9** was determined to be D-mannitol after hydrolysis, acetylation and identification of one of the reaction products as hexaacetyl D-mannitol by comparison of the ^{1}H NMR spectroscopic data and specific optical rotation with that of an authentic sample. Therefore, the structure of the new metabolite **9** was elucidated as 1,6-diacetyl-2,5-bis(2-isocyano-3-methyl-2-butenoyl)-D-mannitol.

From another fraction, a mixture of several compounds having structural characteristics similar to those of **9** was obtained and further separated by prep TLC, as described in the Experimental, to afford components with R_t = 18.3 min (**10**) and 14.7 min (Fig. 2). Although HPLC analysis suggested a chromatographically homogeneous material, preliminary inspection of the ^{1}H NMR spectroscopic data of the component with R_t = 18.3 min (**10**) indicated that this sample contained a mixture of at least two compounds (complex spectrum showing a polyol backbone with two sets of signals for some protons). In addition, this spectrum showed two doublets at δ_H 1.14 and 1.04 coupled to a methine proton at δ_H 2.38 as well as two methyl groups at δ_H 2.3 and 2.2, similar to those observed in **9**. That is, one of the 2-isocyano-3-methyl-2-butenoyl fragments attached to the polyol was replaced with a 2-isocyano-3-methylbutanoyl moiety, whereas the acetyl groups and the backbone remained intact. This conclusion was consistent with the $[M + 1]^+$ *m*/*z* 483 obtained for this sample (LC–MS–ESI). As well, the polyol backbone of **10** was determined to be D-mannitol after hydrolysis and acetylation of a mixture of **9** and **10**, as reported for metabolite **9**. Therefore, the inseparable mixture **10** contained two epimers at C-2′ (due to the acidity of H-2′) and is proposed to be 1,6-diacetyl-2-(2-isocyano-3-methyl-2-butenoyl)-5-(2-isocyano-3-methylbutanoyl)-D-mannitol and named maculansin B (**10**).

Similarly, inspection of the ^{1}H NMR spectroscopic data of component with R_t = 14.7 min showed a spectrum more complex than that of **10**, containing several signals for each proton and thus suggesting that this sample contained a mixture of structural isomers likely due to the presence of two 2-isocyano-3-methylbutanoyl moieties and absence of an acetyl substituent. As in the case of metabolite **10**, the mannitol backbone appeared to remain intact; however, due to the complexity of the spectra, structural assignments could not be made unambiguously and thus structures are not proposed. Therefore, the main components of PDB cultures of virulent *L. maculans* are maculansins A (**9**), B (**10**) and a currently inseparable mixture of related metabolites not fully characterized.

The 2-isocyano-3-methyl-2-butenoyl fragment is rare in natural products, to the best of our knowledge only antibiotic A32390-A (**11**) was reported to contain it (Marconi et al., 1978). This antibiotic, isolated from the culture broth of a *Pyrenochaeta* species, is an inhibitor of dopamine-β-hydroxylase and is antimicrobial against fungi and gram-positive bacteria. Similarly, brassicicolin A (**12**), isolated from cultures of *Alternaria brassicicola*, a fungal pathogen of *Brassica* species, was the first natural product reported to contain 2-isocyano-3-methylbutanoyl fragments and also shown to have potent in vitro activity against the gram-positive bacteria (Gloer et al., 1988). Maculansins A (**9**) and B (**10**) together with A32390-A (**11**) and brassicicolin A (**12**) constitute a very unique group of fungal metabolites derived from D-mannitol that display strong and diverse biological activities.

2.2. Bioactivity

2.2.1. Maculansin A (*9*)

Elicitor activity results were considered positive when droplets of a metabolite solution applied to leaves of 2-week-old plants (4-leaf stage) elicited production of phytoalexins (detected by HPLC analysis of leaf extracts). Toxicity was scored by measurement of leaf lesions (necrosis and/or chlorosis, rated according to the damage index number, where the number is directly proportional to the size of the lesion, as described in the Experimental). The bioactivity of maculansin A (**9**) was compared with that of the toxin sirodesmin PL (**1**, isolated as previously reported by Pedras et al., 1990). Maculansin A (**9**) did not elicit the production of phytoalexins in *B. juncea* cv. Cutlass or *B. napus* cv. Westar (Figs. 3 and 4). However, the damage caused by **9** on the leaves

