Genetic variation of flavonoid defence compound concentrations in Oat (*Avena sativa* L.) entries and testing of their biological activity

**S Bahraminejad**¹,², **R E Asenstorfer**¹, **IT Riley**¹,³, **P Zwer**³, **C.J. Schultz**¹ and **O Schmidt**¹

¹ School of Agriculture and Wine, University of Adelaide, Glen Osmond, SA 5064, Australia  
² The College of Agriculture, Razi University, Kermanshah, Iran  
³ South Australian Research and Development Institute, Urrbrae, SA 5064, Australia

**Abstract.** Flavonoids are a diverse group of plant secondary metabolites that contribute to many biological functions including plant defence. We examined flavone-C-glycoside concentration in a number of oat entries and found significant variation. The biological activity of flavone-C-glycosides isolated from oat cv. Quoll was tested on fungal and bacterial growth. Although the crude extract inhibited all species of *Pyrenophora* except than *P. avenae* DAR 33699, this activity could not be attributed to the flavone-C-glycosides. The active antifungal compounds remain to be identified.

**Introduction**

Oats are resistant to a number of important crop diseases and are grown in crop rotation to limit the detrimental effects caused by the accumulation of pathogens (Soriano *et al.* 2004). For example, oats are resistance to stem rust (*Puccinia graminis*), crown rust (*Sebesta* *et al.* 1991), *Gaeumannomyces graminis* var. *tritici* (*Maizel* *et al.* 1963), barley yellow dwarf virus (*Gray* *et al.* 1993), *Helmenthosporium* sp. (*Sebesta* *et al.* 2001). Oats are effective in lowering the population of nematodes for following crops (*Hollaway* 2002) and altering the soil microflora to suppress pathogens such as *Verticillium* sp. (*Konagai* *et al.* 2005). To improve the resistance of oats to pathogens, an understanding of the mechanisms involved is necessary. Resistance of oats to disease is mostly due to two major groups of secondary metabolites, flavonoids and saponins (*Osbourn* 2003, *Soriano* *et al.* 2004).

Flavonoids are a large group of polyphenolic compounds and important secondary metabolites in plants (*Harborne* and *Williams* 2000). These polyphenolics provide many functions like induction of beneficial microorganisms (*Shimada* *et al.* 2000), plant protection against UV (*Estiarte* *et al.* 1999) and plant defence (*Dillon* *et al.* 1997, *Simmonds* 2001, *Soriano* *et al.* 2004, *Weidenborner* and *Jha* 1997). In oats, the phenylpropanoid pathway genes encoding phenylalanine ammonia lyase (PAL) and 4-coumarate CoA ligase are induced by powdery mildew, *Erysiphe graminis* (*Zhang* *et al.* 1997). *Soriano* *et al.* (2004) identified three oat flavonoids from both shoots and roots of oats, cv. Quoll that have anti-nematode activity. These flavonoids are all flavone-C-glycosides and were shown by mass spectrometry to be *O*-methyl-apigenin-C-hexoside-*O*-deoxyhexoside, apigenin-C-hexoside-*O*-pentoside and luteolin-C-hexoside-*O*-pentoside.

Flavone and flavone glycosides are known to inhibit pathogens and insects in other crops (*Lee* *et al.* 1998, *Weidenborner* and *Jha* 1997). The aims of this study were twofold, 1) to determine the genetic variation in of flavone-C-glycoside concentrations in the oat shoots and 2) to test crude extracts and fractions of the oat cv. Quoll for antimicrobial activity.
**Materials and Methods**

**Plant material and culture methods**

One hundred and fifteen entries of oats from 13 countries were screened in a pilot experiment. Plants were grown in steam pasteurised (65°C for 45 min) soil containing slow release fertilizer. Hoaglands solution and tap water were applied on alternate days. Twenty-one entries covering the range of constitutive level of flavonoids were selected from the pilot experiment. Four plants per pot were grown in a randomised block design (two replicates) in the glasshouse. Finally, five of the twenty-one entries were grown (three replicates, randomised block design) in a growth chamber (22/18°C, 12 h diurnal cycle).

