METABOLITES CORRELATED WITH CEREAL CYST NEMATODE RESISTANCE IN OATS (*AVENA SATIVA*) IDENTIFIED USING SINGLE SEED DESCENT LINES

S. Bahraminejad[1,3], R.E. Asenstorfer[1], K.J. Williams[2], Y. Hayasaka[4], P.K. Zwer[2], I.T. Riley[1,2] and C.J. Schultz[1]*

[1] *School of Agriculture, Food and Wine, University of Adelaide, Glen Osmond, SA 5064, Australia*
[2] *South Australian Research and Development Institute, Glen Osmond, SA 5064, Australia*
[3] *School of Agriculture, University of Razi, Kermanshah, Iran*
[4] *The Australian Wine Research Institute, PO Box 197, Glen Osmond, SA 5064, Australia*

Summary. Secondary metabolites in plants are widely believed to be important for plant resistance to a range of pests and pathogens, but identifying key metabolites remains a challenge. To test whether the phenotypic extremes of a single seed descent (SSD) mapping population can be used to identify important metabolites, we analysed extracts from selected individuals of an SSD mapping population. The *Avena sativa* population was segregating for cereal cyst nematode (CCN), *Heterodera avenae*, resistance and resistance to the stem and bulb nematode, *Ditylenchus dipsaci*. CCN data were collected over three years, 2000, 2001 and 2002. Reversed-phase high performance liquid chromatography provided a profile of compounds from root extracts, allowing us to compare SSD individuals with high CCN count to individuals with a low CCN count. Several avenacins were positively identified by mass spectrometry, but were not correlated with CCN count. There was no significant correlation between flavonoids in oat roots and shoots with either CCN count or stem and bulb nematode resistance. However, three fractions recovered from root tips had a significant correlation with CCN count. Two of the fractions contained putative avenacins that have not been previously reported, whilst the third fraction contained a compound that was too unstable to characterise. This study highlights the potential of SSD populations for identification of bioactive compounds.

Key words: Avenacins, *Heterodera avenae*, *Ditylenchus dipsaci*, secondary metabolites, tolerance.

The cereal cyst nematode (CCN), *Heterodera avenae* Woll., is one of the most destructive root pathogens of cereals and is widely distributed in many cereal-growing areas. The grain yield losses in individual wheat fields in south-eastern Australia have been estimated to average 8% (Eastwood *et al.*, 1991) but can approach 100% in heavily infested fields (Bonfil *et al.*, 2004). The losses on intolerant barley were reported to be up to 30% (Kretschmer *et al.*, 1997).

Oats (*Avena sativa* L.) is one of eight major crops (Walsh *et al.*, 2003) producing in excess of 27 million tons annually worldwide (http://www.fao.org/). It suffers a variety of diseases and pests including CCN. Crops losses due to CCN can reach up to 81% in a heavily infested field when an intolerant cultivar is sown (Zwer *et al.*, 2005). Despite the intolerance of oats to CCN, growing an oat crop after wheat and barley crops is an effective way to reduce the population of root lesion nematode, *Pratylenchus thornei* Sher *et* Allen (Hollaway, 2002) and reduce the number of cysts in CCN-infested soils (Barry *et al.*, 1974).

Interactions between plants and nematodes are complex and controlled by multiple genes, as demonstrated by the segregation of tolerance and resistance in plant mapping populations (Safari *et al.*, 2005; Williams *et al.*, 2006). Resistance to nematodes is defined as the ability of the plant to inhibit and prevent the reproduction of nematodes whereas tolerance to nematodes is the capacity of the plant to withstand and grow and yield well in the presence of nematodes (Rathjen *et al.*, 1998).

Plants react to nematode invasion in several ways, including the expression of defence genes that lead to synthesis of proteins and secondary metabolites, and these responses are widely believed to provide plants with resistance (Huang and Barker, 1991; Oka *et al.*, 1997; Soriano *et al.*, 2004a). The resistance may be achieved by constitutive and/or induced levels of different phytochemicals. Methyl jasmonate (MJ) has been found to provide protection in oats against CCN and root lesion nematode (*Pratylenchus neglectus* Rensch) by reducing the number of invading nematodes and increasing plant mass (Soriano *et al.*, 2004b). This protective effect of MJ against CCN has been attributed to induced flavone-*C*-glycosides (FCG) (Soriano *et al.*, 2004b).

