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Characterisation of *Avena fatua* populations with resistance to multiple herbicides

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Summary

Avena fatua (wild oat) populations with resistance (R) to one or more herbicides have been described in numerous cropping systems worldwide. We previously reported that the R3 and R4 wild oat populations from Montana, USA, were resistant to four herbicides representing three different modes of action: tralkoxydim [acetyl-CoA carboxylase (ACCase] inhibitor), imazamethabenz and flucarbazone [acetolactate synthase (ALS) inhibitors] and difenzoquat (growth inhibitor). We now quantify resistance levels of these populations to triallate [very long chain fatty acid (VLCFA) biosynthesis inhibitor], pinoxaden (ACCase inhibitor) and paraquat (photosystem I inhibitor). Glasshouse dose–response experiments showed that, compared with the means of two susceptible (S) populations, the R3 and R4 populations were 17.5- and 18.1-fold more resistant to triallate, 3.6- and 3.7-fold more resistant to pinoxaden, respectively, and 3.2-fold (R3) more resistant to paraquat. Pre-treatment of R plants with the cytochrome P450 inhibitor malathion partially reversed the resistance phenotype for flucarbazone (both populations), imazamethabenz (R4), difenzoquat (R4) and pinoxaden (R3), but not for tralkoxydim, fenoxaprop-P-ethyl or triallate. Target site point mutations known to confer resistance to ALS or ACCase inhibitors were not detected via DNA sequencing and allele-specific PCR assays in R plants, suggesting the involvement of non-target site resistance mechanism(s) for these herbicides. Together, our results complete the initial characterisation of wild oat populations that are resistant to seven (R3) or six (R4) herbicides from five or four mode of action families respectively.

Keywords: weed management, plant stress response, agronomic weedy species, cytochrome P450 monooxygenase, xenobiotic

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Introduction

Herbicide resistance is a rapidly growing worldwide problem that causes significant crop yield losses, increases production costs and threatens our ability to successfully manage weed populations (Heap, 2014a). Resistance can be due to target site mutations that reduce herbicide binding or non-target site mechanisms (e.g. enhanced rates of herbicide metabolism or sequestration). Weedy species with resistance to multiple herbicides has been best studied in the Australian *Lolium rigidum* Gaudin (rigid ryegrass) populations that are resistant to 11 different modes of action (Yu & Powles, 2014) and the *Alopecurus myosuroides* Huds. (black grass) populations resistant to six modes of action (Délye *et al.*, 2011). In *L. rigidum*, a combination of target site mutations, reduced translocation and enhanced metabolism has apparently been

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introgressed into certain multiple herbicide resistant 'MHR' populations (Busi *et al.*, 2011) and, in both species, resistance is conferred by more than one gene (Letouze & Gasquez, 2001; Preston, 2003). In the USA, populations of another monocotyledonous species *Echinochloa phyllopogon* (Stapf.) Koss (syn *E. oryzicola* Vasing) are resistant to nine herbicides and resistance is correlated with induced expression of several cytochrome P450 monooxygenase (hereafter P450) genes (Iwakami *et al.*, 2014).

Preliminary evidence for enhanced herbicide metabolism in plants is traditionally based on reversal of the resistance phenotype by pre-treatment with P450 inhibitors such as malathion (Kreuz & Fonné-Pfister, 1992), tetcyclasis or piperonyl butoxide. These and similar experiments have led to more recent transcriptome surveys of resistant weeds (Duhoux & Délye, 2013; Gaines *et al.*, 2014), which have identified several P450s potentially involved in herbicide resistance.