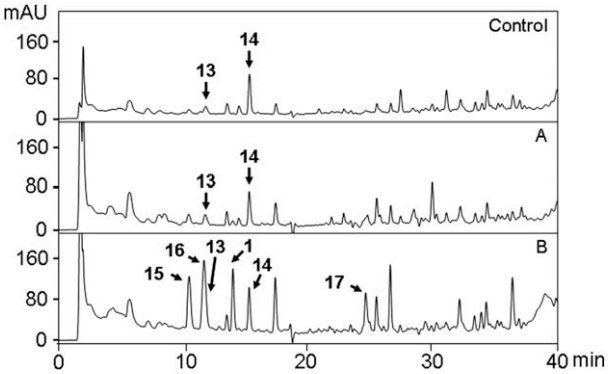


Fig. 3. HPLC chromatograms of extracts of leaves of brown mustard (*Brassica juncea* cv. Cutlass, resistant to blackleg) treated with: (A) maculansin A (**9**) and (B) sirodesmin PL (**1**); numbered peaks identified as: indole-3-acetonitrile (**13**), 1-methoxy-indole-3-acetonitrile (**14**), 1-methoxy-indole-3-methanol (**15**), spirobrassinin (**16**), cyclobrassinin (**17**).

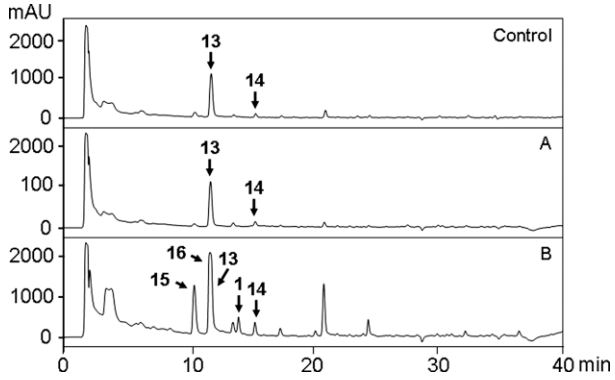


Fig. 4. HPLC chromatograms of extracts of leaves of canola (*Brassica napus* cv. Westar, susceptible to blackleg) treated with: (A) maculansin A (**9**) and (B) sirodesmin PL (**1**); numbered peaks identified as: indole-3-acetonitrile (**13**), 1-methoxy-indole-3-acetonitrile (**14**), 1-methoxy-indole-3-methanol (**15**), spirobrassinin (**16**).

of different crucifer plants appeared to depend on the plant species. For example, **9** at 0.1 mM caused damage (2.2) on resistant brown mustard (*B. juncea* cv. Cutlass), but no damage on either susceptible canola (*B. napus* cv. Westar) or resistant white mustard (*Sinapis alba* cv. Ochre); at higher concentration (1 mM), similar damage on the three plant species was observed (Fig. 5). Contrary to maculansin A (**9**), sirodesmin PL (**1**, 0.2 M) caused similar leaf lesions in all plant species and induced the production of phytoanticipins (indole-3-acetonitrile (**13**), 1-methoxy-indole-3-acetonitrile (**14**), 1-methoxy-indole-3-methanol (**15**)) and phytoalexins (spirobrassinin (**16**) and cyclobrassinin (**17**)) in leaves of brown mustard (Fig. 3) and canola (Fig. 4), as determined by HPLC analysis and direct comparison with authentic samples.

2.2.2. 2,4-Dihydroxy-3,6-dimethylbenzaldehyde (*8*)

Although 2,4-dihydroxy-3,6-dimethylbenzaldehyde (**8**) was shown to have root and hypocotyl growth inhibition effects on lettuce seedlings (Jiao et al., 1994), it was important to determine if it affected root growth of the host plants of *L. maculans*. Thus, the effect of **8** on seeds of brown mustard (resistant) and canola (susceptible) incubated in media amended with **8** (0.5 mM) was determined. A strong inhibitory effect was observed for both species; while the root length of treated canola seeds was 0.3 ± 0.1 cm, control roots measure 1.9 ± 0.7 cm (Fig. 6). Similarly, roots of treated brown mustard measure only 0.2 ± 0.1 cm, while control roots measured 4.9 ± 1.2 cm (seeds were incubated in darkness for 7 days). That is, **8** inhibited 6-fold and 25-fold the root growth of canola and brown mustard, respectively. This is the first time that a metabolite of *L. maculans* is reported to inhibit root