To search for constitutive compounds associated with nematode resistance (or susceptibility), we determined the concentrations of flavonoids and other metabolites from individuals of a single seed descent (SSD) population of oats. We hypothesised that by selecting individuals at the phenotypic extremes of the SSD population, it may be possible to analyse as few as

* Corresponding author e-mail: carolyn.schultz@adelaide.edu.au

twenty lines rather than the 100-200 lines that are found in most mapping populations. Three partially purified fractions from roots were found to have a significant negative correlation with CCN count.

MATERIALS AND METHODS

Plant material. A single-seed descent (SSD) population of 170 lines was generated by the oat Breeding Group, SARDI, Australia, from a cross between 'Potoroo' and 'Mortlock'. This population is segregating for several different disease resistance loci including resistance to CCN and stem nematode (SN), *Ditylenchus dipsaci* (Kühn) Filipjev, (unpublished data). 'Potoroo' is resistant and tolerant to CCN and 'Mortlock' is moderately susceptible and intolerant to CCN. 'Potoroo' is also moderately resistant and moderately tolerant to stem nematode, but 'Mortlock' is very susceptible and very intolerant to stem nematode (Wallwork, 2007). A laboratory-based bioassay (five inoculations of 100 J_2 at 3-day intervals) provided an indicator of CCN resistance, based on the number of white CCN females (CCN female count) developing on the outside of the root ball (Lewis *et al.*, 1999) in five replicates. For stem nematode resistance, the symptoms caused by the nematode (three inoculations of 100 J_4 at 4-day intervals) were scored from 0 (no symptoms) to 5 (dead plants) (Taylor and Szot, 2000) in five replicates. A number of SSD lines were selected from the population based on either high or low CCN female counts. CCN female counts (2000, 2001 and 2002 growing seasons) and SN resistance data (2001) were collected under natural conditions (Vanstone *et al.*, 2008) with supplementary irrigation in pots as part of an independent research project by John Lewis, SARDI, and were kindly provided by Dr Kevin Williams, SARDI.

Plant culture and maintenance. The seeds of SSD lines along with parental lines were sown in a complete randomised block design in three replicates in a growth chamber (22 °C light/18 °C dark, 12 h cycle). Eight seeds of each line were sown into a pot containing steam pasteurised (65 °C for 45 min) University of California potting mix and Osmocote® (Scotts, Australia), a slow release fertilizer. Plants received Hoagland's solution (Beltrano *et al.*, 1998) every other day and tap water on alternate days. After three weeks, plants were harvested. Shoots and roots were separated and frozen immediately in liquid nitrogen and stored at -80 °C until extraction.

Flavonoid extraction and assessment by HPLC. Samples stored at -80 °C were freeze-dried and ground to a fine powder using a coffee grinder (Breville, Australia). Sixty mg of ground sample was extracted with 10 ml methanol (HPLC grade) for 12 h by shaking on an orbital mixer at 130 rpm. Then, 3 ml milli-Q (Millipore, Billerica, MA USA) water was added to 7 ml of the methanolic extract and lipids were removed with 10 ml n-hexane mixed at 130 rpm for 12 h. Eight ml of the methanolic phase was evaporated in a vacuum concentrator (SpeedVac®, Savant Instruments Inc. Hicksville, NY, USA). The residues of shoot and root samples were dissolved in 0.4 and 0.2 ml 45% v/v methanol in water, respectively, and immediately analysed by HPLC.

Separation was achieved on a C18 column (Platinum EPSC18 100A, 7 × 53 mm; Alltech Associates Inc, Deerfield, Illinois, USA) by gradient elution, at a flow rate of 0.5 ml/min at 55 °C in Solvent A (2.5% formic acid), increasing from 0 to 40% Solvent B (1% formic acid: 60% acetonitrile: 39% water) over 18 min, then from 40 to 100% Solvent B over 3 min, and finishing with 100% Solvent B for 1 min. Compounds were monitored at both 254 and 340 nm (Bahraminejad *et al.*, 2008). Peak areas were compared with rutin standards (Sigma-Aldrich, St Louis, MO, USA).

