Detoxification of Oat Leaf Saponins by *Septoria avenae*

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ABSTRACT

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Isolates of *Septoria avenae* and related fungi were analyzed for pathogenicity to oats and wheat and found to be either pathogenic on oat or wheat. Two compounds were identified in methanolic extracts of oat leaves that inhibited the growth of wheat-attacking isolates. Analysis of these compounds by thin-layer chromatography and fast atom bombardment-mass spectrometry supported their identification as the steroidal saponins 26-desglucoavenacosides A and B. Oat-attacking (but not wheat-attacking) isolates of *Septoria* were able to detoxify these saponins by enzymatic hydrolysis of the sugar chain attached at carbon 3. An enzyme that carried out this hydrolysis was purified from the culture filtrate of *S. avenae* f. sp. *avenae*. This enzyme (avenacosidase) was capable of removing both L-rhamnose and D-glucose molecules from the C-3 sugar chain of the saponins. The enzyme had a molecular mass of 110 kDa, an isoelectric point between pH 3.8 and 4.1, and optimal β-D-glucosidase activity at pH 5.4.

Additional keywords: *Avena sativa*, preformed inhibitors, α-L-rhamnosidase, speckled blotch.

Saponins are steroidal or triterpenoid glycosides found in many different plant species (7,12,23). Many saponins have potent antifungal properties and, hence, it has been suggested that these compounds may play a role in protecting plants against attack by fungal pathogens (20). The major mechanism of toxicity of saponins to fungi is believed to be due to their membraneolytic action. Saponins complex with membrane sterols causing the formation of pores and, hence, the loss of membrane integrity (20). Successful pathogens of saponin-containing plants may, therefore, need to tolerate or detoxify the saponins of their hosts (20). A number of fungal pathogens produce hydrolytic enzymes that detoxify saponins by the removal of sugar molecules (12,20,25). Several of these enzymes have been isolated and characterized (15,21,22,24).

The ability of the cereal root pathogen *Gaeumannomyces graminis* to infect oats is dependent on enzymatic detoxification of triterpenoid saponins found in oat roots, avenacins (2). Detoxification of the major antifungal avenacin, avenacin A-1, involves a specific fungal β-D-glucosidase (known as avenacinase) that removes D-glucose molecules from the sugar chain at the C-3 position of the saponin (5,22). Avenacinase-minus fungal mutants generated by targeted gene disruption are unable to infect oat roots, but are still fully pathogenic to wheat, which does not contain avenacins (2). Similar saponin detoxification mechanisms have been reported for several fungal pathogens of tomato plants that encounter the steroidal glycoalkaloid α-tomatine (20). To date, only one of these tomatinase enzymes, that of *Septoria lycopersici*, has been characterized at both the biochemical and the genetic levels. This enzyme detoxifies α-tomatine by cleavage of a D-glucose from the sugar chain at the C-3 position (6,21,24) and has recently been demonstrated to be closely related to avenacinase of *G. graminis* var. *avenae* (21).

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Whereas the triterpenoid avenacin saponins occur only in oat roots, oat leaves contain a different class of saponins known as avenacosides (Fig. 1) (30,31). Avenacosides A and B are glycosylated steroidal molecules derived from nuatigenin and accumulate in oat leaves at concentrations up to 4 mg g^{-1} of fresh weight (16,18). They each have two sugar chains, one at position C-3 and one at C-26 of the steroid molecule, and are referred to as bisdesmosidic. The bisdesmosidic avenacosides do not themselves show any antifungal activity (16), presumably because the glucose at C-26 increases the polarity of the steroid tail, thereby, preventing complex formation with the sterols in the fungal membrane. Damage to oat leaf tissue, e.g., after wounding by fungal pathogens, results in the formation of the antifungal monodesmosidic 26-desglucoavenacosides A and B (26-DGAs A and B) (16). This activation of the avenacosides results from removal of the D-glucose molecule at the C-26 position (Fig. 1) by a specific oat β-D-glucosidase (avenacosidase) (16,19). The C-3 sugars of the avenacosides are unaffected by the oat enzyme.

Toxicity of the oat leaf saponins towards fungal plant pathogens has been studied by Lüning and Schlösser (17). The oat leaf pathogens *Drechslera avenae* and *S. avenae* are relatively resistant to 26-DGAs A and B when fungal mycelia were incubated with the saponins in vitro and amino acid leakage was measured (17). *D. avenae* is capable of enzymatic detoxification of 26-DGAs A and B by hydrolysis to the aglycone nuatigenin (17).

In this work, we investigated the role of avenacosides in the interaction between oats and *S. avenae* A. B. Frank, causal agent of speckled blotch of oat (32). Two formae speciales of *S. avenae* have been identified: *S. avenae* f. sp. *tritici*, which is pathogenic on wheat (27), and *S. avenae* f. sp. *avenae*, which is pathogenic on oats (32). Several isolates were collected and originally assigned to *S. avenae* f. sp. *avenae* and *S. avenae* f. sp. *tritici* (although during the course of this work, it became apparent that the wheat-attacking isolates were more likely to be *S. nodorum*). We determined the pathogenicity of this isolate collection to oat and wheat

and found a correlation between pathogenicity to oat and ability to detoxify oat leaf saponins. The detoxification involved hydrolysis of 26-DGAs A and B by an extracellular fungal enzyme that we named avenacosidase. The mechanism of saponin hydrolysis is described, as well as purification of the avenacosidase of an oat-attacking isolate of *S. avenae*.