Avena fatua L. (wild oat) is one of the ten worst annual weeds of temperate agricultural regions in the world (Holm *et al.*, 1991). *Avena fatua* and *Avena sterilis* L. populations that are resistant to one or more herbicides have been reported in many locations, including the USA (Kern *et al.*, 1996a), Canada (Beckie *et al.*, 2012), Latin America (Cruz-Hipolito *et al.*, 2011), Australia (Ahmad-Hamdani *et al.*, 2013) and elsewhere (Heap, 2014b). We recently reported that the R3 and R4 *A. fatua* populations from Montana, USA, were resistant to tralkoxydim, imazamethabenz, flucarbazone and difenzoquat (Lehnhoff *et al.*, 2013a,b). The objectives of the current studies were to further characterise the herbicide resistance spectra of R3 and R4 and to begin investigating potential physiological mechanism(s) of resistance.

Materials and methods

Population selection

The R3 and R4 populations were derived from seeds collected in 2006 from two *A. fatua* populations not controlled by 60 g a.i. ha^{-1} pinoxaden in two malt barley (*Hordeum vulgare* L.) production fields separated by *c.* 8 km in Teton County, Montana, USA. Field-collected seeds (about 90% of which were resistant to 60 g a.i. ha^{-1} pinoxaden, data not shown) were separately subjected to two generations of recurrent group selection (about 50 plants in each generation) by spraying with the same dose of pinoxaden (Axial XL, 50.3 g a.i. L^{-1}, EC, Syngenta; 60 g a.i. ha^{-1}) to remove susceptible (S) individuals and obtain the R3 and R4 populations. The S1 population was derived from seeds of untreated plants bordering an adjacent malt barley production field and was confirmed to be 100% susceptible in glasshouse screens to field use rates of all herbicides used in these studies (data not shown). A second susceptible population S2 is the non-dormant inbred SH430 line used in seed dormancy research (Johnson *et al.*, 1995).

Plant growth, herbicide treatment application and data analyses

All plants were grown in the glasshouse under a 16-hr photoperiod of natural sunlight supplemented with mercury vapour lamps (165 μmol m^{-2} s^{-1}) at 25 ± 4°C in standard glasshouse soil mix [1:1:1 (by vol) Bozeman silt loam: Sunshine Mix no. 1 (Sun Gro Horticulture, Bellevue, WA, USA):perlite]. Plants were treated with herbicide at either the 2.5- to 3-leaf stage (paraquat, imazamethabenz, flucarbazone, fenoxaprop-P-ethyl and tralkoxydim) or the 4-leaf stage (difenzoquat and pinoxaden) using a moving nozzle sprayer in 94 L ha^{-1} of water at 275.6 kPa. For biomass determinations, shoots were harvested 21 days after herbicide application, dried at 50°C for 7 days and dry weights recorded as g per plant. Dose–response analyses for difenzoquat, imazamethabenz, flucarbazone and tralkoxydim were presented in Lehnhoff *et al.* (2013a), and these herbicides were also included in the malathion reversal experiments. Paraquat (Gramoxone SL, 239.6 g a.i. L^{-1}, EC, Syngenta Crop) dose–response assays were conducted using doses of 0, 28, 84, 280, 840 and 1680 g a.i. ha^{-1} paraquat plus 0.25% (v/v) X-77 surfactant (Loveland Products Inc, Greeley, CO, USA). Pinoxaden dose–response assays were conducted using 0, 6, 18, 60, 180 and 360 g a.i. ha^{-1} pinoxaden. Both assays were conducted twice with four replications of each herbicide dose. Triallate (Far-GO, 479.2 g a.i. L^{-1}, EC, Gowan Co) dose–response assays were conducted as described in Kern *et al.* (1996b). Treatment solutions were applied in 1 μL droplets to the coleoptiles of uniformly sized, 3-day-old seedlings in Petri dishes and shoot lengths recorded 7 days after treatment (DAT). In the first set of experiments, triallate treatment solutions of 0, 50, 75, 100, 150, 200 and 250 mg a.i. L^{-1} were used. The second set of experiments was conducted using 0, 10, 25 and 35 mg a.i. L^{-1} for S plants and 0, 300, 350 and 400 mg a.i. L^{-1} for R plants. Both experimental sets were conducted twice with seven replications of each herbicide dose.

