Phytochemistry, Vol. 25, No. 9, pp. 2069–2073, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

DISTRIBUTION OF AVENACINS A-1, A-2, B-1 AND B-2 IN OAT ROOTS: THEIR FUNGICIDAL ACTIVITY TOWARDS 'TAKE-ALL' FUNGUS

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(*Received* 27 *November* 1985)

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

Abstract—Oat roots contain a group of four major *in situ* inhibitors of the 'take-all' fungus *Gaeumannomyces graminis*, avenacins A-1, A-2, B-1 and B-2: these are trisaccharide-bearing triterpenes esterified (A-1, B-1) with *N*-methylanthranilic acid or (A-2, B-2) benzoic acid. Tests using the more virulent var. *avenae*, which can attack oats as well as the more susceptible wheat, show the *N*-methylanthranilate esters to be considerably more fungicidal. Avenacin contents (0.22–1.0 mg/g dry weight) and composition (47–60% A-1, 5–7% B-1; 30–43% A-2, 3–6% B-2) for eleven species and varieties of oat roots (at 77 days) are recorded. Young roots of oats, *Avena sativa* (var. Peniarth) have a high content of A-1 (73%) relative to A-2 (14%) which gradually shifts to a more even distribution of A-1 (55%) to A-2 (44%) as the root ages. Total avenacins content of young (3 day) root tips is very high (12.8 mg/g dry wt) and can be estimated at ~8 μg/root tip: the remainder of the young root has 5 mg/g dry wt of avenacins. The nutritional status of the var. *avenae* fungus is important in determining its vulnerability to avenacins. The latter provide oat roots with little defence against *Fusarium* attack.

INTRODUCTION

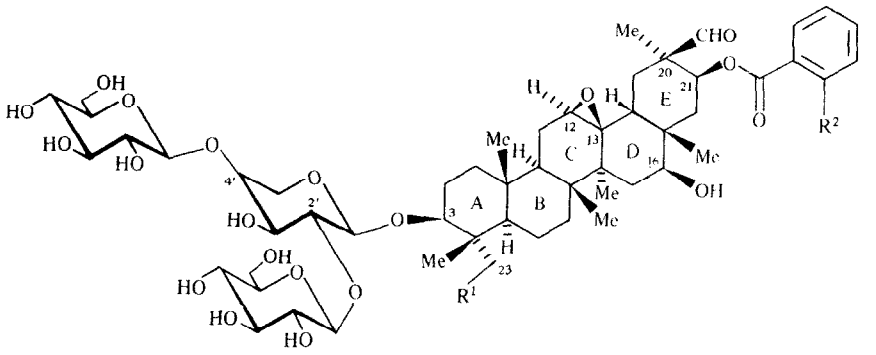
Along with 'eyespot' (*Pseudocercosporella herpotrichoides*) 'take-all' is one of the most destructive of the stem-base diseases of cereals. It is caused by the fungus *Gaeumannomyces graminis* (Sacc) Arx et Olivier (formerly *Ophiobolus graminis*) and is a widespread disease throughout the world, attacking mainly wheat where it infects the roots and tiller bases and is extremely difficult to eradicate with synthetic fungicides. Barley and rye are also attacked but oats are resistant to the fungal variant (var. *tritici* Walker, or Ggt for short) which usually attacks wheat. Since the fungus has low competitive ability and does not survive over long periods without a host, infected wheatland may be cleansed by planting oats. The biology and control of 'take-all' has been the subject of a recent monograph [1] and this should be consulted for details of these aspects. Our concern in this and the following paper [2] is with the distribution in the oat plant of antifungal resistance, fungal interactions, and their phytochemistry.

The resistance of oats was attributed by Goodwin and Pollock [3] to a fluorescent compound 'root-tip glycoside' different from the fluorescent scopoletin which also occurs in oat-roots. The distribution of this antifungal agent within the oat root system was studied by Turner [4] who confirmed the high root-tip concentration, as did Lüning and Schlösser [5] who used an assay based on haemolysis: they found similar haemolytic substances in a range of oat varieties [5]. Apart from demonstrating that oat root sap contained a non-diffusable compound which was toxic to Ggt, Turner [6, 7] made the important observation that a fungal variant existed which had the ability to attack oats (var. *avenae*, or Gga for short). The fungus was described in detail and shown to be less susceptible to oat root juice, apparently producing an enzyme capable of degrading the active materials to less toxic forms by release of sugar. Other authors [1] have confirmed this important difference between the two forms of *Gaeumannomyces graminis*.