Searching for metabolites associated with CCN resistance and/or susceptibility. Fifteen resistant and fifteen susceptible SSD lines were selected from the 170 SSD lines based on the CCN female counts in the 2000, 2001 and 2002 growing seasons (Table I). Forty seeds of each SSD line (in three replicates) were germinated in a misting cabinet on a mesh support. After five days the root tip of each main root was excised (approximately 0.7 cm) with a sterile surgical blade and transferred into a 1.5 ml microfuge tube containing 150 ml 50% methanol (HPLC grade) in batches of twenty for each line in each replicate. Samples were frozen in liquid nitrogen and then stored at -80 °C.

Metabolite extraction from oat roots and HPLC analysis. Each frozen batch of root tips was ground in a tube containing 200 µl 50% methanol in water using a plastic homogenizer. The material was pelleted by centrifugation and re-extracted with 4 × 250 µl 50% methanol. Each time the sample was centrifuged at 16,450 *g* for 5 min and the supernatant was collected and pooled. Samples were air-dried using a speedy vac and the final residue was dissolved in 45% methanol (80 µl) and centrifuged at high speed to remove any insoluble material.

Reversed-phase (RP)-HPLC was performed with a C-18 column (Sphersorb ODS-2, 5 µm, 4.6 mm × 150 mm; Waters, Milford, MA) using a gradient elution applied at a flow rate of 0.8 ml/min at 40 °C. Separation started with 95% Solvent A (water) and 5% Solvent B (acetonitrile), and followed with a gradient from 5 to 70% Solvent B over 17 minutes, and then increased to 100% over 1 min and held at 100% for 2 min. Finally buffer B was reduced to 5% over 1 min and the column re-equilibrated for 7 min. Compounds were monitored with UV detection at 223, 255 and 340 nm. The extraction and HPLC analysis for each SSD line was performed on the same day to minimise possible degradation. The mean and standard error of the data for each

line were calculated using Excel 2000. Genstat (Version 6.1: VSN International Ltd, Oxford, UK) was employed to determine the correlation between the areas under the peak for each line and the CCN female count. Significant peaks from HPLC separation were collected, accumulated, concentrated and subjected to mass spectrometric analysis.

Liquid chromatography-mass spectrometry (LC-MS). MS was carried out with an API-300 triple quadrupole mass spectrometer equipped with an electrospray (ES) ion source (MDS-Sciex, Concord, ON, Canada). Compounds in the active fractions of the crude extract were separated using a reversed-phase column (Synergi Hydro-RP, 4 mm, 150 × 2 mm; Phenomenex, Torrance, CA) with a flow rate of 200 µl/min and an injection volume of 20 µl in 50% methanol in water. Gradient elution (Solvent A: 2.5% formic acid in water; Solvent B: formic acid:acetonitrile:water (1:60:39 v/v/v)) was performed by increasing Solvent B from 0 to 20% over 10 min, then 20% to 60% over 8 min, followed by 60 to 100% over 7 min, held steady for 10 min and finally decreasing to 20% Solvent B over 1 min. The mass spectrometer was operated in either positive or negative ion scan mode and scanned from *m/z* 150 to *m/z* 2000 with a step size 0.2 Da and dwell time of 0.25 msec. The elec-

Table I. Cereal cyst nematode (CCN) white female count data used to identify extreme individuals in single seed descendent lines of oats.