**Extraction and High Performance Liquid Chromatography (HPLC) analyses**

Shoots were harvested 3 weeks after sowing and stored at -80°C. Samples were freeze-dried and finely ground. Extraction, analysis and quantification of flavone-C-glycosides were performed according to Soriano *et al.* (2004). Reverse-phase HPLC was performed with a C-18 column (Waters Sphersorb ODS-2, 4.6×150mm). Isocratic elution was achieved with methanol-water (55/45% v/v) mobile phase at 1 ml/min at room temperature monitored at UV 254 nm. Alternatively separation was achieved on a C18 column (Altech Platinum EPSC18 100A, 7×53mm) by gradient elution applied at a flow rate 0.5ml/min at 55°C with solvent A (2.5% formic acid) and solvent B (1% formic acid: 60% acetonitrile: 39% water), increasing solvent B from 0 to 40% solvent B over 18 min, from 40 to 100% solvent B over 3 min, steady at 100% for 1 min and then reducing to 0% over 3 min. Compounds were monitored at 254 and 340 nm. Rutin standards were used for quantification (Soriano *et al.* 2004).

**Pathogens and culture methods**

Eight fungi and four bacteria were used to test antimicrobial activity of oat extracts. *Fusarium graminearum, Rhizoctonia solani, Pyrenophora avenae* (DAR 33699 and DAR 32137), *P. teres f. teres, and P. teres f. maculata* were grown on potato dextrose agar until the mycelium half covered the plate. *Micosphaerella pinodes* was grown similarly but with a 12 h diurnal cycle and *P. tritici-repentis* was grown on V8 medium. 300 µl of 5-day-old broth cultures of bacteria were adjusted to 5×10⁸ cells/ml. Soft buffer agar was mixed with the suspension of bacteria and then poured over the Kings B medium for *Pseudomonas syringae pv. syringae, P. pisi* and M523 medium for *Rathayibacter tritici* and “Corynebacterium agropyri”.

**Bioassay for antimicrobial activity**

For the fungi, bioassays used autoclaved paper disc (6 mm diameter) loaded with 5 aliquots of 10 µl 45% crude methanolic extract cv. Quoll shoots that had been analysed by HPLC. Each loaded paper disc was placed at about 15 mm from the margin of mycelium and incubated at 25°C. The radius of inhibition was recorded. For the bacteria, loaded paper discs were placed on the inoculated soft buffer agar. Plates incubated at 25°C for about 3 days. The diameter of inhibition was recorded. Controls discs were treated with 45% methanol.

**High voltage paper electrophoresis (HVPE)**

Preparative HVPE was used to fractionate shoot extracts (300 µl). Separation was achieved using a 0.1 M borate buffer (pH 10) at 300 V for 45 min on cellulose paper (Soriano *et al.* 2004). Relative mobility (Rm) was compared with anionic orange G and neutral 2-deoxyadenosine standards. Bands were detected with UV light at 254 and 366 nm. Fractions were recovered by eluting with 45% methanol, dried and desalted before bioassays.
Results and Discussion
Genetic variation in flavone-C-glycosides concentration
We performed a pilot study of the flavonoids in shoot tissue from 115 oat entries. The 115 entries were chosen to include important cultivars in South Australian breeding programs (e.g., Quoll and Potoroo) and cover accessions from several countries and a broad spectrum of climates. As reported by Soriano et al. (2004), oat shoots contain a major peak at about $R_t=4.30$ min when monitored at 254 nm and these flavonoids were shown to be flavone-C-glycosides. In the pilot screening of 115 oat entries the level of flavone-C-glycosides ranged from 1.32 to 12.83 mg (rutin equiv.)/g dry weight (data not shown). These values are slightly higher than has been reported for flavone glycosides in durum wheat shoot [(1.26 mg/g dry weight, Cavaliere et al. 2005).

Twenty-one of the 115 entries were selected for further analysis to include entries with high, medium and low levels of flavonoids. The mean value of the replicates for each of the 21 entries was compared using Duncan’s test (Figure 1). Concentrations of 254 nm absorbance compounds varied from with 3.48 (cv. Amagalon) to 5.81 mg (rutin equiv.)/g dry weight (line IA91406-1).