MATERIALS AND METHODS

Fungal isolates and growth conditions. A total of 11 different fungal isolates were used in these experiments. These isolates were originally classified as *S. avenae* (32) (S143/76/1, S210/80, and S212/80 were supplied by T. W. Hollins, Plant Breeding International, Cambridge; CBS 288.69 was from Centraal Bureau Schimmelcultures, Baarn, the Netherlands; and WAC 1288, WAC 1292, WAC 1293, WAC 8378, WAC 5741, WAC 5742, and WAC 5743 were supplied by R. Loughman, Department of Agriculture, Western Australia, South Perth, Australia). The isolates were maintained on Czapek Dox V-8 juice agar at 20°C as described by Cooke and Jones (3). Sporulation was induced by transferring 7-day-old cultures from dark to near-ultraviolet light (F15W/350BL; Sylvania) with a dark/light cycle of 8 h/16 h. Eight of the 11 isolates sporulated under these conditions. All isolates were purified by isolation of single spores or by two successive rounds of hyphal tip isolation before use in these experiments. Fungal isolates were stored as mycelial colonies on potato-dextrose agar slopes under mineral oil at 4°C.

Pathogenicity tests. Pathogenicity of the isolates was determined using a detached-leaf pathogenicity assay as described by Johnston and Scott (14). Plant material used in the assays was cultivated hexaploid oat (*Avena sativa* cv. Image), the diploid oat species *A. strigosa* (John Innes accession number 02080), and cultivated wheat (*Triticum aestivum* cv. Norman). Plants were grown in the greenhouse with 16 h of light at a temperature of 15 to 20°C. Three-centimeter leaf sections were detached 1 cm from the tip of primary leaves of 12-day-old seedlings and placed on water agar containing 150 mg liter^{-1} of benzimidazole in plastic boxes. Four leaf sections of each plant species were inoculated with 3 μl of a suspension of pycnidiospores (1×10^6 ml^{-1}) in water containing 0.1% Tween 20 (vol/vol). For nonsporulating isolates, a suspension of young mycelia in water containing 0.1% Tween 20 (vol/vol) was used for the inoculations. Control leaf sections were inoculated with 3 μl of 0.1% Tween 20 in water (vol/vol). Symptoms were assessed visually at 7-days postinoculation, and the diameter of the necrotic lesion was measured.

Avenacoside preparation, quantification, and identification. Avenacosides A and B were obtained from 12-day-old, etiolated oat seedlings grown at 20°C (*A. sativa* cv. Image) by extracting whole leaves for 1 h with 3 volumes of hot methanol (65°C). For the isolation of 26-DGAs A and B, oat leaves were homogenized in 1 volume H_2O (wt/wt) using a blender and incubated for 1 h at room temperature. This allowed complete conversion of bisdesmosidic avenacosides A and B into the monodesmosidic forms. The homogenized oat leaves were subsequently extracted for 1 h with 3 volumes of hot methanol (65°C). After centrifugation for 15 min at 5,500 × *g* and filtration through Whatman No. 3 MM paper, the clear supernatants containing either avenacosides A and B or 26-DGAs A and B were concentrated in a rotary evaporator. Concentrated, methanolic oat leaf extracts were dried down onto acid-washed sand, which was applied to a silica G-60 column for separation of the saponins. Purification of avenacosides A and B was achieved on a 15-g silica G-60 column (column diameter, 1.5 cm) equilibrated and eluted with a solvent system of chloroform/methanol/water-70/35/5.5 (avenacosides A and avenacoside B eluted in fractions between 80 to 110 ml and 120 to 160 ml, respectively). 26-DGAs A and B were separated using a 130-g silica G-60 column (column diameter, 2.5 cm) equilibrated and eluted with chloroform/methanol/water-65/15/2 (26-DGA A and 26-DGA B eluted in fractions between 1,250 to 1,500 ml and 2,750 to 3,500 ml, respectively). Purification of the products of enzymatic hydrolysis of 26-DGA B was performed on a column with 20 g of silica G-60 (column diameter, 1.5 cm) equilibrated and eluted with chloroform/methanol/water-65/15/2. Collected fractions were analyzed by thin-layer chromatography (TLC) (silica G-60, 0.25 mm; E. Merck AG, Darmstadt, Germany) developed with chloroform/methanol/water-70/35/5.5. The chromatograms were stained by spraying with a *p*-anisaldehyde/sulfuric acid/acetic acid solution (1/1/48) and baking at 130°C for 5 min. Structural analysis of 26-DGAs A and B was performed using fast atom bombardment-mass spectrometry (FAB-MS). Methanolic solutions of the saponins were ap-