To determine the effect of malathion pre-treatment on herbicide efficacy, S1, S2, R3 and R4 plants were sprayed with malathion (Ortho Malathion 50; 500 g a.i. L^{-1}; EC, Chevron Chemical/Ortho; 1000 g a.i. ha^{-1}) plus 0.25% (v/v) X-77 surfactant either alone, or at the same dosage 3 h prior to herbicide application.

Control plants were treated with water containing surfactant only. Herbicides and treatment rates used in this study were as follows: imazamethabenz (ASSERT, 299.5 g a.i. L^{-1}, EC, Nufarm Inc; 500 g a.i. ha^{-1}) plus surfactant, flucarbazone (Everest, 70%WDG, WDG, Arysta LifeScience North America LLC; 30 g a.i. ha^{-1}) plus surfactant, tralkoxydim (Achieve Liquid, 400 g a.i. L^{-1}, EC, Dow AgroSciences LLC; 202 g a.i. ha^{-1}), difenzoquat (Avenge 200C, 200 g a.i. L^{-1}, EC, Amvac Chem Corp; 840 g a.i. ha-1), pinoxaden (60 g a.i. ha^{-1}), fenoxaprop-P-ethyl (Acclaim Extra, 68.3 g a.i. L^{-1}, EC, Bayer CropScience; 90 g a.i. ha^{-1}) and triallate. For all herbicides except triallate, doses were chosen that caused 50% visual injury to R plants (see Results/Dose–Response Assay section for paraquat, pinoxaden and triallate for the dose causing 50% injury (ED_{50}), and Lehnhoff *et al*., 2013a; for other herbicides.) Triallate ED_{50} doses of 7 and 125 mg a.i. L^{-1} for S and R plants, respectively, were used. For triallate treatments, malathion was applied at the above dose 3 h prior to triallate and shoot lengths were recorded as before. With the exception of pinoxaden, none of the formulated herbicides used in these studies contained safeners. There were four replications of each treatment and the experiment was conducted four times, except for the imazamethabenz and tralkoxydim treatments, which were conducted twice.

Data analysis

Data from experimental repeats of each herbicide dose–response assay were combined after ANOVA analysis showed that there were no interactions among rate, population and experimental repeat (pinoxaden, $F_{3,176}$ = 1.77, P = 0.15; Gramoxone, $F_{3,176}$ = 1.38, P = 0.25; triallate (first set of doses), $F_{3,376}$ = 1.24, P = 0.30; and triallate (second set of doses), $F_{3,152}$ = 1.12, P = 0.34). Both sets of triallate doses were further combined to analyse a complete data set. Similarly, the Fligner–Killeen test for homogeneity of variances showed that S1 and S2 dose–response data could be combined. Dose–response data were analysed using the drc package in R (R version 3.0.0, The R Foundation for Statistical Computing), and four-parameter Weibull models (for triallate and pinoxaden; Equation 1) and a three-parameter log logistic model (for paraquat; Equation 2) were fitted to the data as selected by the drc modelFit function.

$$y = c + (d - c)\exp\{-\exp[b(\log(x) - e)]\} \qquad (1)$$

$$y = \frac{d}{1 + \exp[b(\log(x) - \log(e))]} \qquad (2)$$

For both equations, y is the biomass response (dry weight for paraquat and pinoxaden, or shoot length for triallate), x is the dose, c and d are the lower and upper limits, respectively, b is the relative slope around e, and e is the ED_{50} (Streibig, 1988). Relative population differences in ED_{50} values for each herbicide (based on a t-statistic with $P \leq 0.05$) were compared using the selectivity indices (R/S ratio; Knezevic *et al*., 2007), which are the ratios between two ED_{50} values from dose–response curves.