The chemistry of the protective antifungal components of oat roots has been slow to develop. Maizel *et al.* [8, 9] isolated a fungicidal compound, avenacin, and recognized it as a triterpenoid glycoside esterified by *N*-methylanthranilic acid. Work was continued by Tschesche *et al.* [10] who revised the functionalities and made valuable advances on the nature of the carbohydrate, though the type of triterpene could not be discerned. This group also reported a second compound, claimed to be a disaccharide, with the CH_2OH of the avenacin structures replaced by methyl. These two substances are reported to be the most haemolytic saponins known [11].

Recently a detailed study of the isolation, chemistry and structures of the antifungal agents of oat roots [12–14] has shown that four major compounds, all having the same trisaccharide attachment, are present (Table 1). The structures of the four avenacins are **1**–**4***: the pair (A-1 and B-1) containing anthranilate residues are strongly fluorescent, but A-2 and B-2 lack this property. These compounds are pre-formed inhibitors [15, 16] and as such are distinguished from phytoalexins: a phytoalexin avenalumin I (**5**) [17] has however been identified as being produced from fungal infected oat-leaves, and avenacosides [18–20] from the same source are apparently also to be placed in this class [21, 22].

*It seems likely, from its description, that 'avenacin' of the earlier literature is the same, or very similar, to our avenacin A-1.



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 $R^1 = H$, $R^2 = H$

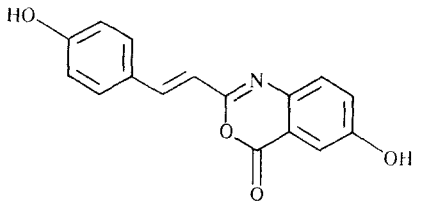


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Table 1. The avenacins of oat roots

	mp	Mol. formula (M)*
Avenacin A-1 (**1**)	228–233°	$C_{55}H_{83}O_{21}N$ (1093)
Avenacin A-2 (**2**)	237–239°	$C_{54}H_{80}O_{21}$ (1064)
Avenacin B-1 (**3**)	glass	$C_{55}H_{83}O_{20}N$ (1077)
Avenacin B-2 (**4**)	glass	$C_{54}H_{80}O_{20}$ (1048)

*From FAB-mass spectrometry.

RESULTS AND DISCUSSION

With the four pure avenacins **1**–**4** available, a more definitive study of their occurrence and role in oat plants under fungal attack has become possible. First, the distribution and content of avenacins within healthy *Avena* spp. was studied and the results are summarized in Table 2. Oat varieties and the related grass *Arrhenatherum elatius* were grown hydroponically under similar conditions, usually for 77 days, and then analysed for avenacins using C_{18}-reversed phase HPLC eluting with 75% methanol. All the oats tested contained the same four avenacins in approximately similar proportions: the 23-hydroxy compounds are the major components and *N*-methylanthranilates are formed rather more plentifully than benzoates. Actual amounts of avenacins varied from 0.22 mg/g dry weight of roots from spring oats at 77 days to 1.00 mg/g dry weight of roots from winter oats (entries 6 and 7). Entries 10 and 11, however, relate to 10-day-old material and it is clear that on a dry weight basis the avenacin content is much higher in young than in old roots (compare entries 9 and 10). The grass *Arrhenatherum elatius* contained only traces of avenacin A-1.

Table 3 shows the total amounts of avenacins and the total amounts of A-1 + B-1 and A-2 + B-2 for oats of various ages. These compounds require careful chromatography for separation and estimation of the components of the pairs but this has been done for the two subgroups and analysis of the four component mixture is given in this and other Tables. Dry oat seeds contain only traces of avenacin A-1 but at the very early stage of 3–4 days avenacins are maximal on a mg/g dry weight basis: ageing up to a 210-day period of growth shows a pattern of general decline in content of the avenacins. There is also a gradual shift in the composition of the avenacin mixture with age. Young roots have a high percentage of A-1 (73%) relative to A-2 (14%) which gradually shifts to a more even distribution of A-1 (50%) to A-2 (44%) as the root ages. There is also some decline in the B-1 level whilst B-2 remains steady as the most minor avenacin.

In Table 4 the total avenacin content of root tips (2–3 mm) from 3-day-old roots is found to be 12.76 mg/g dry weight (about 8 μg/tip). The remainder of the root analysed at 5.01 mg/g confirming the high concentration in the growing tip. The oat root tips also have a higher concentration of avenacin A-1 relative to A-2 (75:20) than is the case for the rest of the roots (59:34); B-1 and B-2 remain minor components.