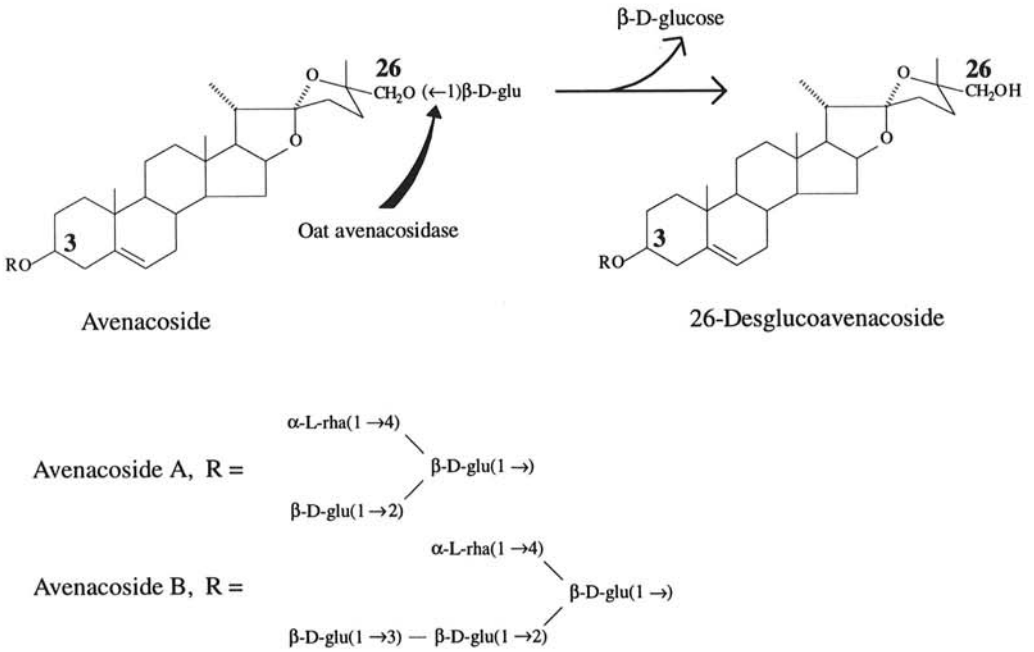

Fig. 1. Activation of the bisdesmosidic saponins avenacosides A and B by the oat enzyme avenacosidase. Avenacosidase removes a glucose molecule from position C-26 of the saponin to give the monodesmosidic 26-desglucoavenacosides A and B, which are antifungal. Avenacosides A and B differ only in the presence/absence of a third glucose molecule attached to the sugar chain at C-3.

plied to a drop of glycerol on the copper probe tip of the fast atom bombardment attachment coupled to a Kratos MS9/50TC mass spectrometer (Kratos, Manchester, United Kingdom). The sample was bombarded with a fast atom beam of xenon produced by an Ion Tech NF gun (neutral fine; Ion Tech Ltd., Teddington, United Kingdom) operating at 9 kV (nominal). FAB-mass spectra were recorded in both negative and positive modes.

Quantification of the active 26-DGAs A and B was performed using red blood cell haemolysis as described before (16,18).

Fungal growth inhibition. Radial growth-inhibition assays were performed on potato-dextrose agar in 50-mm petri dishes. The growth medium was supplemented with different amounts of a crude methanolic extract of oat leaves containing a mixture of 26-DGAs A and B (1% oat leaf extract (vol/vol) was equivalent to approximately 12.5 μg ml^{-1} of 26-DGA A and 37.5 μg ml^{-1} of 26-DGA B as determined by TLC and red blood cell haemolysis). Methanol concentration in the medium was adjusted to 1% (vol/vol) for all treatments. Radial growth was measured in duplicate at 11-days postinoculation, and the experiment was repeated once.

The toxicity of components of the oat leaf extract towards different fungal isolates was tested using TLC bioassays. The oat leaf extract, as used in the radial growth-inhibition assay, purified avenacosides A and B, and 26-DGAs A and B were fractionated by TLC as described above. Three identical TLC plates were developed. One chromatogram was stained with *p*-anisaldehyde. The two remaining chromatograms were sprayed with a nutrient solution (20 g of sucrose, 2 g of casamino acids, 2 mg of $ZnCl_2$, 2 mg of $MnSO_4.4H_2O$, 1.5 g of $MgSO_4.7H_2O$, and 110 mg of KCl in 1 liter of 0.0025 M K_2HPO_4/KH_2PO_4 buffer, pH 6.0) containing spores of either a wheat-attacking or an oat-attacking isolate of *Septoria* (S212/80 and WAC 1293, respectively) at 1×10^6 ml^{-1}. The TLC plates were incubated in the dark at 20°C for 10 days in large plastic boxes containing water-soaked filter paper. Antifungal compounds were identified by a clear white zone that was free of fungal growth.

Analysis of fungal culture filtrates and avenacosidase purification. Culture filtrates of 11 isolates of *S. avenae* were obtained from 100-ml cultures that had been grown in Jermyn's media (13) for 5 days at 22°C with shaking at 200 rpm. Cultures were inoculated either with spores (5×10^7 $liter^{-1}$) or with a homogenate of mycelia derived from fungal colonies grown on Czapek Dox V-8 juice agar for 3 weeks (one colony per 100 ml of liquid culture). Proteins were precipitated from the culture filtrates using ammonium sulfate (80% saturation), dissolved in water, and dialyzed against 20 mM NaH_2PO_4/Na_2HPO_4, pH 6.5, as described before (21). The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany). 26-DGA-hydrolyzing activity was determined using a haemolysis assay as described below.

TABLE 1. Pathogenicity of *Septoria* isolates to wheat or oat

Isolate	Length of necrotic lesion (mm)[a]		
	Avena sativa	*Avena strigosa*	*Triticum aestivum*
S143/76/1	0	0	4.8 ± 1.4[b]
S210/80	0	0	5.4 ± 1.6
S212/80	0	0	5.5 ± 1.4
CBS 288.69	5.6 ± 2.8	5.4 ± 1.3	0
WAC 1288	4.2 ± 0.7	3.2 ± 0.7	0
WAC 1292	6.0 ± 2.6	7.7 ± 1.0	0
WAC 1293	6.6 ± 1.0	7.4 ± 1.7	0
WAC 8378	6.8 ± 0.5	5.9 ± 0.6	0
WAC 5741	5.3 ± 0.5	8.8 ± 1.3	0
WAC 5742	4.5 ± 0.6	6.7 ± 1.0	0
WAC 5743	4.5 ± 0.6	6.0 ± 2.2	0

[a] Average length of necrotic lesions (mm) 7-days postinoculation as obtained from two experiments (eight infected leaves total).
[b] Standard deviation.