Analysis of data from malathion/herbicide experiments used the response variable of percentage change of shoot length (for triallate) or plant biomass (all other herbicides). Square root-transformed data from experimental repeats with homogeneity of variances (Fligner–Killeen test) were combined, and ANOVA was used to determine differences in response to malathion for each population, with experimental replication included as a random effect. Tukey's honestly significant difference test was used to evaluate population–malathion differences. The herbicide susceptibility change for each herbicide and population after being treated with malathion was calculated as:

$$\frac{PC_{\text{without malathion}} - PC_{\text{with malathion}}}{PC_{\text{without malathion}}} \times 100 \qquad (3)$$

where PC = percentage change in plant biomass or shoot length (for triallate) relative to the control plants. Data analyses were performed in R.

ACC1 CT domain and ALS amplification and sequencing

For *ACC1* and *ALS* sequencing, genomic DNA was isolated from two plants of each R and S population using the DNeasy Plant Mini kit as recommended by the manufacturer (Qiagen, Germantown, MD, USA). In addition, total RNA was isolated from four plants of each population as previously described (Kern *et al*., 2005). cDNA was synthesised and contaminating DNA was removed in a reaction containing 2 μg total RNA, 1x SuperScript® RT buffer (40 mM Tris-HCl [pH 7.9], 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$) (Invitrogen, Carlsbad, CA, USA) and 2 U of RQ1 DNase (Promega Corp., Madison, WI, USA). The mixture was incubated at 37°C for 30 min and heat inactivated at 65°C for 10 min in the presence of 2 mM EGTA (pH 8.0). To test for DNA contamination, 1 μL of the DNase-treated RNA was subjected to PCR analysis under the conditions described below with primer pair Ta54227-F and Ta54227-R (Table 1). DNA-free RNA was reverse-transcribed with SuperScript® III Reverse Transcriptase (Invitrogen) using

Table 1 DNA sequencing and PCR primers used in these studies

Primer	5′–3′ Sequence	Source
Test for DNA contamination		
Ta54227-F	5′-CAAATACGCCATCAGGGAGAACATC-3′	Paolacci *et al.* (2009)
Ta54227-R	5′-CGCTGCCGAAACCACGAGAC-3′	Paolacci *et al.* (2009)
Amplification and sequencing of *ACC1*		
AFACCF16	5′-AACCCTGAACGTGGATTTAAGTAC-3′	Christoffers *et al.* (2002)
AFACCR5	5′-GTCTGCAACATCTGAGAGCAAC-3′	Christoffers *et al.* (2002)
AFACCR3	5′- CAACTGCATGTGGGAGCTG -3′	Christoffers *et al.* (2002)
ACC2F	5′-GACCAATAGACAGACCTGTTG-3′	This study
SQCT- a1R	5′-TCCTCTGACCTGAACTTGATCTC-3′	Liu *et al.* (2007)
WT-F1	5′-TTAGCTCTTCTGTTATAGCGCACA-3′	Liu *et al.* (2007)
HR-Ft1	5′-GATGGACTAGGTGTGGAGAACT-3′	Liu *et al.* (2007)
HR-FLeu1781	5′-GATGGACTAGGTGTGGAGAACC-3′	Beckie *et al.* (2012)
ACCWT1781	5′-GATGGACTAGGTGTGGAGAACA-3′	This study
HR-RCys1999	5′-TTGGTAGCTGAATCTGGAAAA3-3′	Liu *et al.,* 2007
ACCWT1999	5′-TTGGTAGCTGAATCTGGAAAC-3′	This study
HR-RCys2027M	5′-CCCACCAGAGAAGCCTCTG-3′	Beckie *et al.* (2012)
HR-Rcys2027	5′-CCCACCAGAGAAGCCTCTA-3′	Liu *et al.* (2007)
ACCWT2027	5′-CCCACCAGAGAAGCCTCTC-3′	This study
HR-RAsn2041	5′-TTGATCCAGCCTGCAGAT-3′	Liu *et al.* (2007)
Amplification and sequencing of *ALS*		
ALS1F_M	5′-CGCCGACATCCTCGTCGA-3′	Beckie *et al.* (2012)
ALS1B_M	5′-ATCTGCTGCTGGATGTCCTT-3′	Tan *et al.* (2007)
ALS2F_M	5′-CCTTCTTCCTCGCGTCCTC-3′	Tan *et al.* (2007)
ALS2B_M	5′-ACCTGGATAGCATATTGCGG-3′	Beckie *et al.* (2012)
ALS3F_M	5′-AGCAGAAGAGGGAGTTTCCTC-3′	Tan *et al.* (2007)
ALS3B_M	5′-CACCTTCCATGATAATGTCCTTAA-3′	Beckie *et al.* (2012)