Two fungi were tested for bioassaying the four avenacins. The first fungus *Fusarium avenaceum* grew well on a minimal agar medium but it was found (Table 5) that

Table 2. Nature and content of avenacins in the roots of *Avena* species and cultivars*

Species or cultivar	Age (days)	Wt (g) of dry roots	Wt (g) of MeOH ext.	% of dry wt	Avenacins (mg/g dry wt)			Avenacins % in mixture			
					A-1 + B-1	A-2 + B-2	Total	A-1 (1)	B-1 (3)	A-2 (2)	B-2 (4)
1. *A. abyssinica*	77	1.57	0.33	21.0	0.19	0.16	0.35	49	6	39	6
2. *A. brevis* var *turgida*	77	1.94	0.50	25.8	0.23	0.14	0.37	56	6	35	3
3. *A. byzantina* C. Koch	77	2.11	0.57	27.0	0.27	0.21	0.48	50	6	40	4
4. *A. fatua* L. (spring wild oat)	77	0.80	0.19	23.8	0.36	0.25	0.61	52	7	36	5
5. *A. sativa* L. (Ethiopia)	77	1.96	0.39	19.9	0.30	0.22	0.52	53	5	38	4
6. *A. sativa* fyne (spring)	77	1.64	0.40	24.4	0.14	0.08	0.22	57	6	34	3
7. *A. sativa* Maris Osprey (winter)	77	1.14	0.28	24.6	0.52	0.48	1.00	47	5	43	5
8. *A. Sativa* var nuda (Parkers huskless)	77	1.59	0.34	21.4	0.28	0.18	0.46	55	6	36	3
9. *A. sativa* (Peniarth)	77	1.83	0.41	22.4	0.31	0.21	0.52	55	5	37	3
10. *A. sativa* (Peniarth)	10	2.52	1.06	42.0	2.16	0.97	3.13	65	4	28	3
11. *A. sterilis* L.	10	0.07	0.01	14.0	1.65	1.35	3.00	53	2	44	1
12. *A. strigosa* Schreber (bristle or small oat)	77	1.51	0.34	22.5	0.51	0.27	0.78	59	6	32	3
13. *A. vaviloviana*	77	0.69	0.19	27.5	0.20	0.10	0.30	60	7	30	3
14. *Arrhenatherum elatius* (L.) Beauv ex J. & C. Presl	77	1.92	0.24	12.5	very faint trace of A1						

*Greenhouse grown during May–June: the amount of root varies considerably with the variety.

Table 3. Avenacin content of *Avena sativa* (var. Peniarth) roots of different ages

Age (days)	Root length (cm)	Dry wt (g)	MeOH ext. (g)	Avenacins (mg/g dry wt)			Avenacins % in mixture			
				A-1 + B-1	A-2 + B-2	Total	A-1	B-1	A-2	B-2
Dry seed	0	120	8.48	← Possibly slight trace of A-1 →						
3–4	2–3	0.94	0.48	4.3	0.96	5.26	73	10	14	3
10		2.52	1.06	2.16	0.97	3.13	65	4	28	3
12	Up to 15	1.76	1.26	1.60	0.75	2.35	60	6	28	3
28	>20	0.24	—	0.92	0.75	1.67	51	4	42	3
77		1.83	0.41	0.31	0.21	0.52	55	5	37	3
210		0.79	—	0.37	0.33	0.70	50	3	44	3

Table 4. Avenacin content of root tips and of roots with tips excised*

	Dry wt (g)	MeOH ext. (g)	Avenacins (mg/g dry wt)			Avenacins % in mixture			
			A-1 + B-1	A-2 + B-2	Total	A-1	B-1	A-2	B-2
Root tips	0.24	0.09	10.0	2.76	12.76	75	3	20	2
Remainder of roots	0.41	0.27	3.1	1.91	5.01	59	3	34	4
Total root	0.65	0.36	5.7	2.2	7.9	69	3	25	3

*Seedlings were 3 days old with roots 0.5–2 cm long; 158 seeds gave 384 tips since many seeds produced more than one root. Total avenacins/root tip ~8 μg.

Table 5. Growth of *Fusarium avenaceum* (isolate 1 of Table 1)* on minimal agar medium containing avenacins

μg/ml of medium	Mean diam. (mm) after 4 days growth at 24–26°				
	0	20	33	100	200
Avenacin A-1	28 (±1.0)	26.9 (±2.4)	27.8 (±1.8)	27.8 (±1.3)	25.4 (±0.9)
Avenacin A-2	28 (±1.0)	27.0 (±0.5)	27.2 (±2.3)	28.0 (±1.5)	27.2 (±0.8)
Avenacin B-1	28 (±1.0)	27.2 (±1.3)	29.5 (±2.5)	28.7 (±1.3)	27.2 (±1.2)
Avenacin B-2†	16.3 (±2.3)	14.3 (±1.2)	17.0 (±2.0)	16.2 (±1.2)	16.7 (±0.7)

*See preceding paper.
†Measured on a different occasion.

none of the four avenacins had any appreciable toxicity towards this particular fungus up to 200 μg/ml of medium. This is in agreement with reports that this fungus successfully colonizes oat roots [11, 22]. The second fungus, Gga, grew very poorly on this minimal medium (Table 6, entry 6) and all subsequent experiments were therefore carried out using potato dextrose agar. Comparison of entry 6 with entry 5 of the Table illustrates the marked effect of nutritional status and toxicity. Entries 1–4 shows that there are distinct differences in toxicity between the four pure avenacins: the *N*-methylanthranilate-containing A-1 and B-1 are considerably more effective in inhibiting growth of the Gga fungus than the two benzoate esters A-2 and B-2. The proportion of these two groups will therefore be of importance in determining the resistance of oat roots to fungal attack.