Larger preparations for the purification of 26-DGA-hydrolyzing enzymes involved a 2-liter culture filtrate of *S. avenae* isolate WAC 1293. The filtrate was chilled to 4°C and incubated for 30 min with protease inhibitors (2 mM ethylenediaminetetra-acetic acid disodium salt, 50 μM phenylmethylsulfonylfluoride, 1 μm of benzamidine hydrochloride, 0.5 μM phenanthroline, 0.5 μM aprotinin, 2 μM leupeptin, and 1.5 μM pepstatin A). Purification of a 26-DGA-hydrolyzing enzyme involved ammonium sulfate precipitation, followed by successive free-flow isoelectric focusing (RF3 protein fractionator; Rainin Instruments Co., Woburn, MA), anion-exchange high-performance liquid chromatography (HPLC) (TSK DEAE-5PW column; TosoHAAS, Montgomeryville, PA), and gel filtration HPLC (TSK G3000 SW XL column; TosoHAAS) as described before for purification of the tomatinase enzyme of *S. lycopersici* (21). With each purification step, fractions with 26-DGA-hydrolyzing activity were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; using Rainbow molecular weight markers [Amersham International plc., Little Chalfont, United Kingdom]) and assayed for β-D-glucosidase and α-L-rhamnosidase activity, using *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) and *p*-nitrophenyl α-L-rhamnopyranoside (*p*NPR) as substrates, as described below.

Enzyme assays. 26-DGA-hydrolyzing activity was determined qualitatively and quantitatively. For a qualitative assay, protein samples were incubated with a mixture of 26-DGAs A (15 μg) and B (15 μg) in 0.1 ml of 20 mM NaH_2PO_4/Na_2HPO_4, pH 6.2. After incubation for 1 h at 37°C, the samples were freeze-dried, extracted with methanol, and analyzed by TLC. Hydrolysis of the saponins results in less polar products with higher mobility when separated on TLC. Red blood cell lysis has been used to quantify 26-DGAs A and B, and these two saponins caused complete haemolysis at 9 and 3 μg ml^{-1}, respectively (16). Enzymatic hydrolysis of 26-DGAs A and B was correlated with a reduction of haemolytic activity for these compounds (J. P. Wubben, M. J. Daniels, and A. E. Osbourn, *unpublished data*). Therefore, 26-DGA-hydrolyzing activity was quantified by measuring the amount of haemolytic activity present in a mixture of a known amount of 26-DGAs A and B after incubation with an enzyme preparation.

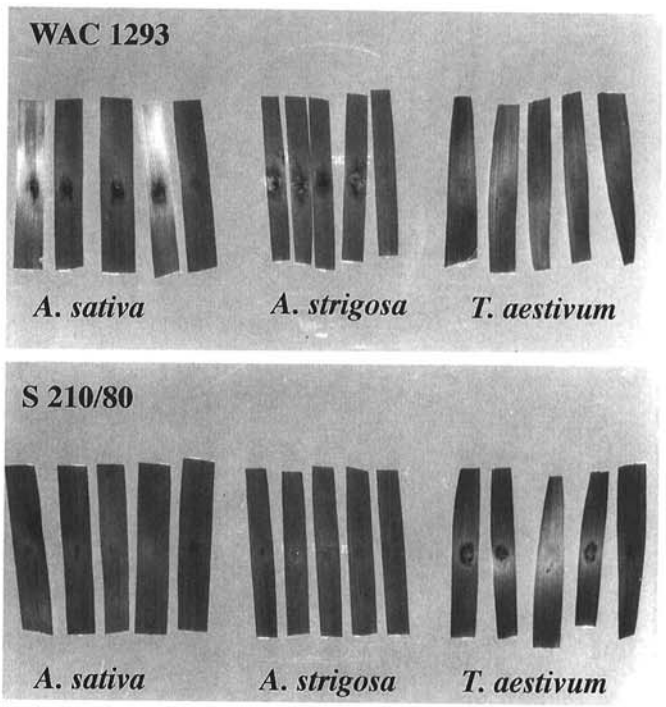

Fig. 2. Detached leaf pathogenicity assay showing pathogenicity of *Septoria* isolates to oat (WAC 1293) or wheat (S210/80). For each panel of five leaves, the first four leaves were inoculated with fungal spores and the fifth leaf was mock-inoculated.

Protein samples were incubated for 16 h at 37°C with a mixture 26-DGAs A (2 µg) and B (6 µg) in 0.2 ml of 20 mM NaH_2PO_4/Na_2HPO_4, pH 6.2. Titration of haemolytic activity of the saponin mixture using increasing amounts of enzyme is a measure for 26-DGA-hydrolyzing activity.

β-D-glucosidase and α-L-rhamnosidase activities were determined using *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) and *p*-nitrophenyl α-L-rhamnopyranoside (*p*NPR) as substrates. The assays were performed by incubating the enzyme with substrate for 1 h in 0.1 ml of 5 mM *p*NPG (or *p*NPR) and 100 mM NaOAc (pH 5.0) at 37°C. Activity was determined by measuring optical density at 420 nm after the addition of 0.9 ml of 100 mM KOH.

RESULTS

Pathogenicity of fungal isolates to oat and wheat. Eleven isolates of *S. avenae* were tested for pathogenicity to oat (*A. sativa* and *A. strigosa*) and wheat (*T. aestivum*) (Table 1, Fig. 2). All isolates showed characteristic leaf blotch symptoms on either oat or wheat (Fig. 2). The data clearly show that the isolates can be grouped based on their pathogenicity as either oat- or wheat-attacking (Table 1). The lengths of pycnidiospores produced by the oat-attacking isolates WAC 1292, WAC 1293, and WAC 8378 (30 to 50 µm, average 37 µm), which were identified as *S. avenae* f. sp. *avenae*, confirmed the original classification. The oat-attacking isolates WAC 5741, WAC 5742, and WAC 5743 (spore lengths: 25 to 46 µm, average 34 µm) were originally assigned to *S. avenae* f. sp. *tritici*, because they were isolated from wheat plants. However, our pathogenicity assays suggest that these isolates are also *S. avenae* f. sp. *avenae*. The lengths of the pycnidiospores of the wheat-attacking isolates S210/80 and S212/80 (17 to 25 µm [average 20 µm] and 18 to 27 µm [average 24 µm], respectively) suggest that these isolates are not *S. avenae* f. sp. *tritici*. These isolates are more likely to be *S. nodorum* (14). Fungal isolates S143/76/1, CBS288.69, and WAC1288 did not sporulate under the growth conditions used.