2.5 μM oligo$(dT)_{20}$ primer (Integrated DNA Technologies, Coralville, IA, USA) following the manufacturer's protocol. PCRs contained 50 ng of either genomic DNA or cDNA, 200 nM each primer, 200 μM dNTPs, 2 mM $MgCl_2$, 1x Platinum® Taq buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl) and 1 U Platinum® Taq DNA polymerase (Invitrogen). An initial denaturation step at 94°C for 4 min was followed by 34 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 90 s. A final 72°C incubation for 7 min was performed, and the resulting PCR products were agarose gel purified (QIAEX II Gel Extraction Kit; QIAGEN) prior to sequencing.

PCR amplification of a 1236-bp region of the plastidic *ACC1* gene containing the carboxyltransferase (CT) domain was performed using forward and reverse primers AFACCF16 and AFACCR5, respectively (Christoffers *et al.*, 2002; Table 1), and the products directly sequenced (Molecular Research Core Facility, Idaho State University, Pocatello, ID, USA) using primers AFACCF16, AFACCR3 (Christoffers *et al.*, 2002), ACC2F or SQCT-α1R (Liu *et al.*, 2007; Table 1). Double peaks on sequencing chromatograms were scored as homologous polymorphisms as defined by Beckie *et al.* (2012). Sequences from each population were aligned to published *A. fatua ACC1* sequences using ClustalW2 software (Larkin *et al.*, 2007).

For *ALS* sequencing, primer pairs ALS1, ALS2 and ALS3 (Tan *et al.*, 2007; Beckie *et al.*, 2012; Table 1) were used to PCR amplify a 1701-bp region of *ALS* containing 22 possible point mutations known to confer herbicide resistance (Beckie *et al.*, 2012; Han *et al.*, 2012) and the PCR products sequenced using the *ALS* amplification primers.

The possibility of single nucleotide polymorphisms (SNPs) conferring ACCase inhibitor resistance was further examined using PCR assays with allele-specific primers. PCR amplifications were performed using 50 ng genomic DNA from two plants from each S and R population, with an initial denaturation at 95°C for 45 s, followed by 34 cycles of 95°C for 30 s, annealing at the temperatures listed below for 45 s, 72°C extension for 90 s and followed by a final extension at 72°C for 120 s. Primer pairs (Beckie *et al.*, 2012; Liu *et al.*, 2007; Table 1) targeting non-synonymous SNPs that create the following amino acid substitutions and their annealing temperatures were as follows: Ile1781Leu/Leu (63°C), Trp1999Cys/Ser (62°C), Trp2027Cys/Cys (64°C), Ile2041Asn (62°C), Asp2078Gly (65°C), Cys2088Arg (65°C) and Gly2096Ser (66°C). PCRs were repeated using wild-type primers (Table 1) to ensure that a lack of amplification with non-synonymous SNP primers was not due to high annealing temperatures. The Cys2088Arg PCR fragment was

digested with 10 U of *Hha*I and the resulting products visualised on a 1% agarose gel.