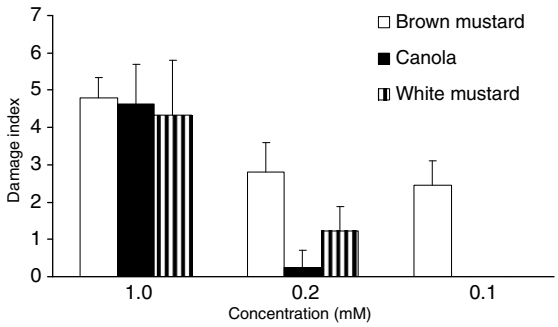


Fig. 5. Phytotoxicity of maculansin A (**9**) on brown mustard (*Brassica juncea* cv. Cutlass, resistant), canola (*B. napus* cv. Westar, susceptible) and white mustard (*S. alba* cv. Ochre, resistant).

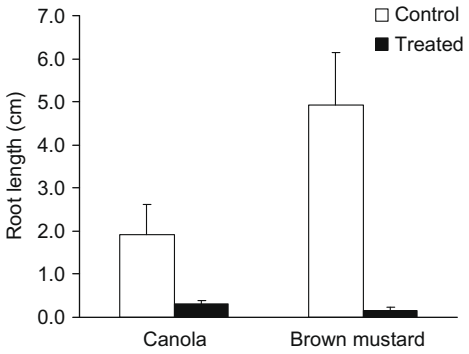


Fig. 6. Root length of seedlings of canola (*Brassica napus* cv. Westar, susceptible) and brown mustard (*B. juncea* cv. Cutlass, resistant) treated with metabolite **8** at 0.5 mM.

growth of susceptible and resistant host plants. It is particularly intriguing that the inhibitory effect on the resistant plant is much higher than that caused in susceptible plants.

The somewhat selective phytotoxicity of **9** is inconsistent with the pathogenicity of *L. maculans*, because **9** caused more damage on resistant plants (*B. juncea* cv. Cutlass) than on susceptible plants (*B. napus* cv. Westar). Moreover, the damage caused by **9** suggested that it is able to diffuse in the leaf tissue, causing irregular lesions, similar to those caused by the pathogen and the toxin depsilairdin (Pedras et al., 2004). On the other hand, the damage caused by sirodesmin PL (**1**) is a defined circular lesion, indicating immediate lyses of leaf cells.

3. Conclusions

Altogether our past (Pedras et al., 2007) and current results show that culture conditions have a great impact on the production of secondary metabolites by virulent *L. maculans*. The diversity of metabolite structures determined by medium composition is particularly intriguing. One wonders which of these metabolites, if any, affect the interaction of *L. maculans* with its host plants. Our multiple attempts to identify fungal metabolites in infected plant tissues have yet to produce consistent and convincing results. Nonetheless, considering that *L. maculans* seems to be expanding its host range to infect brown mustard as well (isolates Laird 2 and Mayfair 2 are virulent on brown mustard, Pedras et al., 2007), maculansins could be useful in chemotaxonomic studies for grouping the various isolates. In this context, a question arises whether maculansin type metabolites are also produced by isolates Laird 2 and Mayfair 2, as this may explain the higher phytotoxicity of maculansin A (**9**) on brown mustard and warrants further investigations. Finally, it is also worthy to note that maculansin A (**9**) causes obvious damage on leaf tissues but does not elicit phytoalexins, an outcome very useful for the pathogen.

4. Experimental

4.1. General

All chemicals were purchased from Sigma–Aldrich Canada Ltd., Oakville, ON; solvents were HPLC grade and used as such. Organic extracts were dried with Na_2SO_4 and solvents removed under reduced pressure in a rotary evaporator. Flash column chromatography (FCC) was carried out using silica gel grade 60, mesh size 230–400 Å. Prep TLC was carried out on silica gel plates, Kieselgel 60 F_{254} (20 × 20 cm × 0.25 mm); compounds were visualized under UV light. Specific rotations $[\alpha]_D$ were determined at ambient temperature on a polarimeter using a 1 ml, 10 cm path length cell; the

units are 10^{-1} deg cm^2 g^{-1} and the concentrations are reported in g/100 ml.