Inhibition of fungal growth by oat leaf extracts. The effect of oat leaf extracts on the growth of oat- and wheat-attacking isolates of *S. avenae* was determined by a radial growth assay (Fig. 3). Different amounts of methanolic extract from the primary leaves of oat seedlings, containing 26-DGAs A and B, were added to potato-dextrose agar. Three fungal isolates (S143/76/1, S210/80, and S212/80) were clearly inhibited in growth relative to the controls with increasing amounts of oat leaf extract added (Fig. 3). The other eight isolates were either unaffected in growth or even stimulated in growth by the oat leaf extract (Fig. 3). The diameters of fungal colonies for the controls were as follows: S143/76/1, 26 mm; S210/80, 31 mm; S212/80, 28 mm; CBS288.69, 15 mm; WAC1288, 10 mm; WAC1292, 19 mm; WAC1293, 20 mm; WAC8378, 15 mm; WAC5741, 15 mm; WAC5742, 10 mm; and WAC5743, 16 mm.

Purification and characterization of avenacosides from oat leaf extracts. The bisdesmosidic avenacosides A and B and their corresponding monodesmosidic forms (26-DGAs A and B) were isolated from 12-day-old oat seedlings (*A. sativa* cv. Image). These four compounds have different mobilities on TLC because of different numbers of sugar molecules attached to the aglycone (Fig. 4, lanes 3 to 6). R_f values for the different compounds were avenacoside A, 0.29; avenacoside B, 0.19; 26-DGA A, 0.50; and 26-DGA B, 0.40 (Fig. 4A, lanes 3, 4, 5, and 6, respectively). A yellow compound copurified with 26-DGA A (visible as the lower band of the two in lane 5). The occurrence of this compound in 26-DGA A preparations has also been reported by other workers (16). Partial structure confirmation of the higher mobility compound in lane 5 and of the compound in lane 6 were performed using FAB-MS. The compound from lane 5 (R_f value 0.50) gave the following spectra (h = hexose, d = deoxyhexose, and w = water): m/z899(100), $M\text{-}H^-$; m/z753(9), $M\text{-}H\text{-}d^-$; m/z737(52), $M\text{-}H\text{-}h^-$; m/z591(100), $M\text{-}H\text{-}d\text{-}h^-$; m/z923(5), MNa^+; m/z901(20), MH^+; m/z593(100), $MH\text{-}d\text{-}h^+$; m/z431(90), $MH\text{-}d\text{-}2h^+$; and m/z413(95), $MH\text{-}d\text{-}2h\text{-}w^+$. These spectra indicate the presence of a molecule with M_r of 900 with a trisaccharide moiety containing two hexoses and one deoxyhexose and confirm both a hexose and a deoxyhexose in terminal positions. The sugar sequence was attached to an aglycone with a mass of 430, which corresponds to either nuatigenin or isonuatigenin. Analysis of the compound from lane 6 resulted in the following spectra: m/z1061(67), $M\text{-}H^-$; m/z915(37), $M\text{-}H\text{-}d^-$; m/z899(22), $M\text{-}H\text{-}h^-$; m/z753(13), $M\text{-}H\text{-}h\text{-}d^-$;

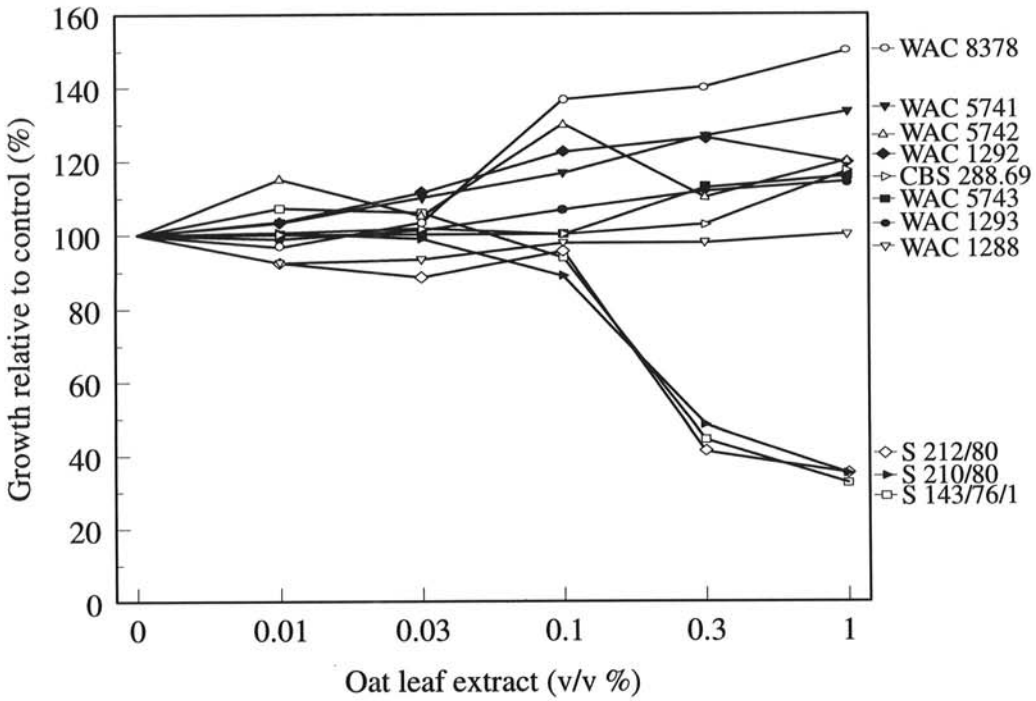

Fig. 3. Effect of oat leaf extract on radial growth of *Septoria* isolates (measured at 11-days postinoculation). Growth is presented relative to the growth on medium containing methanol alone (1% vol/vol). The graph shows means from a total of four different measurements of two experiments.

m/z737(12), M-H-2h$^-$; m/z591(13), M-H-2h-d$^-$; m/z1085(2), MNa$^+$; m/z1063(4), MH$^+$; m/z431(82), MH-3h-d$^+$; and m/z413(100), MH-3h-d-w$^+$. These spectra indicate the presence of a molecule with M_r of 1,062 with a tetrasaccharide moiety containing three hexoses and one deoxyhexose and confirm a terminal deoxyhexose and a dihexose moiety. The sugar sequence was attached to an aglycone with a mass of 430. These spectra confirm that the compounds in lanes 5 and 6 are likely to be 26-DGA A and 26-DGA B, respectively, based on the comparison with published structures for the avenacosides (30,31).

