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PATHOGENICITY OF 'TAKE-ALL' FUNGUS TO OATS: ITS RELATIONSHIP TO THE CONCENTRATION AND DETOXIFICATION OF THE FOUR AVENACINS

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Key Word Index—*Avena sativa*; Gramineae; *Gaeumannomyces graminis*; *Fusarium avenaceum*; 'take-all'; oats; avenacins; triterpenoids; detoxification.

Abstract—Data for inhibition of the growth of *Gaeumannomyces graminis* var. *tritici* (Ggt) and var. *avenae* (Gga), *Phialophora radicicola* and *Fusarium avenaceum*, caused by avenacins, are presented. The avenacins found in all oat species examined are sufficient in quantity to totally suppress growth of wheat 'take-all' (Ggt), even old roots containing 25 μg/g (fr. wt). Fungal variants that can also attack oats [var. *avenae* (Gga)] show considerable variations in their tolerance to avenacin A-1, EC_{50} values being 5–80 μg/ml. Nevertheless, all Gga isolates maintained some growth at avenacin A-1 concentrations as high as 200 μg/ml and it is this ability to grow, albeit slowly, at high concentrations that is the critical difference between Gga and Ggt strains. The pathogenicity towards oats of a range of isolates of Gga is related to the fungicidal activity of avenacins. Gga pathogenicity is shown to increase with poor nutrition of the oat hosts (poor illumination, lack of minerals). Fungal detoxification of avenacins produces mono-deglucosylavenacin A-1, bis-deglucosylavenacin A-1 and, in one case, tris-deglycosylavenacin A-1. Ggt strains left avenacin A-1 almost unaffected giving only traces of mono-deglucosyl product. Gga strains bring about mono- and bis-deglucosylation whilst *Fusarium avenaceum* causes mainly bis-deglucosylation. Mono-deglucosylavenacin is shown to be less inhibitory to Gga than is avenacin A-1, whilst the bis-deglucosyl compound is still less inhibitory.

INTRODUCTION

In the previous paper [1] it was shown that there were substantial differences in toxicity among the four avenacins towards the fungus *Gaeumannomyces graminis* var. *avenae* (Gga, isolate No. 5), those esterified with *N*-methylanthranilic acid being the more toxic. However, it was also found that there are large variations in the fungicidal activity of a pure avenacin towards various *G. graminis* isolates and investigation of this is an important objective of this paper. The fungal isolates employed in this and the previous paper [1] are listed in Table 1. Besides *G. graminis*, sources of *Fusarium* and *Phialophora* are included. Strains of the wheat fungus *G. graminis* var. *tritici* were used, but isolates of the var. *avenae* which attack oats were of special interest. *Gaeumannomyces graminis* is known to decline in virulence in prolonged axenic culture. Isolate 6 was maintained by repeated subculturing on potato dextrose agar (PDA) whereas isolates 7–9 were passaged through oats and re-isolated as mycelium from surface-sterilized root pieces. A number of single (10–16) and mixed (17–20) ascospores were recovered from perithecia formed on the lowest internode of infected oat plants, and were included along with Gga derived from spring oats and *Agrostis palustris*. Several of the latter proved particularly virulent.

RESULTS AND DISCUSSION

Collected together in Table 2 are data for 50% (EC_{50}) and 100% (EC_{100}) inhibition of growth on potato dextrose medium of a number of the fungal species and isolates listed in Table 1. Data are complete for the important avenacin A-1 and a few observations on other avenacins are shown, to give some impression of the overall picture. The cultures of *Gaeumannomyces graminis* have a considerable range of sensitivity to avenacin A-1. The two *tritici* (Ggt) isolates (Nos 3 and 4 of Table 2), which attack wheat but not oats, are inhibited in growth by low concentrations of avenacin A-1, whilst the *avena* (Gga) strains are much more tolerant. The pathogenicity of isolate No. 5 towards oats was moderate to weak in early experiments, producing stem blackening and perithecia, but after repeated subculturing (isolate 6) the virulence declined further and produced no disease symptoms (Tables 3 and 4). This is reflected in Table 2 where the ability to tolerate avenacin A-1 has fallen from an original EC_{50} value of 15 μg/ml to only 5 μg/ml. However, despite this adverse change in EC_{50} value isolate 6 continued to grow slowly on high avenacin A-1 concentrations and EC_{100} was > 200 μg/ml. The EC_{100} value may be compared with that for the two Ggt entries (Nos 3 and 4) where the EC_{100} is 25 μg/ml. This ability to grow slowly despite high adverse avenacin concentrations (a similar effect applies to the avenacins other than A-1) would appear to be the critical difference between the Gga and Ggt strains. With lower avenacin concentrations the Gga strains show regular growth with the edge of the mycelium well-defined. With high concentrations growth becomes irregular, a few hyphae penetrating up to twice the distance beyond the main mycelial front: this is presumably an indicator of variability within the culture.