Line #	CCN Count				Expt 1[1]	Expt 2[2]
	2000	2001	2002	Mean		
			High CCN count			
10	6.80	7.00	9.20	7.67	+	+
22	13.80	4.40	10.00	9.40	+	+
35	8.20	3.60	10.40	7.40	-	+
74	13.40	3.60	11.40	9.47	-	+
78	7.70	7.60	9.40	8.23	+	+
84	6.30	7.80	7.00	7.03	+	+
93	5.70	15.00	13.20	11.30	+	+
95	12.80	14.00	5.00	10.60	+	+
111	14.00	13.25	8.20	11.82	-	+
115	15.70	10.80	11.60	12.70	+	+
152	11.60	6.40	6.00	8.00	-	+
157	12.90	5.00	8.80	8.90	+	+
174	17.90	5.40	18.00	13.77	-	+
190	16.80	8.60	8.40	11.27	+	+
192	7.90	9.80	6.80	8.17	+	+
			Low CCN count			
11	0.00	0.00	0.60	0.20	+	-
13	0.40	0.00	0.00	0.14	+	+
15	0.10	0.00	0.20	0.10	+	±
20	0.00	0.00	0.50	0.17	+	+
23	0.00	0.00	2.80	0.93	+	-
28	0.00	0.00	0.25	0.08	+	+
29	0.10	0.00	0.00	0.03	+	+
30	0.30	0.00	0.20	0.17	+	+
34	1.50	0.00	1.25	0.92	+	-
47	0.00	0.00	0.00	0.00	-	+
80	0.10	0.00	0.20	0.10	-	+
83	0.10	0.00	0.00	0.03	-	+
92	0.00	0.00	0.00	0.00	-	+
108	0.00	0.40	1.00	0.47	+	-
114	0.00	0.00	0.00	0.00	-	±
116	0.30	0.00	0.00	0.10	-	+
125	0.00	0.00	0.00	0.00	-	+
126	0.00	0.00	0.40	0.13	-	+
189	0.10	0.00	0.40	0.17	-	+

[1] Experiment 1 examined FCG concentration in whole roots (Table II).
[2] Experiment 2 examined metabolites from root tips by RP-HPLC (Table IV and V).
+ Included in experiment; - Not included in experiment; ± Grown for Experiment 2, but not analysed due to high standard error and/or low recovery of all metabolites.

Table II. Concentrations of flavone-*C*-glycosides (FCGs) in the roots of single seed descendent lines of oats with different cereal cyst nematode (CCN) female counts, based on mg (rutin equiv.)/g dry weight.

Line #	Experiment 1 FCG data			
	FCG1	FCG2	FCG3	Total FCGs
	High CCN count			
10	0.0062	0.0299	0.0348	0.0709
22	0.0053	0.0224	0.0300	0.0577
78	0.0077	0.0356	0.0354	0.0787
84	0.0085	0.0342	0.0346	0.0773
93	0.0045	0.0336	0.0351	0.0732
95	0.0057	0.0281	0.0362	0.0700
115	0.0018	0.0201	0.0247	0.0466
157	0.0069	0.0214	0.0331	0.0614
190	0.0063	0.0266	0.0391	0.0720
192	0.0091	0.0326	0.0419	0.0836
	Low CCN count			
11	0.0052	0.0231	0.0309	0.0592
13	0.0089	0.0325	0.0429	0.0843
15	0.0075	0.0246	0.0294	0.0615
20	0.0056	0.0256	0.0349	0.0661
23	0.0092	0.0274	0.0337	0.0703
28	0.0075	0.0404	0.0391	0.0870
29	0.0031	0.0279	0.0379	0.0689
30	0.0073	0.0326	0.0346	0.0745
34	0.0041	0.0260	0.0250	0.0551
108	0.0042	0.0214	0.0334	0.0590
	Parental			
'Potoroo'	0.0101	0.0366	0.0502	0.0969
'Mortlock'	0.0066	0.0247	0.0314	0.0627

FCG1, luteolin-*C*-hexoside-*O*-pentoside; FCG2, apigenin-*C*-hexoside-*O*-pentoside;
FCG3, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside; nt, not tested.

trospray needle, orifice and ring potentials were set at 5000 V, 30 V and 250 V for positive mode and 4300 V, -30 V and 250 V for negative mode, respectively. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 12 units, respectively. The samples were monitored using a diode array detector (DAD, HP 1100; Agilent, Santa Clara, CA) at 254 nm and 340 nm. The mass spectral data was processed using Multiview (Version 1.2b3; PE-Sciex) or Analyst (Version 1.4; Applied Biosystems/MDS Sciex) software. Retention times and mass spectra were compared with avenacin A-1 and avenacin B-1 standards, provided by Anne Osbourn, John Innes Centre, UK.