TLC plates similar to the one shown in Figure 4A were sprayed either with spores of a wheat- or an oat-attacking isolate of *Septoria* (isolates S212/80 and WAC 1293, respectively). Clear inhibition of growth of isolate S212/80 because of compounds with R_f values around 0.50 and 0.40 was observed (Fig. 4B, lanes 1, 2, 5, and 6), suggesting that 26-DGAs A and B had clear antifungal activity towards S212/80 in this bioassay. The bisdesmosidic avenacosides A and B did not result in any growth inhibition (Fig. 4B, lanes 3 and 4). In addition to avenacosides A and B, the presence of other avenacoside-like saponins in oat leaves has been described (31). These could account for the appearance of additional inhibition zones observed in the crude oat leaf extract (Fig. 4B, lanes 1 and 2), although clearly it appears that 26-DGAs A and B are the major antifungal compounds detectable under these conditions. No fungal growth inhibition was observed on the TLC sprayed with the oat-attacking isolate WAC 1293 (data not shown).

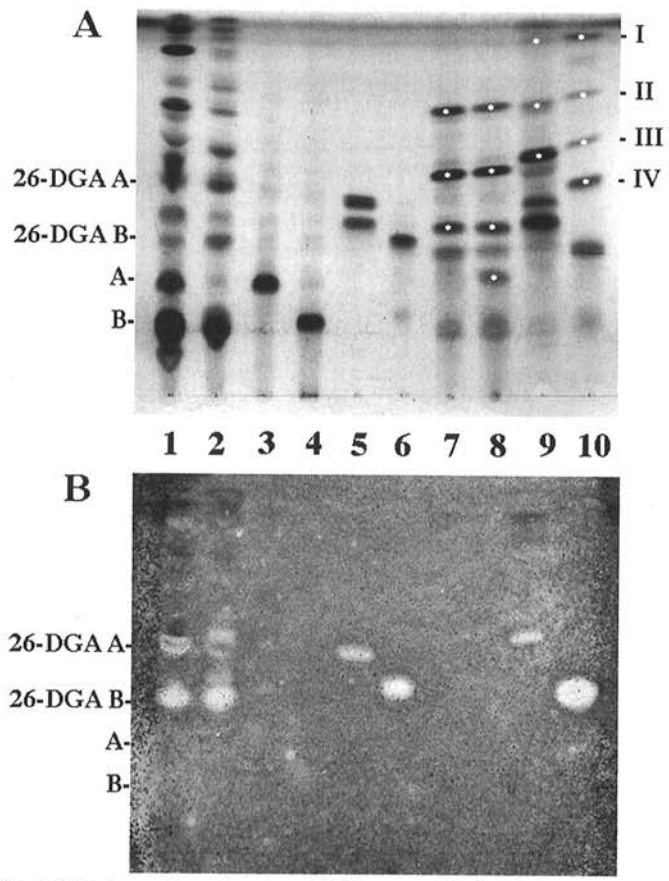

Fig. 4. Thin-layer chromatograms of different oat leaf-saponin preparations before and after enzymatic hydrolysis with concentrated culture filtrate of an oat-attacking isolate of *Septoria avenae* (WAC 1293) sprayed with **A,** *p*-anisaldehyde or **B,** spores of the wheat-attacking isolate S212/80. Lane 1, crude methanolic extract of whole oat leaves; lane 2, crude methanolic extract of homogenized oat leaves; lane 3, avenacoside A (A); lane 4, avenacoside B (B); lane 5, partially purified 26-desglucoavenacoside A (26-DGA A); lane 6, partially purified 26-desglucoavenacoside B (26-DGA B); lane 7, products of enzymatic hydrolysis of avenacoside A; lane 8, products of enzymatic hydrolysis of avenacoside B; lane 9, products of enzymatic hydrolysis of 26-DGA A; and lane 10, products of enzymatic hydrolysis of 26-DGA B. The products of saponin hydrolysis are indicated by white asterisks. **B,** The bioassay shows inhibition of fungal growth in lanes 1, 2, 5, 6, 9, and 10.

Analysis of fungal culture filtrates. Ammonium sulfate-concentrated culture filtrates of the *Septoria* isolates were assayed for the ability to hydrolyze 26-DGAs A and B. The amount of 26-DGAs A and B remaining after incubation with the culture filtrates was determined using red blood cell lysis. Only culture filtrates of oat-attacking isolates reduced the amount of 26-DGAs A and B in the incubation mixture (data not shown). For the wheat-attacking isolates, up to 50 times more total protein was unable to give a detectable hydrolysis of 26-DGAs A and B as measured by red blood cell lysis (data not shown).