The *ACC1* and *ALS* partial gene sequences described above were also examined for resistance-conferring mutations from 401 and 109 contig assemblies, respectively, from six additional S1 and R4 plants subjected to RNA high-throughput sequencing (Supporting Information). Thus, the total number of plants subjected to DNA sequencing and allelic PCR screens were 22 S and 22 R plants for *ACCase* and 18 S and 18 R plants for *ALS* genes.

Results

Dose–response assays

Dose–response assays were conducted to compare R and S plant responses to triallate, paraquat and pinoxaden treatment (Fig. 1). For triallate, ED_{50} values for R3 and R4 seedlings were 123 and 127 mg a.i. L^{-1}, respectively, as compared with the S mean ED_{50} value of 7 mg a.i. L^{-1} (Fig. 1A). R3 and R4 ED_{50} values for paraquat were 331 and 156 g a.i. ha^{-1}, respectively, with the S mean value of 102 g a.i. ha^{-1} (Fig. 1B). For pinoxaden, R3 and R4 ED_{50} values were 61 and 63 g a.i. ha^{-1}, respectively, with the S mean value of 17 g a.i. ha^{-1} (Fig. 1C). These data were subsequently used to calculate R/S ratios, which for R3 and R4 were 17.5 and 18.1 (triallate), 3.2 and 1.5 (paraquat), and 3.6 and 3.7 (pinoxaden) respectively (Table 2). For all herbicide treatments except R4 plants treated with paraquat, ED_{50} values of R3 and R4 plants were significantly ($P \leq 0.05$) greater than the mean values of S plants.

Herbicide and P450 inhibitor treatments

S and R plants were treated with seven herbicides, both with and without malathion pre-treatment, to determine whether the inhibition of some P450 activities increased herbicide susceptibility (Table 3). Malathion treatment alone did not significantly ($P \leq 0.05$) alter S or R plant dry weights or shoot lengths (for triallate) (data not shown). Malathion did not significantly ($P \leq 0.05$) improve herbicide efficacy on S populations except for S2, where plant dry weight was decreased after flucarbazone treatment. Of the seven herbicide treatments shown in Table 3, malathion pre-treatment did not significantly ($P \leq 0.05$) alter the efficacy of imazamethabenz, tralkoxydim, difenzoquat, fenoxaprop-P-ethyl or triallate on either R population. For R4, malathion pre-treatment showed a trend of improved efficacy for imazamethabenz ($P = 0.064$) and difenzoquat ($P = 0.096$). Malathion pre-treatment also significantly ($P \leq 0.001$) improved the efficacy of flucarbazone treatment on R4