Table 1. List of fungal isolates

Isolate no.	Species	Host	Source
1	*Fusarium avenaceum* (Corda ex fries) Saccardo	*Dianthus* sp. roots	CMI 145562
2	*Fusarium avenaceum* (Corda ex fries) Saccardo	Oats	ATCC 36875
3	*Gaeumannomyces graminis* var. *tritici* Walker (Ggt)	Cereal roots	ATCC 28229
4	*Gaeumannomyces graminis* var. *tritici.*	Wheat	Isolated at Nottingham from infected wheat/ex MAFF Cambridge, 1984
5	*Gaeumannomyces graminis* var. *avenae* (Gga)	Oats	ATCC 15419
6	Gga as isolate 5, after repeated sub-culture on potato dextrose agar	Oats	Nottingham, 1984
7	Gga as isolate 6, passaged through oats and reisolated (Re I-1)	Oats	Nottingham, 1984
8	Gga as isolate 6, passaged twice through oats and reisolated (ReI-2/1)	Oats	Nottingham, 1984
9	Gga as isolate 6, passaged twice through oats and reisolated (ReI-2/2)	Oats	Nottingham, 1984
10–16	Gga as isolate 6, but reisolated as single ascospore cultures and initially designated ASA 1–7	Oats	Nottingham, 1984
17–20	Gga as isolate 6, but reisolated possibly as mixed ascospore cultures and initially designated ASM 1–4	Oats	Nottingham, 1984
21–23	Gga-1–3*	Spring oats	Dr. D. Hornby (ex B. C. Clifford, Wales, 1969)
24–27	Gga-5, 9, 6 and 7* respectively	*Agrostis palustris*	Dr. D. Hornby (ex P. T. W. Wong)
28	Gga-180†	Wheat roots	Dr. D. Hornby (ex P. T. W. Wong, S. Australia 1977)
29	*Phialophora radicicola* var. *graminicola* Deacon	Grass roots	CMI 187783

*Numbers from M. Holden and A. Hornby (1981) *Trans. Br. Mycol. Soc.* **77**, 107–118.
†Provisionally classified as Ggt until found to attack oats in the greenhouse (D. Hornby).

Table 2. Concentrations of avenacins causing 50% (EC_{50}) and 100% (EC_{100}) inhibition of growth after 6 days at 24–26° in potato dextrose medium

Fungus	Isolate no.*	EC_{50} (μg/ml)				EC_{100} (μg/ml)			
		A-1	A-2	B-1	B-2	A-1	A-2	B-1	B-2
Ggt	3	1.5	7	3	8	~ 25	50–100	~ 25	~ 100
Ggt	4	3	5	—	—	25	25–50	—	—
Gga	5	15	50	7	50	> 200	> 200	> 200	> 200
Gga	6	5	—	—	—	> 200	—	—	—
Gga	21	4.5	—	—	—	> 100	—	—	—
Gga	24	17	—	—	—	> 100	—	—	—
Gga	25	8.7	55	—	—	> 200	> 200	—	—
Gga	26	23	—	—	—	> 200	—	—	—
Gga	27	20	—	—	—	> 200	—	—	—
Gga	28	80	—	—	—	> 100	—	—	—
Phialophora radicicola	29	10	46	5	25	~ 50	> 100	~ 50	> 100
Fusarium avenaceum	2	> 200	> 200	> 200	> 200	> 200	> 200	> 200	> 200
Fusarium avenaceum	1	> 200	> 200	—	—	> 200	> 200	—	—

*Numbers in this column refer to those given in Table 1.

The vigour of the weakened isolate 6 could be increased by two passages through oats (see isolates 7, 8 and 9 in Tables 3 and 4). However measurements of growth on potato dextrose agar (PDA) showed no increased tolerance to avenacin A-1 (EC_{50} for isolates 6, 7 and 8 were 4.8, 4.4 and 4.8 μg/ml respectively) and factors other than the latter appear to be involved. Isolate 6 mycelium was tested for adaptation to avenacin A-1 by maintaining it on 50 μg/ml of avenacin A-1 in PDA for 4 weeks but at the end of this period there was no appreciable change in the EC_{50} value (initial 4.8; final 5.0 μg/ml).

Table 5 of the previous paper showed that the avenacins

Table 3. Pathogenicity of Gga (isolate 6) compared with pathogenicity of reisolated material after one or two passages through oats, and with ascospore isolates

Fungus	Isolate no.†	Shoot* Mean max. stem ht (cm)	Shoot* Fr. wt (g)	Shoot* Dry wt (g)	Shoot* Infection score‡ (mean)	Root* Fr. wt (g)	Root* Dry wt (g)	Root* Infection score§ (mean)
Gga	6	47.2	1.036	0.248	0.0	0.625	0.096	0
Gga (ReI-1)	7	48.2	1.079	0.273	0.0	0.554	0.103	0
Gga (ReI-2/1)	8	40.3	0.675	0.183	1.0	0.469	0.084	0
Gga (ReI-2/2)	9	36.2	0.451	0.193	2.2P	0.191	0.068	2P
Gga (single spore)	10	38.4	0.433	0.168	1.8P	0.595	0.061	1/2P
Gga (single spore)	11	45.8	0.801	0.201	0.1	0.580	0.096	0
Gga (single spore)	12	34.3	0.353	0.171	2.3P	0.154	0.056	2P
Gga (single spore)	13	36.0	0.291	0.109	2.0P	0.231	0.054	2P
Gga (single spore)	14	43.8	0.648	0.200	1.4P	0.346	0.069	1/2P
Gga (single spore)	15	43.2	0.695	0.196	2.0	0.555	0.103	0
Gga (single spore)	16	42.9	0.540	0.201	1.5	0.633	0.080	0
Gga (mixed spore)	17	39.4	0.513	0.174	1.4	0.293	0.050	1
Gga (mixed spore)	18	35.9	0.304	0.126	3.0P	0.140	0.044	2P
Gga (mixed spore)	19	47.4	1.063	0.254	0.0	0.584	0.094	0
Gga (mixed spore)	20	44.8	1.035	0.196	1.0	0.571	0.099	0
Phialophora	29	46.0	1.017	0.243	0.0	0.404	0.052	1

*Weights (g) and heights (cm) relate to oat plants after 90 days growth.
†See list of isolates (Table 1).
‡On a scale of 1–5, based on darkening at the base of the stem and the presence of perithecia (P).
§Scored 0 (healthy roots), 1 (slightly discoloured), 2 (badly discoloured), P (perithecia present on roots).