Nuclear magnetic resonance (NMR) spectra (^{1}H, ^{13}C, HMQC-heteronuclear multiple quantum coherence, HMBC-heteronuclear multiple bond coherence) were recorded on Bruker Avance 500 spectrometers. High resolution (HR) electron impact (EI) mass spectra (MS) were obtained on a VG 70 SE mass spectrometer employing a solids probe. LC–MS–ESI data were obtained on an Agilent 1100 HPLC system coupled to an LC/MSD ion trap detector with electrospray ionization. Chromatographic separation was carried out at room temperature using an Eclipse XSB C-18 column (5 μm particle size silica, 150 mm × 4.6 mm) equipped with an on-line filter. The mobile phase consisted of a gradient of 0.2% HCO_2H in H_2O (A) and 0.2% HCO_2H in CH_3CN (B); linear gradient elution starting with 75:25 of A/B to 25:75 of A/B in 35 min, to 100% B in 5 min and a flow rate 1.0 mL/min. Other conditions were as previously reported (Pedras et al., 2006).

HPLC–DAD 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 Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), having an in-line filter. Mobile phase: 75% H_2O-25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient, and at a flow rate 1.0 ml/min.

4.2. Fungal isolates and culture conditions

Canadian virulent isolate of *L. maculans* IBCN 57 (BJ 125) was grown in Erlenmeyer flasks (250 ml) containing PDB (100 ml), inoculated with fungal spores (10^8 spores/100 ml) and incubated on a shaker at 120 rpm, at 23, 25, 27, and 29 °C. Culture samples (20 ml) of 4-day-old or 7-day-old cultures were filtered to separate the broth from mycelia. The broth was extracted with EtOAc (20 ml × 3) and the EtOAc layer was dried over Na_2SO_4 and concentrated. The residue was dissolved in CH_3CN (1.0 ml) and analyzed by HPLC.

4.3. Isolation and characterization of maculansins A (**9**) and B (**10**)

PDB cultures of *L. maculans* (5 l) were prepared as reported above and grown in an incubator at 27 °C to maximize production of **9**. After four days, the cultures were combined and filtered, the broth was extracted with EtOAc, and the EtOAc layer was concentrated to yield 360 mg of residue. The residue was separated by FCC (RP-18, CH_3CN–H_2O, 10:90 to 40:60, gradient elution) to yield 30 fractions (15 ml per fraction). Fractions 8–10 were combined and further purified by FCC (MeOH–CH_2Cl_2 0:100 to 5:95, gradient elution) to give compound **8** (10.5 mg). Fractions 15 to 18 were combined (45.1 mg) and further purified by prep TLC (MeOH–CH_2Cl_2, 5:95) to yield compound **9** (9.7 mg) and a mixture of the component with R_t = 14.7 min (mixture of isomers structural type similar to **9**). Fractions 22–25 contained a mixture of maculansin type metabolites that were further separated by prep TLC to yield an epimeric mixture of maculansin B (**10**, 1.6 mg).

Maculansin A (9). HPLC R_t = 16.4 min. HRMS–EI: *m*/*z* 480.1749, $C_{22}H_{28}N_2O_{10}$, calcd. 480.1744. MS–EI: *m*/*z* 480 (3%), 465 (22%), 115 (100%). for ^{1}H and ^{13}C NMR spectroscopic data see Table 1. FTIR (KBr): 3460, 2960, 2923, 2116, 1740, 1732, 1222 cm^{-1}. UV (MeOH): λ_{max} (log ε) 231 (4.2) nm; $[\alpha]_D = -71$ (*c* 0.10, $CHCl_3$).