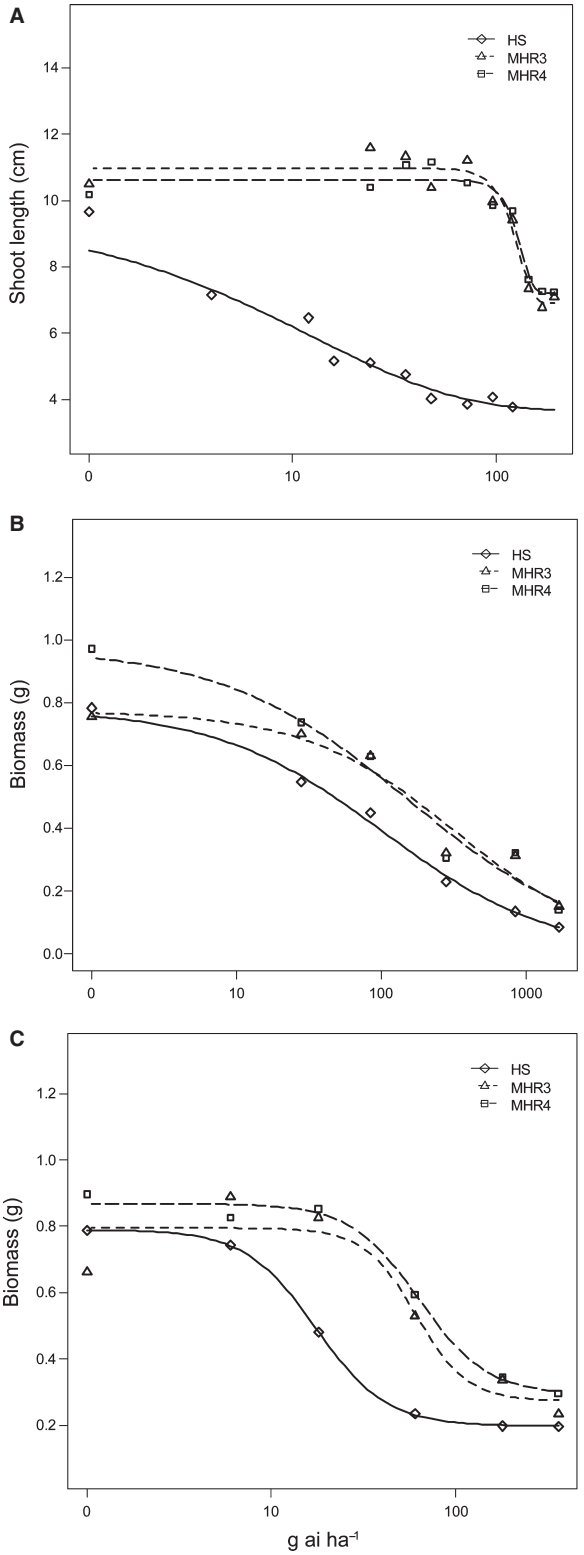

Fig. 1 Dose–response assays of herbicide resistant (R3, R4) and susceptible (HS) *Avena fatua* plants treated with (A) triallate, (B) paraquat or (C) pinoxaden. For triallate, 3-day-old seedlings were treated with the indicated doses and shoot lengths recorded 7 days after treatment (DAT). For paraquat and pinoxaden, 3-leaf stage plants were treated with the indicated doses and shoot dry weights recorded 21 DAT.

Table 2 Mean herbicide doses (ED_{50} in g a.i. ha^{-1} ± [SE]) required for 50% biomass or shoot length (for triallate) reduction and R/S ratios for herbicide susceptible (S) and herbicide resistant (R3 and R4) *Avena fatua* plants. ED_{50} and probability values (P) were calculated by the ED and selectivity index functions, respectively, in the R 3.0.0 (2013) drc package

Herbicide	S ED_{50}	R3 ED_{50}	*P*†	R/S‡	R4 ED_{50}	*P*§	R/S¶
Paraquat	102 (35)	331 (141)	<0.001	3.2	156 (64)	0.314	1.5
Triallate*	7 (1.2)	123 (4.7)	<0.001	17.5	127 (5.2)	<0.001	18.1
Pinoxaden	17 (2.7)	61 (14)	<0.001	3.6	63 (16)	<0.001	3.7

*Units for triallate are mg a.i. L^{-1}.
†*P* value indicates level of significance of ED_{50} differences between R3 and S biotypes.
‡Resistant (R3) to susceptible (S) ratio of dose required for ED_{50}.
§*P* values indicate level of significance of ED_{50} differences between R4 and S biotypes.
¶Resistant (R4) to susceptible (S) ratio of dose required for ED_{50}.

and showed a trend of improved efficacy on R3 ($P = 0.096$). R3 plants were visibly injured by malathion + flucarbazone (Fig. 2), but a proliferation of post-treatment tillering caused an increase in dry weight that compensated for initial plant injury. Pinoxaden efficacy was improved by malathion pre-treatment for R3 ($P = 0.003$) but not for R4.