The effect of fungal culture filtrate on 26-DGAs A and B was further analyzed using TLC. Ammonium sulfate-concentrated culture filtrate of an oat-attacking isolate of *S. avenae* (WAC 1293) was incubated with avenacoside A, avenacoside B, 26-DGA A, or 26-DGA B for 16 h at 37°C. Analysis by TLC revealed new products appearing resulting from enzymatic hydrolysis of avenacosides A and B (Fig. 4A, lanes 7 and 8) and 26-DGAs A and B (Fig. 4A, lanes 9 and 10). TLC of the reaction mix of enzymatic hydrolysis of avenacoside A showed several compounds with higher mobility than avenacoside A. Based on the green appearance of the avenacosides after staining with *p*-anisaldehyde, three major bands were assigned as products of saponin-hydrolysis (Fig. 4A, lane 7, indicated by asterisks; R_f values 0.44, 0.57, and 0.74, respectively). Similarly, hydrolysis of avenacoside B resulted in four new saponin products of which the top three had similar R_f values to the products seen after hydrolysis of avenacoside A (Fig. 4A, lane 8, indicated by asterisks; R_f values 0.31, 0.44, 0.57, and 0.74, respectively). Comparable results were obtained for the enzymatic hydrolysis of 26-DGA A and 26-DGA B (Fig. 4A, lanes 9 and 10, respectively). Hydrolysis of 26-DGA A resulted in three major products with R_f values of 0.64, 0.74, and 0.93 (Fig. 4A, lane 9). Hydrolysis of 26-DGA B gave four major products with R_f values of 0.55 (IV), 0.64 (III), 0.74 (II), and 0.93 (I) (Fig. 4A, lane 10). The latter four were purified by silica G-60 chromatography and analyzed by FAB-MS. The re-

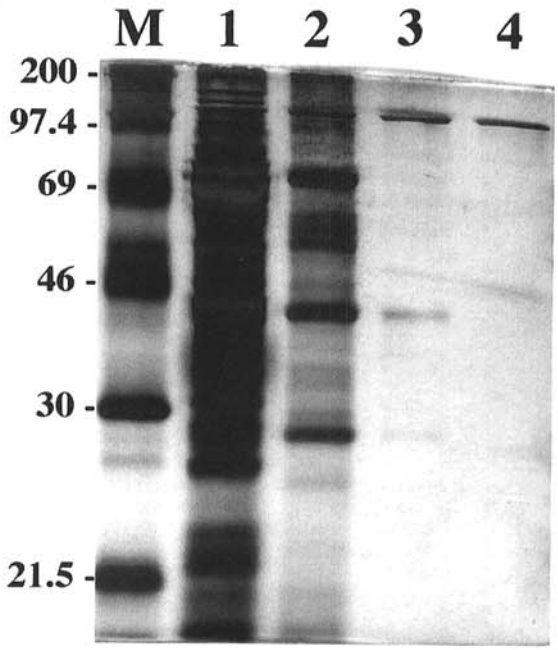

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing fractions containing avenacoside-hydrolyzing activity from the *S. avenae* isolate WAC 1293 at different stages of the purification. Lane M, molecular weight markers; lane 1, dialyzed sample after ammonium sulfate precipitation; lane 2, after free-flow isoelectric focusing; lane 3, after DEAE anion-exchange high-performance liquid chromatography; and lane 4, after size-exclusion chromatography. The gel was stained by silver staining.

sulting spectra gave the following information for the different compounds: (I) m/z431(100), MH^+; (II) m/z591(100), $M\text{-}H^-$; m/z593(100), MH^+; m/z431(61), $MH\text{-}h^+$; and m/z413(82), $MH\text{-}h\text{-}w^+$; (III) m/z 753(100), $M\text{-}H^-$; m/z591(11), $M\text{-}H\text{-}h^-$; m/z755(42), MH^+; m/z431(68), $MH\text{-}2h^+$; and m/z413(100), $MH\text{-}2h\text{-}w^+$; and (IV) m/z915(100), $M\text{-}H^-$; m/z939(6), MNa^+; m/z917(5), MH^+; m/z755(7), $MH\text{-}h^+$; m/z431(67), $MH\text{-}3h^+$; and m/z413(100), $MH\text{-}3h\text{-}w^+$. Compound I had a molecular mass of 430, which is the same as the aglycone nuatigenin. Compounds II, III, and IV differed by 162 mass units or multiples of 162 from each other, suggesting they each differed by one glucose molecule. Furthermore, compound IV differed from 26-DGA B by 146 mass units, suggesting that it was a nuatigenin with three linked glucose moieties. This was most likely the first major product of hydrolysis of 26-DGA B as the result of removal of L-rhamnose (M_r 146). Initial removal of L-rhamnose was more likely than removal of a terminal D-glucose, since the product of the latter, 26-DGA A, was hardly observed upon enzymatic hydrolysis of 26-DGA B. Furthermore, the least polar product from the hydrolysis of avenacosides A and B (Fig. 4A, lanes 7 and 8, R_f value 0.74) had a lower R_f value than the aglycone nuatigenin (Fig. 4A, lane 10; compound I, R_f value 0.93), suggesting that the glucose at position 26 was still present. Therefore, the fungal avenacoside-hydrolyzing enzyme only removed sugars from the chain at carbon 3 and not carbon 26. Under the conditions of these assays, none of the products of enzymatic hydrolysis of avenacosides A and B and 26-DGAs A and B showed antifungal activity in the TLC bioassays, either sprayed with the wheat-attacking isolate S212/80 (Fig. 4B) or with the oat-attacking isolate WAC 1293 (data not shown).