Table 4. Pathogenicity of Gga isolates compared with Phialophora and Ggt

Fungus	Isolate no.†	Shoot* Mean max. stem ht (cm)	Shoot* Fr. wt (g)	Shoot* Dry wt (g)	Shoot* Infection score‡ (mean)	Root* Fr. wt (g)	Root* Dry wt (g)	Root* Infection score§ (mean)
Gga	6	64	4.74	0.74	0	1.58	0.16	0
Gga (ReI-2/2)	9	51	1.48	0.58	4P	1.01	0.13	3P
Gga-1	21	64	4.23	0.68	0	1.52	0.16	0
Gga-2	22	63	3.04	0.62	1P	1.26	0.19	1
Gga-3	23	66	4.57	0.89	0/1	1.48	0.15	0/1
Gga-5	24	All dead			5P			3P
Gga-9	25	52	3.16	0.68	1	1.29	0.19	1
Gga-6	26	All dead			5P			3P
Gga-7	27	All dead			5P			3P
Gga-180	28	57	3.75	0.67	0	1.19	0.15	0
Ggt	4	65	4.79	0.75	0	1.93	0.18	0
Phialophora	29	56	3.11	0.56	0	0.89	0.11	1
Control	—	59.5	4.51	0.75	0	1.28	0.14	0

*Weights (g) and heights (cm) relate to oat plants after 90 days growth at 21°, 16 hr day (1000 lux fluorescent tubes with normal daylight from 9-11-84 to 11-2-85). Plants watered weekly with mineral salts.
†See list of isolates (Table 1).
‡On a scale of 1–5, see Table 3. P = perithecia present.
§On a scale of 1–3 with 3 indicating dark brown—dying: see Table 3.

were not toxic to *Fusarium avenaceum* up to 200 μg/ml of basic agar medium and the results in Table 2 of this paper using PDA medium and two fungal strains (isolates Nos 1 and 2) bear this out. *Phialophora radicola* (isolate 29) from grass roots shows an intermediate range of susceptibility between Ggt and Gga.

Our work on the relationship between avenacin content and pathogenicity of *Gaeumannomyces* required growth experiments using infected oats and these are summarized in Tables 3 and 4. The evaluation of the virulence of strains was also required in connection with the chemical detoxification work discussed later. Initial experiments

employed groups of young oat seedlings kept in contact with mycelial mats for 4 days, followed by growing on hydroponically. Root and shoot fresh and dry weights were measured after a total growing time of 9 days [2]. However, even when using highly pathogenic strains (isolates 21–28) no significant differences were found. The method was felt to be unsatisfactory and a longer term approach was substituted.

In this, oat seeds (Peniarth) were pre-germinated for 3 days on damp blotting paper and then planted 2 cm deep in sterile sand on top of a 6 mm agar disc cut from the edge of a growing fungal culture. Four seeds were planted per pot and two pots were included for each fungal isolate. Culture pots were maintained in a growth room at $19 \pm 1°$ (16 hr day, 2000 lux daylight fluorescent tubes) for 90 days. Pots were watered daily and fed at weekly intervals with half-strength Hoagland's solution. Once seeds had formed, fresh and dry weights of shoots and roots were measured and each plant was scored for stem base blackening and the presence or absence of perithecia (Table 3). Conditions for Table 4 were similar though in these experiments there was 1000 lux illumination and winter daylight.

In parallel experiments using sterilized compost in place of sand the effects of inoculation on oat growth proved much less severe, indicating that the growth medium significantly influences disease development: this has been reported previously by other authors [3]. Table 4, with data obtained using oats under good nutritional conditions, shows that isolates No. 24, 26 and 27 are highly virulent causing poor growth in a few weeks and finally death. Isolate 9, which is the weak isolate 6 passaged twice through oats, has greatly increased in virulence: others such as isolates 28 and 4 score zero on infection. Table 5 shows the same set of fungi as in Table 4 but on oats grown under poorer nutritional conditions (1000 lux light but no daylight, no provision of mineral salts). It is apparent that more severe infections have occurred, e.g. compare isolates 28 and 22 in Tables 4 and 5. In the inoculation test the Gga isolate 6, attenuated by subculture, was as virulent as isolate 9 which had been twice passaged through oats.

It was shown in the preceding paper that all types of oats tested contained all four avenacins in approximately the same proportions so that although *N*-methyl-anthranilate esters (A-1 and B-1) are appreciably more toxic than the benzoate esters (A-2 and B-2) this will cause little difference between oat varieties. The amounts of avenacins found in all the oats examined [1] were more than sufficient to suppress growth of wheat 'take-all' (Ggt): a rough estimate in old roots, where the concentration is low, gave a value of 25 μg/g of fresh root and even this would account for lack of pathogenicity towards oats.

The fungal variants (Gga) which attack oats (Tables 3–5) are found to vary considerably in their tolerance to avenacin A-1 with EC_{50} values ranging from 5 to 80 μg/ml (Table 2). However, all isolates proved able to maintain some growth on concentrations as high as 200 μg/ml. Our estimates indicate that levels of avenacin may be as high as 800 μg/g in fresh young root tips but decline rapidly with increasing age and distance from the tip. Growth of Gga on all but the youngest roots should therefore be possible.