RESULTS

Single seed descent mapping population segregating for CCN resistance. To test whether the phenotypic extremes of the 'Mortlock' × 'Potoroo' SSD population can be used to identify metabolites important for CCN resistance, individuals with high CCN counts and individuals with low CCN counts were selected (Table I). The most susceptible lines had mean CCN counts (over the 3 years) of between seven to thirteen females, whereas the most resistant individuals had CCN counts less than one female. In many of the susceptible lines, for example lines 22, 74, 157 and 174, the CCN count was lower in 2001 than in 2000 and 2002.

Flavone-C-glycoside concentration and nematode resistance. Flavonoids were extracted from the roots of the parental lines and twenty SSD lines chosen on the basis of extremes of CCN female count (Table II) and the shoots of twelve SSD lines chosen on the basis of extremes in stem nematode scores (Table III). For each sample, the concentrations of the three known flavonoids (Soriano *et al*., 2004a; Bahraminejad *et al*., 2008), luteolin-*C*-hexoside-*O*-pentoside (FCG1 at R_t = 10.3), apigenin-*C*-hexoside-*O*-pentoside (FCG2 at R_t = 10.8) and *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside (FCG3 at R_t = 11.1) were quantified, and they were always approximately 350-fold higher in the shoots (Table III) than in roots (Table II). The FCG data were compared with the CCN female counts for each of the three years, 2000, 2001 and 2002, and the means of counts in the three years. There was significant correlation neither between the concentration of FCGs in the roots and CCN female count nor between the concentration of FCGs in the shoots and resistance to stem nematode (data not shown).

Other metabolites and CCN resistance. To determine if other metabolites are involved in CCN resistance we extracted metabolites from the root tips of 30 SSD lines (Table I). Two out of 30 lines were removed from analysis due to high standard error and/or low recovery of all metabolites. Methanolic extracts were analysed by HPLC and 16 peaks were identified (Fig. 1A). The area under the peak for each of the peaks recognised by HPLC was determined for each of the SSD lines in three replicates. Peaks 1, 2 and 8 (data not shown) were ignored because they were consistent in both resistant and susceptible lines based on visual inspection.

Peaks 11 (R_t = 13.6 min; *m/z* 1065.8 (MH^+), 609.6 (MH^+-456.2); λ_{max} 232, 280 nm), 15 (R_t = 14.6 min; *m/z* 1094.8 (MH^+), 638.4 (MH^+-456.4); λ_{max} 255, 357 nm) and 16 (R_t = 15.3 min; *m/z* 1078.8 (MH^+), 622.8 (MH^+-456); λ_{max} 227, 255, 360 nm) were identified using standards and LC-MS as the saponins avenacin A-2, avenacin A-1 and avenacin B-1, respectively (Crombie *et al*., 1986; Begley *et al*., 1986). These were not correlated with CCN female counts.

Three of the peaks were significantly higher in the CCN resistant lines compared to the CCN susceptible lines, based on the mean CCN female counts (Tables IV and V). In contrast, no significant correlation was found between CCN tolerance and any of the peak areas (Table IV).

Table III. Concentrations of flavone-*C*-glycosides (FCGs) in the shoots of single seed descendent lines based on mg (rutin equiv.)/g dry weight and their stem nematode scores.

Selection number	Stem nematode score (2001)	FCG data			
		FCG1	FCG2	FCG3	Total FCGs
		Intermediate score			
10	1.75	2.81	11.73	9.56	24.10
23	2.25	3.39	11.19	11.51	26.09
30	0.75	3.07	11.68	10.79	25.54
35	2.25	2.75	9.73	11.43	23.91
47	1.50	2.96	9.67	11.53	24.16
73[1]	1.50	3.23	11.67	10.30	25.20
		High Score			
78	4.50	2.27	11.46	8.95	22.68
93	4.67	2.18	8.86	11.89	22.93
112	4.67	3.17	10.43	12.67	26.27
128[1]	5.00	3.50	9.79	11.73	25.02
134[1]	4.75	2.48	11.21	11.17	24.86
175[1]	4.75	3.66	11.37	9.50	24.53
		Parental			
'Potoroo'	nt	3.11	11.68	9.90	24.69 *
'Mortlock'	nt	2.89	9.63	11.06	23.58 *

[1] This line is not included in Table I because the CCN female count was not high or low (data not shown).
FCG1, luteolin-*C*-hexoside-*O*-pentoside; FCG2, apigenin-*C*-hexoside-*O*-pentoside;
FCG3, *O*-methyl-apigenin-*C*-deoxyhexoside-*O*-hexoside.
* difference is significant ($P<0.05$)
nt, not tested.