EXPERIMENTAL

Growth and analysis of oat roots for avenacins. The majority of the oat varieties were kindly provided by Dr. R. J. Giles of the Scottish Research Institute (Pentland field). Samples were grown on wire-mesh covered with muslin cloth with a supply of constantly aerated water beneath. After 11 weeks, the roots were excised, freeze-dried, ground and extracted twice with 80% MeOH in H_2O and then twice with MeOH. After filtration, the extracts were evaporated to dryness below 40° and redissolved in a small amount of MeOH. The soln was purified by chromatography on a dry silica column, eluting first with $CHCl_3$ and then with a mixture of $CHCl_3$–MeOH–H_2O (12.5:6.5:1). All fractions were monitored by TLC using silica and eluting with $CHCl_3$–MeOH–H_2O (13:6:1). The combined avenacin fractions were finally separated by C_{18}-reversed phase HPLC, eluting with

Table 6. Growth of *Gaeumannomyces graminis* var *avenae* (Gga) (isolate 5 from Table 1 of ref. [2]) on avenacins contained in potato dextrose agar medium

	Mean diameter (mm) after 4 days growth at 24–26°					
μg/ml of avenacin	0	25	50	100	150	200
1. Avenacin A-1	36.9 (±2.9)	9.5 (±3.0)	6.7 (±1.8)	4.0 (±1.5)	4.2 (±0.8)	3.0 (±0.3)
2. Avenacin A-2	36.9 (±2.9)	28.7 (±0.7)	17.2 (±1.2)	10.7 (±0.8)	6.3 (±0.8)	5.5 (±0.5)
3. Avenacin B-1	36.9 (±2.9)	14.0 (±1.5)	9.2 (±1.3)	5.8 (±1.7)	3.2 (±0.8)	3.0 (±1.5)
4. Avenacin B-2	36.9 (±2.9)	19.3 (±2.3)	16.5 (±1.0)	10.7 (±1.8)	8.0 (±1.0)	7.2 (±0.7)
5. Avenacin A-1	31.4 (±2.0)	18.7 (±0.7)	11.4 (±2.9)	5.5 (±1.5)	—	4.2 (±0.8)
6. Avenacin A-1*	15.5 (±0.5)	1.6 (±1.4)	1.7 (±1.3)	0.3 (±0.7)	—	0 (±0)

*Medium was basic agar in this experiment.

75% MeOH in H_2O. For quantitation, peak areas from a refractive index detector were compared with the responses of the four pure avenacins. Avenacin A-1 was also estimated after elution by absorbance measurement at the UV peak λ_{355} nm.

For young seedlings, oats (Peniarth) were grown on moist filter paper in trays covered with a glass sheet under normal light and temp.

Avenacin A-1, A-2, B-1 and B-2 standards. The pure avenacins were isolated as described elsewhere [12–14] (see Table 1). UV data useful for quantitation are: avenacin A-1: λ_{max}^{EtOH}: 223 (ε 25250), 255 (7900), 357 (5500) nm; avenacin A-2: λ_{max}^{EtOH}: 223 (ε 14700), 274 (1200), 281 (1100) nm; avenacin B-1: λ_{max}^{EtOH}: 223 (ε 23300), 253 (7900), 356 (5300) nm; avenacin B-2: λ_{max}^{EtOH}: 228 (ε 14600), 274 (1300), 281 (1250) nm.

Experiments on growth of fungi. Cultures were maintained on potato dextrose agar (PDA) medium and subcultured at intervals. For experiments with avenacins, Petri dishes (4.5 cm diameter) were filled with PDA medium (3 ml) to which the appropriate concentration of avenacin in sterile water had been added. At least three replicates were used for each concn. A 4 mm disc cut from the actively growing edge of a culture was placed centrally in each dish and growth was maintained at 24–26°. Radial growth measurements were made daily, taking the average of two diameters at right angles to each other. Results are reported as mean diameters in mm after subtraction of the original 4 mm inoculum.

Acknowledgements—We thank the A.F.R.C. for support, and Mr. L. J. Smith and Mr. A. Spibey for technical help. Out thanks are due to Dr. R. J. Giles for the provision of oat varieties.

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