The mechanism by which Gga resists the action of 'avenacin' was originally investigated by Turner [4] who found chromatographic evidence that tolerant fungi are able to bring about enzymic deglucosylation. Waiyaki and Schlösser [5] have extended this work, also using TLC, reporting that *Fusarium avenaceum* isolates which are pathogenic to oats contain a β-glucosidase and an α-arabinosidase active against 'avenacins', hydrolysing it to

Table 5. Pathogenicity of Gga, Ggt and Phialophora isolates under poor nutritional conditions

		Shoot†			Root†	
Fungus*	Isolate no.‡	Fr. wt (g)	Dry wt (g)	Infection score§ (mean)	Fr. wt (g)	Dry wt (g)
Gga	6	0.158	0.098	4P	0.075	0.023
Gga (ReI-2/2)	9	0.198	0.100	4P	0.890	0.293
Gga-1	21	0.378	0.098	0	2.36	1.23
Gga-2	22	0.147	0.067	4P	1.085	0.425
Gga-3	23	0.247	0.085	2	1.658	0.847
Gga-5	24	Dead		5P		
Gga-9	25	0.457	0.180	0	3.042	1.813
Gga-6	26	Dead		5P		
Gga-7	27	Dead		5P		
Gga-180	28	0.495	0.118	2	1.843	0.480
Ggt	4	0.278	0.073	0	1.550	0.353
Phialophora	29	0.375	0.085	0	1.802	0.738
Control	—	0.512	0.115	0	1.925	0.798

*Same fungi as in Table 4 but nutrition of oat plants now poor.

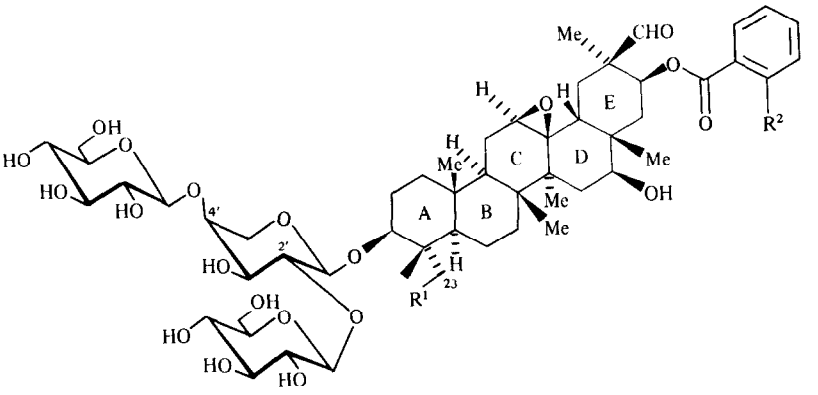
†Weights (g) relate to oat plants after 75 days growth at $19 \pm 1°$, 16 hr day (1000 lux fluorescent tubes): no addition of mineral salts.

‡See list of isolates (Table 1).

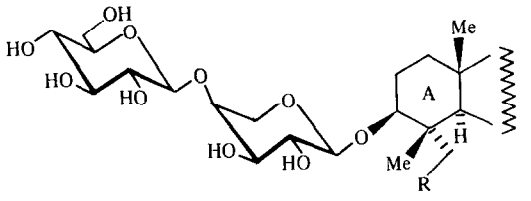
§On a scale of 1–5.

the aglycone. Waiyaki [2] also reports that Gga has β-glycosidase and α-arabinosidase activity giving the aglycone, whilst Ggt has β-glucosidase but no α-arabinosidase activity and removes two glucose residues. Identifications by TLC, though useful when compounds are of known structure and are well characterized, have their limitations and the question of enzymic detoxification was therefore re-examined.

In our work, fungi were grown in unshaken liquid culture on a standard oat medium until a mat of mycelium formed, then transferred after washing to an aqueous solution of the appropriate avenacin (usually the more toxic A-1). The medium was extracted and in large scale experiments the main products were isolated and characterized. In smaller scale experiments products were compared by TLC (usually two systems) and reversed-phase HPLC with the isolated pure compounds. The latter included the mono-deglucosyl-A-1, characterized by FAB-MS and spectroscopic data, though because of the small amounts available it has not been possible to state with certainty whether the 2′- or 4′-glucose residue has been removed (i.e. **5** or **6**). The bis-deglucosyl compound (**7**) was similarly characterized. On one occasion only, a trace of tris-deglycosyl-A-1 (i.e. loss of two glucoses and one arabinose) was detected and it was identified by comparison with the 12,13-epoxide (**9**) which we had earlier isolated as a very minor component from extractives of healthy oat roots [6–8]. A claim that *Fusarium avenaceum* and Gga degrades 'avenacin' to 'avenamine' [2] is highly unlikely to be correct. At the time that claim was made the nature and structures of neither 'avenacin' nor 'avenamine' were known. Apart from the possibility that a mixture was being employed under the term 'avenacin', it is probable that 'avenacin' can be broadly equated with our avenacin A-1, whilst the acid hydrolysis product 'avenamine' can be broadly equated with our avenestergenin A-1 (**8**). The latter has undergone an epoxy-ketone rearrangement due to the acidic conditions



1 Avenacin A-1 R^1 = OH, R^2 = NHMe
2 Avenacin A-2 R^1 = OH, R^2 = H
3 Avenacin B-1 R^1 = H, R^2 = NHMe
4 Avenacin B-2 R^1 = H, R^2 = H



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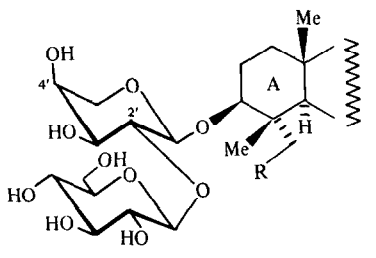