Table IV. Correlation of metabolites from oat roots with cereal cyst nematode (CCN) female counts. (Experiment 2)

Peak	CCN female count				Tolerance
	Mean	2000	2001	2002	2001
P3	**-0.474***	**-0.480****	-0.358 ns	**-0.443***	0.343 ns
P4	-0.106 ns	-0.155 ns	0.057 ns	-0.161 ns	-0.072 ns
P5	0.317 ns	0.268 ns	**0.382***	0.236 ns	-0.207 ns
P6	0.141 ns	0.041 ns	0.214 ns	0.162 ns	0.073 ns
P7	0.046 ns	-0.008 ns	0.109 ns	0.046 ns	0.164 ns
P9	-0.231 ns	-0.154 ns	-0.242 ns	-0.254 ns	-0.227 ns
P10	**-0.456***	**-0.476***	-0.361 ns	**-0.393***	-0.028 ns
P11 [A]	-0.283 ns	-0.179 ns	-0.320 ns	-0.303 ns	0.090 ns
P12	-0.015 ns	0.118 ns	-0.078 ns	-0.118 ns	0.094 ns
P13	**-0.450***	**-0.429***	-0.343 ns	**-0.450***	0.212 ns
P14	-0.034 ns	0.036 ns	0.013 ns	-0.151 ns	0.098 ns
P15 [B]	-0.078 ns	-0.068 ns	-0.055 ns	-0.090 ns	-0.127 ns
P16 [C]	0.003 ns	0.061 ns	-0.064 ns	-0.011 ns	0.255 ns

[A] avenacin A-2; [B] avenacin A-1; [C] avenacin B-1 (see Discussion).
ns,*, **, non-significant and significant at $P<0.05$, $P<0.01$ respectively. Bold data highlight significant correlations.
Data are based on 15 SSD lines with high CCN count and 13 SSD lines with low CCN count (see Tables I and V).

The significant differences between CCN resistant and susceptible lines for Peak 10 (R_t = 13.25 min) and Peak 13 (R_t = 4.03 min) are clearly shown for one of the three replicate samples for the resistant line 92 and the susceptible line 78 (Fig. 1B). The other peak that was significantly different was Peak 3, (R_t = 5 min) (data not shown). The data for these three peaks show a significant negative correlation for 2000 and 2002, but not 2001, and this is presumably due to the low level of invasion obtained in 2001 (Table I). Peak 5 (R_t = 7.1 min) had significant positive correlation with the data of 2001. However, this was not observed in the other years, nor in the mean of the three years, and therefore is unlikely to be real.

Peaks 3, 10 and 13 were separated by HPLC, collected and concentrated for further analysis by LC-MS.