![Figure 1. Constitutive flavone-C-glycoside concentration in oat shoots of 21 entries. Flavonoids were measured based on mg rutin equivalent / g dry weight. Means (n=2) compared by Duncan’s test, differences ($P<0.05$) indicated by different letters.](image)

Analysing the five entries grown in the growth chamber (Figure 2) was carried out by HPLC at 254 and 340 nm. Highly significant correlation ($r=0.975$) was found between data obtained from two measurements, 254 and 340 nm, demonstrating that both wavelengths can be used to estimate flavone-C-glycosides in the oat shoots. However, the measured flavonoid at 254 nm was much lower than 340 nm due to the differences in extinction coefficient at the respective wavelengths. Mean comparison ($P<0.05$) revealed that these entries could be classified in three groups. IA91406-1 had the highest level with 28.6 (rutin equiv.)/g dry weight, and Quoll had the lowest with 18.5 mg (rutin equiv.)/g dry weight (based on 340 nm).
Figure 2. Constitutive flavone-C-glycoside concentration in oat shoots. Samples were analysed by HPLC at 340 (□) and 254 (■) nm. Means (n=3) compared by Duncan’s test, differences (P<0.05) indicated by different letters and is the same for both wavelengths.

The difference in ranking of 91Qk195 between the experiments with 21 and 5 entries is possibly due to the effect of environment (Emmons and Peterson 2001). The range in the flavonoid concentration in oat shoots is potentially sufficient to be used in an oat-breeding program to improve cultivar resistance against nematodes (Soriano et al. 2004).

Bioassays for fungal and bacterial pathogens
The fungal and bacterial bioassay showed that the growth of all *Pyrenophora* species, except *P. avenae* DAR 33699, was inhibited by the crude oat shoot extract. The highly specific inhibition of crude extracts was not expected, however only a small number of pathogens were investigated. Inhibition of *P. teres f. teres* and *P. avenae* DAR 32137 by crude extract is shown in Figures 3a and 3b respectively. Other fungi and bacteria were not inhibited by the oat extract (data are not shown).

To determine if flavone-C-glycosides were responsible for the observed inhibition, an extract of oat cv. Quoll shoots was fractionated by HVPE. All fractions were analysed by HPLC (data not shown) and UV-Vis spectrometry. Analysis showed that fractions 4 and 6 had the highest level of flavone-C-glycosides (based on absorption spectrum and HPLC monitoring at 340 nm). Fractions had low or no inhibitory effect to *P. teres f. teres* (Table 1). In contrast, fractions 3b, 3a and 2b had clear inhibitory effect on this fungus. Although flavonoids are believed to be plant defence compounds (Soriano et al. 2004), these bioassays demonstrated that the flavone-C-glycosides from oat cv. Quoll had no antimicrobial effect against the bacteria and fungi tested. Further work is needed to determine the active compound(s) in the crude extract of oat shoots against *Pyrenophora* species. The other major group of secondary metabolites possibly conferring disease resistance are saponins. Future work will focus on identifying the antimicrobial compounds.
Figure 3. Effect of crude extract of oat shoots on a) *Pyrenophora teres* f. *teres* (Methanolic crude extract of the wheat shoot and water were applied against this fungus) and b) *P. avenae* (DAR32137) after two days. In a) water and methanolic extracts of wheat were tested for the efficacy of the experiment.

Table 1. Characteristics of fractions from oat cv. Quoll shoots fractionated by HVPE and assayed against *Pyrenophora teres* f. *teres*. Flavone-C-glycosides quantified at 340 nm.

<table>
<thead>
<tr>
<th>HVPE Fraction</th>
<th>Relative Mobility</th>
<th>flavone-C-glycosides mg (rutin equiv.)/ml</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;</th>
<th>Inhibition radius zone (mm +/- se)</th>
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<tr>
<td>1</td>
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<td>257.8</td>
<td>3.85 ± 0.27</td>
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<td>2a</td>
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<td>0.02</td>
<td>204.8, 272.6</td>
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<td>2b</td>
<td>0.16</td>
<td>0.025</td>
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<td>8.52 ± 1.25</td>
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<tr>
<td>3a</td>
<td>0.22</td>
<td>n.d.</td>
<td>207.8</td>
<td>9.83 ± 1.36</td>
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<tr>
<td>3b</td>
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<td>n.d.</td>
<td>206.1</td>
<td>11.50 ± 0.47</td>
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n.d., not detected

References