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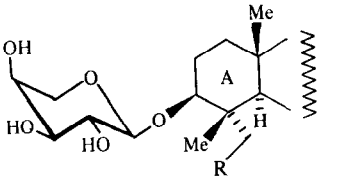

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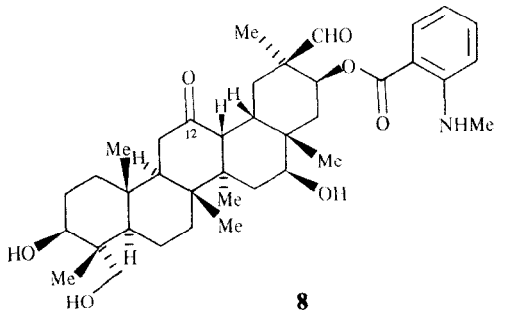

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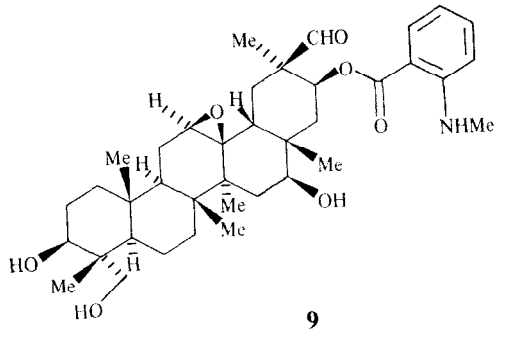

9

of hydrolysis [6–8]. Ketone **8** and epoxide **9** are in fact readily distinguished by TLC [R_f (**8**) 0.60, R_f (**9**) 0.73 silica, eluant hexane–ether, 3 : 1] or by C_{18}-reversed-phase chromatography [6–8]. The products from growth of a series of fungi on avenacins, usually A-1, are shown in Table 6 and these products also show significant differences from those claimed by Waiyaki *et al.* [2, 5].

Both *tritici* variants (Ggt) showed little ability to attack avenacin A-1, giving only traces of mono-deglucosylated material. Although they varied in their ability to degrade avenacin A-1, the major product using Gga isolates (a number of which had high pathogenicity) was almost always the mono-deglucosyl-compound (**5** or **6**) along with lesser amounts of the bis-deglucosyl-compound (**7**). Only on one occasion was the tris-deglycosyl-compound (**9**), mentioned above, detected. Our data do not therefore bear out the claims of Waiyaki referred to earlier [2]. The general deglucosylation activity of the Gga series is much greater than the Ggt. *Fusarium avenaceum* showed considerable ability to deglucosylate avenacin A-1 or B-1 (mainly to the bis-level) and this may be associated with the lack of toxicity of avenacins towards it. When grown on a mixture of avenacins A-1 and A-2 the latter avenacin disappeared more rapidly than the former, suggesting that ease of detoxification may account for the lower fungicidal activity of A-2 relative to A-1. There appears to be a general relationship between the ability of a fungus to attack oat roots and its ability to detoxify avenacin A-1. *Phialophora radicicola* occupies a position intermediate between Gga and Ggt producing a higher proportion of mono-deglucosylation product than Gga.

Using *Fusarium avenaceum* the work was extended from liquid culture studies to work on growing oat plants. *Fusarium avenaceum* attacked the roots of oat seedlings causing discoloured necrotic lesions. Roots were divided into infected and uninfected parts which were analysed separately by TLC as shown in Table 7. The avenacin A-1 degradation products appear to parallel closely those obtained when the fungus is grown in liquid culture on A-1. In an additional experiment avenacin A-1 was found to be present at 2.0 mg/g dry wt of healthy control root whilst in *Fusarium* infected root the corresponding value was 1.4 mg/g. Mono- and bisdeglucosyl-A1 (**5**/**6** and **7**) were absent from the control but were found at levels of 0.47 and 1.4 mg/g respectively in infected roots. The ratio of avenacin A-1 to avenacin A-2 in the controls was 2.2 : 1, but in infected material it was 2.6 : 1 which may be due to

Table 6. Metabolites of fungi grown on avenacins

Fungus	Isolate no.*	Avenacin	Concentration (μg/ml)	Products†
Gga	6	A1	67	Monodeglucosyl-A-1
		A1 + A2	119	Monodeglucosyl-A-1
Gga	21	A1	33	Mono- and bis-deglucosyl-A-1
Gga	22	A1	33	Mono- and bis-deglucosyl-A-1
Gga	23	A1	33	Mono- and bis-deglucosyl-A-1
Gga	24	A1	33	Mono- and bis- and tris-deglycosyl-A-1
Gga	25	A1	33	Mono- and bis-deglucosyl-A-1
Gga	27	A1	33	Mono- and bis-deglucosyl-A-1
Gga	28	A1	33	Unchanged
Ggt	3	A1	10	Mono-deglucosyl-A-1 (trace)
Ggt	4	A1	10	Mono-deglucosyl-A-1 (trace)
Phialophora radicicola	29	A1	26	Mono-deglucosyl-A-1
Fusarium avenaceum	1	A1	67	Mono- and bis-deglucosyl-A-1‡
		A1 + B1	63	Bis-deglucosyl-A-1 and B-1
		A1 + A2	145	Bis-deglucosyl-A-1
Fusarium avenaceum	2	A1	84	Bis-deglucosyl-A-1§

*For isolate numbers see Table 1.
†Recovered substrate obtained in all cases.
‡Major product is the bis.
§A trace of mono also observed.