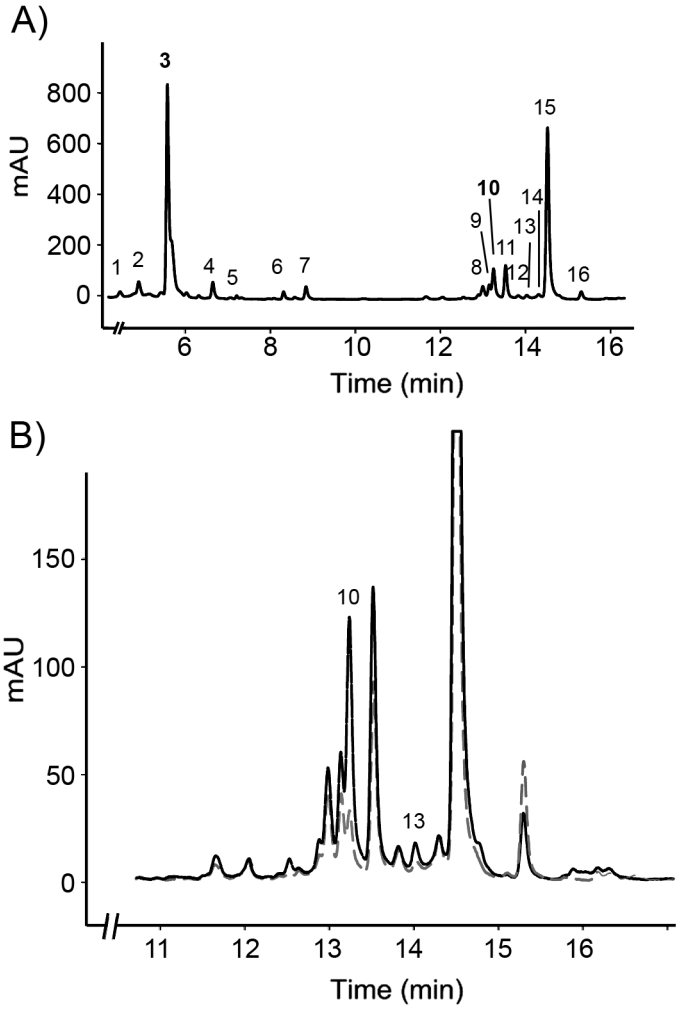

Fig. 1. HPLC chromatogram of methanolic extracts from oat root tips. A: example of the separation of extract from one replicate of 'Mortlock'. Bold numbers represent the peaks that were significantly different between CCN resistant lines and susceptible lines when data for 28 SSD lines were compared. B: a comparison of HPLC chromatograms of the CCN resistant line 92 (solid line) and CCN susceptible line 78 (dashed line). Peaks 10 and 13 were significantly different when data for all three replicates were compared (Table IV).

Based on the similarity of retention times with avenacin A-1 and avenacin B-1 standards, peaks 10 and 13 were probable saponins. The mass spectra of Peak 10 showed ions at *m/z* 1080.8 and 1043.8 for positive ion mode and *m/z* 1079.0 and 1041.8 for negative ion mode, suggesting that two compounds, with molecular masses of 1080 and 1042, coelute (Table VI). In addition, two ions, *m/z* 624.6 and 587.4, were observed in the positive ion mass spectrum, representing a loss of 456 Da (uncharged) from the parent ions of *m/z* 1080.8 and 1043.8, respectively (Table VI). A *m/z* 456 mass loss, or loss of two hexoses and one pentose (Table VI), would be similar to loss of the trisaccharide-glycoside from the avenacins A-1, A-2, B-1and B-2 resulting in their respective aglycone avenestergenins (Crombie *et al.*, 1986; Begley *et al.*, 1986). The ions *m/z* 1080.8 and 1043.8 (MH^+) and the putative aglycones *m/z* 624.6 and 587.4 (MH^+-456) represent saponins that have not previously been identified.

The negative ion mass spectrum of Peak 13 indicated an ion at *m/z* ($M\text{-}H^+$) 1026.0, but the equivalent ion (*m/z* 1028) was not detected in positive mode. Howev-

Table V. Average peak area ± standard error for significant HPLC peaks in Fig. 1.

Line #	Peak area		
	P3	P10	P13
	High CCN count		
10	3229±328	397±30	92±2.1
22	2776±253	313±25	84±1.9
35	4233±393	523±523	111±4.0
74	2672±171	541±26	119±2.4
78	2697±95	232±20	72±4.5
84	3059±113	560±10	122±5.3
93	4187±7321	430±7	93±3.5
95	2795±195	449±26	103±2.7
111	3334±232	272±24	81±5.3
115	2966±685	438±75	118±18.0
157	3655±612	507±20	128±1.8
190	3124±472	301±11	95±13
192	2488±533	524±32	121±5.8
152	4109±78	291±5	83±5.1
174	2225±162	447±14	87±1.1
	Low CCN count		
13	3307±274	710±7	134±0.8
20	3719±245	501±18	131±6.3
28	3937±693	477±28	120±4.1
29	5008±430	313±7	96±3.0
30	3694±450	676±17	130±1.6
47	4979±923	364±16	99±2.7
80	2732±436	613±29	123±10.8
83	2978±550	618±27	116±4.0
92	4269±501	662±17	124±4.7
116	6279±1125	504±17	123±2.9
125	3379±415	453±11	104±5.3
126	3085±54	548±9	106±2.0
189	3978±269	503±17	104±5.4

Table VI. Characterisation of two peaks negatively correlated with the number of white females of the cereal cyst nematode developing on the root ball of oat.