Maculansin B (10). HPLC R_t = 18.3 min. HRMS–ESI: $[M + Na]^+$ *m*/*z* 505.1807, $C_{22}H_{28}N_2O_{10}Na$, calcd. 505.1792. HRMS–ESI–MS/MS: *m*/*z* 505 (100%), 380 (40%), 378 (70%), 253 (27%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.2 (*m*, 1 H), 5.1 (*m*, 1 H), 4.6 (*m*, 2 H), 4.4 (*m*, 2 H), 4.28 (*d*, *J* = 4.2 Hz, 1 H), 3.8 (*m*, 2 H), 3.07 (*d*, *J* = 7.4 Hz, 1 H, OH), 2.93 (*d*, *J* = 8.3 Hz, 1 H, OH), 2.4 (*m*, 1 H), 2.32 (*s*, 3 H), 2.19 (*s*, 3 H), 2.10 (*s*, 3 H), 2.09 (*s*, 3 H), 1.14 (*d*, *J* = 6.8 Hz, 3 H), 1.04 (*d*, *J* = 6.8 Hz, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.5 (2C), 158.8, 116.6, 115.0, 74.0, 72.5, 67.6 (2C), 62.8, 61.9 (2C), 24.8, 21.3 (2C), 20.8 (2C), 19.5, 16.6, (C-1′, -6′, and -1″ were not detected). ^{13}C NMR spectral data were obtained from analysis of HMQC and HMBC correlations.

Chemical degradation of maculansin A (9). Na_2CO_3 (10.0 mg) was added to a solution of maculansin A **9** (8.0 mg, 0.017 mmol) dissolved in MeOH (4.0 ml). The reaction mixture was stirred for 4 h at room temperature then concentrated. The residue was dissolved in H_2O (5 ml), the pH of the solution was adjusted to 2, followed by extraction (CH_2Cl_2). The aqueous layer was concentrated to dryness then pyridine (500 μl) and Ac_2O (500 μl) were added to the residue and the reaction mixture stirred for 5 h at room temperature. After concentration to dryness, the residue was separated (prep TLC, MeOH–CH_2Cl_2, 4:96) to yield a compound (6.7 mg) identical to D-mannitol hexaacetate as determined by ^{1}H NMR, HRMS and specific optical rotation data ($[\alpha]_D$ = 13 (*c* 0.51, $CHCl_3$); authentic D-mannitol hexaacetate $[\alpha]_D$ = 12 (*c* 0.26, $CHCl_3$)).

4.4. Bioactivity assays

Three activity assays were carried out as described below.

Elicitor activity. Canola (*B. napus* cv. Westar, susceptible to *L. maculans*) and brown mustard (*B. juncea* cv. Cutlass, resistant to *L. maculans*) were grown in a growth chamber with 16 h light (fluorescent and incandescent, 450–530 μmol s^{-1} m^{-2})/8 h dark, at 24 ± 2 °C. After two weeks, extracts dissolved in MeOH–H_2O (1:1, v/v) solutions were applied to leaves punctured with a needle (about 20 punctures per leaf). After two days, the leaf tissues were frozen in liquid N_2, crushed with a glass rod and extracted with 50 ml EtOAc. EtOAc extract was dried over Na_2SO_4 and concentrated in a rotary evaporator. The residue was dissolved in 1% MeOH–CH_2Cl_2, applied to a mini silica gel column (Pasteur pipette) and eluted with 1% MeOH–CH_2Cl_2 (5 ml); the eluate was concentrated, the residue was dissolved in CH_3CN (80 μl) and analyzed by HPLC. Control leaves were treated similarly (MeOH–H_2O, 1:1) and analyzed by HPLC. All assays were carried out in triplicate.

Phytotoxic activity. Canola, brown mustard and white mustard (*S. alba* cv. Ochre, resistant to *L. maculans*) were grown as described above. Solutions of either fractions or purified compounds dissolved in MeOH–H_2O (1:1, v/v) were applied to punctured leaves (5 μl, 6 punctures per leaf). After two days, the damaged areas were measured using a stencil having cut out circles with different diameters, and the measured diameters were converted to the damage index, as shown in Table 2. All bioassays were carried out in triplicate.

Root growth inhibition activity. Seeds of brown mustard (20) and canola (20) were sterilized by soaking in Javex (10%) for 10 minutes, washed with H_2O, air dried and incubated in PDB medium (0.9 g/100 ml) amended with compound **8** (0.5 mM). The dishes were sealed with parafilm and kept in darkness at room temperature. After 7 days the root length of seedlings was measured with a ruler.

Table 2
Scale for conversion of damaged area to damage index

Lesion diameter (mm)	Damage index
<1.5	0
1.6–2.3	1
2.4–3.1	2
3.2–3.9	3
4.0–4.7	4
4.8–5.5	5
5.6–6.3	6
6.4–7.0	7

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