Table 7. Fluorescent avenacins and their degradation products in oat roots showing varying amounts of infection by *Fusarium avenaceum*

Degree of infection	Dry wt (mg)	[R_f of fluorescent compounds] in $CHCl_3$–MeOH–H_2O (13:6:1)					in EtOAc	
Not visibly infected	77.3	0.30	0.38			0.90	0.0	0.73
Moderately infected*	40.6	0.30	0.38	0.63‡	0.80	0.90	0.0	0.73
Badly infected†	39.1	0.30	0.38	0.63‡	0.80	0.90	0.0	0.73
Uninfected control	164.5	0.30	0.38			0.90	0.0	0.73
Avenacin A-1		0.30					0.0	
Avenacin B-1			0.38				0.0	
Mono-deglucosyl-A-1				0.63			0.0	
Bis-deglucosyl-A-1					0.80		0.0	
Scopoletin						0.90		0.73

*Light brown.
†Dark brown.
‡In addition there was an unidentified spot at R_f 0.59.

preferential loss of avenacin A-2 as observed in the culture experiments.

In another experiment an isolate of Gga (isolate 27, Table 1), which caused severe infection and finally death of young oat seedlings, was placed in contact with sterile germinated oats. After 36 days the badly infected roots (few remained) were extracted: only a trace of avenacin A-1 and mono- and bis-deglucosylation products of A-1 (in small amount) could be detected. The control showed normal amounts of A-1 and no deglucosylation products. Infected roots from long-term inoculation experiments were also analysed. Here, avenacin content was low, as expected for old roots, but little in the way of deglycosylation products could be found.

Direct experimental evidence that deglucosylation of avenacin A-1 is a detoxification process is given in Table 8. Towards Gga the mono-deglucosylated compound **5** or **6** is less toxic than avenacin A-1 and in turn the bis-deglucosylated compound **7** is less toxic still. Avenestergenin A-1 (**8**) is virtually inactive, but we have had insufficient of the tris-deglycosylated compound, the 12,13-epoxide (**9**), for testing. In the *in vivo* situation the detoxification of avenacin A-1 may well be greater than indicated in Table 8 as the polarity and water solubility of the molecule is reduced on removal of sugars, and the effective antifungal concentration may thus be additionally diminished. There therefore seems to be considerable evidence that the ability of Gga isolates to attack oats is at least attributable in part to its enzymic ability to hydrolyse one or two glucose residues from toxic avenacins, thereby bringing about detoxification. *Fusarium* showed this ability to a still more marked degree and vigorously attacked oat roots, whilst the *tritici*-variant of *Gaeumannomyces graminis* (Ggt) had little if any ability to break down avenacins and could not attack oats. *Phialophora* showed restricted ability to attack oat roots and to detoxify avenacin A-1 by sugar hydrolysis. However it would be unwise to press these correlations too far. Our most virulent isolates, such as 24, 26 and 27, exhibited hydrolytic activity similar to isolates 21, 22 and 23 which were much less pathogenic in pot tests. Ability to detoxify avenacins by sugar hydrolysis, whilst of great importance, must be regarded as only one factor influencing the pathogenicity of Gga isolates.

EXPERIMENTAL

See also the preceding paper [1] for certain details.

Isolation of bis-deglucosylavenacin A-1 (cf. 7) by incubation of avenacin A-1 with Fusarium avenaceum. Twenty flasks each containing oat medium (25 ml) [from Quaker oats (15 g) in H_2O (100 ml)] were inoculated with *Fusarium avenaceum* (isolate 1 of Table 1) previously cultured on oat agar. These were maintained unshaken at 23–24° until the mycelial mat covered the bottom of each flask (10 days). Medium was then decanted and the mats were thoroughly washed with H_2O. Each washed mat was then

Table 8. Growth of Gga (isolate 6 of Table 1) on avenacin A-1 and its deglucosyl-products* after 3 days at 24–26°

μg/ml of medium	% Inhibition of growth 10	25	50	100	150
Avenacin A-1 (**1**)	58	—	67	71	—
Monodeglucosyl-Avenacin A-1 (**5**/**6**)	30	—	31	33	—
Bisdeglucosyl-Avenacin A-1 (**7**)	2	9	25	—	—
Avenestergenin A-1 (**8**)	—	—	—	—	0

*Compounds were dissolved in a minimum of dimethyl sulphoxide (DMSO) and appropriate blanks substracted (DMSO alone is very slightly inhibitory at the highest concentration used).

shaken with sterile H_2O (15 ml) containing 67 μg/ml of avenacin A-1. After two days the liquid was filtered through glass wool and the mycelial mats were washed successively with H_2O, MeOH and $CHCl_3$. The combined filtrates were evaporated at low temp. to yield a brown gummy residue (1.5 g). This was purified by prep. TLC on silica HF254 plates (20 × 20 cm), eluting with $CHCl_3$–MeOH–H_2O (13:6:1) and yielded two major and one minor band, all showing strong blue fluorescence in UV light. In a control experiment using boiled mycelium but treating as above, only recovered avenacin A-1 could be detected.