Purification of an avenacoside-hydrolyzing enzyme. An avenacoside-hydrolyzing enzyme was purified to homogeneity from 2 liters of filtrate of a 7-day-old culture of the oat-attacking isolate WAC 1293, as described in Materials and Methods. After free-flow isoelectric focusing, a single 26-DGA-hydrolyzing activity peak was found in the fractions with a pH value between 3.8 and 4.1. Pooled active fractions were subjected to DEAE anion-exchange chromatography. The avenacosidase activity bound to the column, which was equilibrated with 20 mM NaH_2PO_4/Na_2HPO_4, pH 6.2. Upon elution with a linear gradient of NaCl (0 to 0.4 M), the activity eluted in a single peak around 0.17 M NaCl. A final purification step was performed using a TSK G3000 SW XL HPLC gel filtration column, which resulted in elution of the activity as a single peak. The fraction with the highest activity contained one major protein with an estimated molecular weight of 110,000, as judged by SDS-PAGE analysis (Fig. 5). The purified protein from gel filtration chromatography was capable of removal of all the sugars from 26 DGAs A and B, indicating that it contained both α-L-rhamnosidase and β-D-glucosidase activity. Furthermore, it was also capable of hydrolyzing the sugar molecules at position C-3 of the bisdesmosidic avenacosides A and B (data not shown). Therefore, this enzyme was given the name avenacosidase. The pH optimum for β-D-glucosidase activity for the avenacosidase as determined using *p*NPG as substrate was found to be around pH 5.4.

DISCUSSION

The oat-attacking isolates of *S. avenae* described in this paper were able to detoxify the oat leaf saponins 26-DGAs A and B. Detoxification of these antifungal saponins may be a prerequisite for pathogenicity of *S. avenae* on oat leaves. Wheat-attacking isolates were unable to infect oat or to detoxify 26-DGAs A and B. However, it is likely that these isolates differed from the oat-attacking isolates in a number of other ways, in addition to the inability to detoxify 26-DGAs, since they appeared to be taxonomically distinct from the oat-attacking isolates. Thus, the inability to infect oat need not be associated with the apparent inability to detoxify the foliar oat leaf saponins. Further identification of the different isolates in our collection is required. Ribosomal DNA sequence analysis may help to resolve the relatedness between the different *Septoria* isolates (1).

The results presented here confirm that 26-DGAs A and B are the major antifungal compounds present in oat leaves. In healthy oat leaves, only the inactive avenacosides are present and are contained in the vacuoles of the leaf cells (16,19). Activation of these bisdesmosidic saponins requires the activity of the oat enzyme avenacosidase, which is localized in leaf plastids (8,9,19). Since *S. avenae* is a necrotroph, it will cause disruption of cell integrity during infection allowing the oat avenacosidase enzyme to convert the avenacosides into 26-DGAs. Oat-attacking isolates of *S. avenae* were able to detoxify 26-DGAs by sequential hydrolysis of sugars from the C-3 sugar chain. The first sugar to be released seemed to be L-rhamnose, followed by the two (for 26-DGA A) or three (for 26-DGA B) glucose molecules. Removal of the L-rhamnose alone was adequate to give a substantial reduction in toxicity. It has been demonstrated for other saponins that the presence of a terminal rhamnose is associated with high antifungal activity (29). In addition, removal of the L-rhamnose molecule results in a loss of branching of the sugar chain. The presence of a branched sugar chain is believed to be important for haemolytic and antifungal activity for some saponins (28).

Because of the ability of the purified enzyme of *S. avenae* to hydrolyze both the bisdesmosidic avenacosides and the 26-DGAs, we proposed the name avenacosidase. The *S. avenae* avenacosidase seemed to hydrolyze only the sugar moieties at position C-3 of the saponin and not the glucose molecule at C-26. This specificity was observed before in a crude preparation containing saponin-hydrolases produced by the oat leaf pathogen *Drechslera avenae* (17). Conversely, the oat leaf avenacosidase enzyme only hydrolyzes the glucose at position C-26 of the saponin and not C-3 (16). The purified fungal avenacosidase required both α-L-rhamnosidase and β-D-glucosidase activity for complete hydrolysis of the C-3 sugar chain of the saponins. Similar dual activities for saponin-hydrolyzing enzymes of fungal origin have been found before (26). The fungal avenacosidase enzyme was capable of hydrolyzing *p*NPG, but no activity towards the oat root saponin avenacin A-1 was detected (J. P. Wubben, M. J. Daniels, and A. E. Osbourn, *unpublished data*).

Two saponin-hydrolyzing enzymes of fungal origin have been purified before in our laboratory: avenacinase from *G. graminis* var. *avenae* and tomatinase of *S. lycopersici* (22). Both enzymes had a molecular weight around 110,000 and an isoelectric point around pH 4.6. Furthermore, avenacinase and tomatinase have considerable homology at the nucleotide level and for the predicted amino acid sequences (21). However, they differed in their substrate specificities. Avenacinase revealed hydrolytic activity towards avenacin A-1, whereas tomatinase specifically hydrolyzed α-tomatine. These two saponin-hydrolyzing enzymes are both β-glucosyl hydrolases, belonging to the glycosyl hydrolase family defined as family 3 by Henrissat (10) and Henrissat et al. (11). The *S. avenae* avenacosidase was purified by a similar procedure to that used for the purification of avenacinase and tomatinase. The fungal avenacosidase enzyme had a molecular weight around 110,000, as well, and an isoelectric point between pH 3.8 and 4.1. Whether or not the *S. avenae* saponin-hydrolyzing enzyme also belongs to family 3 glycosyl hydrolases remains to be determined.

The purification of avenacosidase from *S. avenae* should now allow a reverse genetic approach to be taken in order to clone the cognate gene. This requires amino acid sequence analysis of the purified enzyme or specific antibodies raised against the avenacosidase. Cloning of the gene will allow relatedness to avenacinase and tomatinase to be assessed. The cloned avenacosidase gene will also be used to generate vectors for use in transformation-mediated targeted gene disruption experiments. This will allow specific mutants of *S. avenae* to be generated that are defective

only in the avenacosidase production. The effects of this mutation on the ability to tolerate 26-DGAs and on pathogenicity to oat will be tested. For that purpose we have developed a transformation system for *S. avenae*, adapted from a method used for the transformation of *S. nodorum* (4).

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