Peak	Compound	λ_{max} (nm)	$(M+H^+)$ Molecular ion (*m/z*)	$(M-H^+)$ Molecular ion (*m/z*)	$(M+H^+-456^A)$ Putative aglycone ion (*m/z*)	Molecular Weight
10	1	218, 247, 339	1080.8	1079	624.6	1080
	2	nd	1043.8	1041.8	587.4	1043
13	3	198, 256, 347	nd	1026.0	571.6	1027

[A]2×hexose + 1×pentose.
nd, not detected.

er, an ion *m/z* 571.6 was observed in positive mode, resulting from a loss of 456 Da from the expected molecular ion, MH^+ (Table VI). These data suggest that peak 13 also represents a saponin that has not been described previously.

It was not possible to characterise Peak 3 (R_t = 5.2 min) by mass spectrometry due to its instability.

DISCUSSION

The availability of a single seed descent population (SSD) segregating for CCN resistant and tolerance and stem nematode resistance provided an opportunity for identifying compounds that are important for resistance to or tolerance of nematode attack. Three HPLC fractions associated with CCN resistance were found in extracts made from oat roots (Fig. 1). These three fractions were shown to have significant negative correlation with CCN female counts by looking at 28 of 170 individual lines in the population (Table I). The area of each peak was correlated with CCN female counts in 2000, 2001 and 2002 (Table IV). The analysis of data in multiple years is important and strengthens these findings.

The choice of root tips for this part of the study probably contributed to the success with the limited sample size of 28 SDD lines. Root tips are the establishment and the feeding site of CCN (Mor *et al.*, 1992). It was possible to obtain root tips without any damage and contamination because the plants were grown in a misting cabinet suspended in air on mesh.

Peaks 3, 10 and 13 were the peaks negatively correlated with CCN female counts (Fig. 1). The compounds represented by these peaks were partially characterised by UV/Vis and mass spectrometry. Peak 3 (R_t = 5.2 min), with maximum absorption at 198, 221, and 278 nm, was the biggest peak among the significant peaks and showed a 25% increase in resistant lines when compared to susceptible lines. This compound was unstable even when stored at -20 °C and degraded into two smaller peaks in the HPLC chromatogram with similar retention times. It was not possible to further characterise peak 3 due to its instability.

Our data suggest that Peaks 10 and 13 are saponins. They have similar HPLC retention times to avenacin A-1 and avenacin B-1, their UV/vis spectra are similar to avenacin A-1 (λ_{max} 223, 255, 357 nm) and avenacin B-1 (λ_{max} 223, 255, 356 nm) (Begley *et al.*, 1986) and the mass spectrum with a neutral loss of *m/z* 456 suggests the loss of two hexoses and one pentose (Table VI), which is consistent with other saponins found in oats (Crombie *et al.*, 1986). Other peaks identified using standards and LC-MS as the saponins avenacin A-2 (Peak 11), avenacin A-1 (Peak 15) and avenacin B-1 (Peak 16) were not correlated with CCN female counts.

Analysis of flavonoids in this single seed descent population indicated that flavonoids are not major contributors to constitutive resistance to nematodes, based on analysis of whole roots. It is possible that flavonoids are not important in nematode resistance in cultivars 'Mortlock' or 'Potoroo' but are important in other cultivars such as "Quoll", where flavonoids have been reported as providing protection against nematodes (Soriano *et al.*, 2004a).

This study has identified three metabolites in oat roots that may play a role in nematode resistance. Further studies are needed to fully characterise these compounds and to determine whether they have individual and/or synergistic effects in functional tests.

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