The band of R_f 0.3 corresponded to unchanged avenacin A-1. The weak band had R_f 0.44 and the new major band R_f 0.83. After recovery the latter band was further purified by C_{18} reversed phase HPLC on a C_{18} semi-preparative 7.8 mm i.d. steel column (Waters) eluting with 75% MeOH in H_2O at 2 ml/min. This yielded bis-deglucosylavenacin A-1 (**7**, 3 mg) as a white powder, mp 251–254° from MeOH–H_2O, R_t 24.6 min (avenacin A-1 under the same conditions had R_t 14.8 min). It had an FAB mass spectrum (positive ion, matrix glycerol/thioglycerol) giving an $[M+1]^+$ peak at 770.0 ($C_{43}H_{63}O_{11}N$ requires $[M]^+$ 769): the mass spectrum also showed a cleavage product at *m/z* 151 characteristic of the *N*-methylanthranilic acid residue. Compound **7** (in THF soln) had ν_{max} 3600 br (OH), 1693s and 1733s (ester and CHO, no six-membered ketone) cm^{-1}. ^1H NMR (pyridine-d_5 + D_2O): δ 10.18 (1H, *s*, CHO), 8.13 (1H, *dd*, *J* = 1.3, 8.2 Hz, aromatic), 7.55 (1H, complex *t*, aromatic), 6.76 (2H, *d*, *J* = 8.2 Hz, aromatic), 5.69 (1H, *dd*, *J* = 4, 12 Hz, ester H obscured in soln when D_2O present but apparent when absent), 5.03 (1H, *d*, *J* = 7.2 Hz, anomeric arabinose H), 4.66 (1H, *dd*, *J* = 4, 10.3 Hz, CHOH), 4.45 (1H, *t*, *J* = 7.2 Hz, CHO-arabinose), 4.32 (1H, *s*), 4.28–4.15 (4H, *m*, arabinose Hs), 3.78 (1H, *d*, *J* = 11 Hz), 3.64 (1H, *d*, *J* = 11 Hz, CH_2OH), 3.06 (1H, *br s*: without D_2O it appears at 2.92, *d*, *J* = 3.4 Hz, epoxide H), 2.94 (1H, *dd*, *J* = 4.3, 13.7 Hz), 2.83 (3H, *s*, NHMe) 2.35–1.47 (~ 13H, *m*), 1.46 (3H, *s*), 1.33 (3H, *s*), 1.25 (3H, *s*), 1.22 (3H, *s*), 0.88 (6H, *s*) (six triterpene methyls). In a similar spectrum without D_2O being present the NH signal is apparent: δ 6.22 (1H, *br d*, *J* = 5 Hz) and the NHMe signal at 2.83 (3H, *s*) appears as 2.72 (3H, *d*, *J* = 5 Hz).

A similar experiment with *F. avenaceum* (isolate 2), incubating 10 flasks as above, each containing 84 μg/ml of avenacin A-1, yielded recovered avenacin A-1 (1.8 mg) and bis-deglucosylavenacin A-1 (**7**, 1.2 mg). A third compound was isolated (0.8 mg), identified as mono-deglucosylavenacin (**5**/**6**) by comparison (R_f on silica 0.53, $CHCl_3$–MeOH–H_2O, 65:30:3), R_t in HPLC 26.8 min (72.5% MeOH at 2 ml/min) with a sample previously isolated in small amount from uninfected oats. Under the above conditions avenacin A-1 had R_f 0.22 and R_t 18.4 min.

Isolation of bis-deglucosylavenacin B-1 (cf. **7***) by incubation of avenacin B-1 with* Fusarium avenaceum. Using the method described above 15 flasks containing a soln of avenacin A-1/B-1 mixture (63% A-1, 37% B-1 by HPLC) (63 μg/ml) were shaken with *Fusarium avenaceum* (isolate 1). After 3 days products were isolated as before and chromatographed on silica plates to reveal six fluorescent bands. The two of lowest R_f corresponded to unchanged avenacin A-1 and B-1: two of intermediate R_f were very faint and the lower corresponded to the monodeglucoside compound of the A-1 series (cf. **5**/**6**). Two major bands had R_f values of 0.79 and 0.88 (silica, eluant $CHCl_3$–MeOH–H_2O, 65:30:3): the first co-chromatographed with bis-deglucosyl-avenacin A-1. The second band was purified by C_{18}-reversed phase chromatography eluting with 75% MeOH in H_2O/3 ml/min) and had R_t 20.4 min (for comparison the bis-compound of the A-1 series had R_t 15.2 min). Its identity as bis-deglucosylavenacin B-1 was confirmed by a mass spectrum in the positive ion FAB mode: $[M+1]^+$ = 754. Avenacin B-1 with two glucose units hydrolysed ($C_{43}H_{63}O_{10}N$) requires $[M]^+$ 753.

Incubation of Fusarium avenaceum *with avenacin A-2.* Using the method above 30 flasks containing a soln of avenacin A-1/A-2 mixture (50% A-1, 50% A-2 by HPLC) (145 μg/ml) was shaken with *Fusarium avenaceum* (isolate 1) and worked up as before. The usual major product, bis-deglucosylavenacin A-1 (cf. **7**) was readily isolated but careful separation and HPLC investigation indicated no sign of the corresponding A-2 compound. The experiment was repeated with similar results.

Incubation of Gga (isolate 6) with avenacin A-1. The fungus was shaken (4 days) with a soln (67 μg/ml) of avenacin A-1 using the method above (20 flasks). Work-up and prep. TLC on silica gave two bands showing bright blue fluorescence in UV light. The lower corresponded to unchanged avenacin A-1 whilst the upper co-chromatographed with mono-deglucosylavenacin A-1. Reversed phase chromatography (C_{18}-column, eluant 72.5% MeOH in H_2O at 2 ml/min) gave the pure mono-deglucosylated A-1 compound (1 mg). The positive ion FAB mass spectrum showed an $[M+1]^+$ molecular ion at 932 ($C_{49}H_{73}O_{16}N$ requires $[M]^+$ 931): the characteristic ion at *m/z* 151, confirming the *N*-methylanthranilate ester, was present. In the NMR the compound showed ^1H resonances (weak spectrum in pyridine-d_5) at δ 10.23 (1H, *s*, CHO), 8.2 (*d*) and 6.75 (*m*) (aromatics), 5.75 (*dd*, ester H), 4.75–4.1 (*m*), 3.1 (*dd*), 2.97 (1H, *d*, *J* = 3 Hz, epoxide H), 2.78 (*s*, NHMe), 1.47 (*s*), 1.32 (*s*), 1.31 (*s*), 1.18 (*s*), 1.03 (*s*) and 0.9 (*s*) (6 methyl groups). A similar large experiment provided material for evaluation of fungicidal activity.

Incubation of Gga (isolate 6) with avenacin A-2. The fungus was shaken (23 flasks, 4 days) with a soln of avenacin A-1 and A-2 (50% A-1, 50% A-2 by HPLC) (119 μg/ml) following the method above. Extraction and work up allowed isolation of unchanged avenacins, but the A-1:A-2 ratio was now 1:0. Mono-deglucosylavenacin A-1 was also isolated and its identity checked by a mass spectral FAB molecular weight: however, no similar compound corresponding to A-2 could be found.

Incubation of Gga (isolates 21–28) with avenacin A-1. Each isolate was shaken as before in avenacin A-1 soln (33 μg/ml; 7 flasks) and the products were examined by TLC. The results are summarized in Table 6 and the identification of a trace of the epoxide **9** containing no sugar residues is of particular interest (comparison with authentic specimen by R_f on silica with EtOAc as eluant: the ketone **8** was clearly distinguishable).

A large scale experiment using isolate 24 (38 flasks, avenacin A-1 100 μg/ml) gave unchanged avenacin R_t 8.8 min (C_{18} reversed phase HPLC, eluting with 72.5% MeOH in H_2O at 2 ml/min) (3.2 mg), mono-deglucosylavenacin A-1 (cf. **5**/**6**) R_t 13.9 min (2.4 mg), bis-deglucosylavenacin A-1 (cf. **7**) R_t 16.2 (0.2 mg) and tris-deglycosylavenacin A-1 (**9**) (trace).

Incubation of Ggt (isolate 3) with avenacin A-1. Seven flasks were incubated and a weak soln (10 μg/ml) of A-1 employed. After work up a strong band of unchanged avenacin A-1 was found on prep. TLC: there was only a very faint band corresponding to monodeglucosylavenacin A-1 and no sign of bis-compound.

Incubation of Phialophora radicicola *(isolate 29) with avenacin A-1.* Five flasks were incubated with avenacin A-1 soln (26 μg/ml). On work-up, much unchanged avenacin A-1 was detected. There were traces of the mono-deglucosylated compound (cf. **5**/**6**) and a very faint band corresponding to the bis (cf. **7**).

Isolation of compounds from oat roots infected with Fusarium avenaceum. Oat plants were grown in sterilized Hortag (Pan Products Ltd) a medium used for greenhouse shelves which we have found to be very suitable for extracting whole root systems cleanly with minimum damage. *Fusarium avenaceum* (isolate 2) was inoculated into Czapek-Dox medium and left to grow (17 days) until a thick fungal mat covered the surface. Sterilized oats (Peniarth), pregerminated for 4 days on damp blotting

paper, were placed on the surface and grown on for a further 8 days. They were then separated into (1) light-coloured apparently uninfected roots (fr. wt 39.1 mg), (2) moderately infected light brown roots (40.6 mg) and (3) strongly infected dark brown roots (77.3 mg). The control roots weighed 64.5 mg. Each material was extracted by grinding successively with 80% MeOH in H_2O (2 × 50 ml) then MeOH (2 × 50 ml). The extracts were evaporated to small vol. and examined by prep. TLC (Table 7). Bands from (2) and (3) were also combined and their components verified by separation by C_{18} reversed phase HPLC (eluting with 72.5% MeOH in H_2O).

Production of oat roots infected with Gga (e.g. using isolate 27). The fungus was grown on PDA until well-developed. Pots were filled with sand and sterilized by autoclaving, holes 2 cm deep made with a 7 mm diameter sterile glass rod and a 5 mm disc of fungus dropped in. Sterilized Peniarth oats, pregerminated on blotting paper for 48 hr, were placed in each hole on top of the inoculum block and the sand was gently tapped over. They were then grown on under greenhouse conditions for 36 days when severe infection had occurred. Roots from eight plants, all showing severe browning, were extracted, examined by TLC and compared with uninfected controls (see text).

Infection of oats with Gga isolates, Ggt and Phialophora. Oat seeds (Peniarth) were sterilized in 5% 'Domestos' hypochlorite soln for 30 min, and then pregerminated on damp blotting paper for 3 days. The germinated seeds were planted 2 cm deep in sterile sand on top of a 6 mm agar disc cut from the edge of a growing fungal culture. In the experiments of Table 3 four seeds were planted in each pot and two replicate pots were included for each fungal isolate. In the experiments of Table 4, three seeds were used. Culture pots used in Table 3 were maintained in a growth room at 19 ± 1° (16 hr day, 2000 lux daylight fluorescent tubes) for a total period of 90 days. Pots were watered daily and fed at weekly intervals with half-strength Hoagland's soln. For the data in Table 4, greenhouse temperatures were 21° with a 16 hr day under fluorescent tubes (1000 lux) in addition to normal daylight between 9-11-84 and 11-2-85. Conditions for Table 5 were as stated there.

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