Sequence analyses of A. fatua ACC1 CT domains

CT domains of *ACC1* genes from 22 S (GenBank KJ606969 [S1] and KJ606970 [S2]) and 22 R (KJ606971 [R3] and KJ606972 [R4]) plants were sequenced to determine whether they contain SNPs known to confer resistance to ACCase-inhibiting herbicides (Jang *et al.*, 2013; Supporting Information). All non-synonymous amino acid changes in the R populations were similarly detected in one or both of the S populations. Sequence homology comparisons of cDNAs confirmed that all three expressed homologous genes (*ACC1;1*, *ACC1;2* and *ACC1;3*) were detected (data not shown). Only S1 contained the *ACC1;1-1* allele at nucleotide 1149, while only S2 contained a silent SNP at nucleotide 1287 that is not found in the Canadian *A. fatua* populations described in Beckie *et al.* (2012) (Supporting Information).

The ACCase inhibitor resistance-conferring Trp1999Cys mutation was not detected in R plants by allele-specific PCR (Fig. 3), and additional assays using primers based on other resistance-conferring mutations did not detect their presence (data not shown). For the Cys2088Arg mutation, *Hha*I digestion showed that R plant PCR products did not contain the SNP creating a restriction enzyme recognition site (data not shown).

Sequence analyses of A. fatua ALS genes

Sequence analyses of 18 plants each of the R and S populations did not detect any of the 22 mutations known to confer resistance to ALS-inhibiting herbicides in S (GenBank KJ606973 [S1], KJ606974 [S2]) or R (KJ606975 [R3], KJ606976 [R4]) plants (Supporting Information). Although there were polymorphic differences among S and R populations, we found no unique sequences that have not been previously reported as being involved in resistance (Beckie *et al.*, 2012).

Discussion

We previously reported that the R3 and R4 populations are resistant to the ACCase inhibitor tralkoxydim, the growth inhibitor difenzoquat and the ALS inhibitors flucarbazone and imazamethabenz (Lehnhoff *et al.*, 2013a). Together with the herbicides used in the current studies, the resistance spectrum is now extended to seven (R3) and six (R4) herbicides from five and four mode of action families respectively. To our knowledge, this is the first report of *A. fatua* populations with resistance to this number of herbicides.

For most of the herbicides tested here, the current resistance spectra may represent selection by the herbicide groups that have been commonly used for *A. fatua* control in North America. Chronologically, triallate was widely used from about 1966 until the early 1990s and, to a lesser extent, difenzoquat was also used for *A. fatua* control through this period. Producers then adopted the ACCase inhibitor diclofop-methyl and other selective aryloxyphenoxypropionate and cyclohexanedione herbicides throughout the 1980s. Similar adoption of ALS inhibitors such as imazamethabenz and flucarbazone in 1988 and 1998, respectively, and the ACCase inhibitor pinoxaden in 2005, represent a chronological rotation among modes of action, and yet their sequential use apparently exerted sufficient selection for combined resistance to members of all of these herbicide groups. For pinoxaden, complaints about the lack of control in the first year

Table 3 Effect of herbicide and malathion + herbicide treatments on biomass or shoot length (triallate) response of herbicide sensitive (S1, S2) and herbicide resistant (R3, R4) *Avena fatua* populations†

		Biomass [% of untreated control ± (SE)]			
Herbicide	Population	Herbicide alone	Malathion + herbicide	Susceptibility increase (%)‡	*P*§
Imazamethabenz	S1	16.0 (2.0)	11.8 (2.5)	26.9	0.104
	S2	10.4 (0.9)	9.97 (1.6)	4.5	0.623
	R3	84.1 (8.3)	80.2 (5.8)	4.6	0.745
	R4	101 (8.0)	82.9 (3.0)	18.0	0.064*
Flucarbazone	S1	9.6 (1.4)	7.6 (0.9)	21.1	0.308
	S2	11.1 (1.8)	6.2 (1.2)	44.5	0.012**
	R3	73.7 (10.2)	54.5 (9.8)	26.0	0.096*
	R4	96.4 (7.4)	45.0 (8.1)	53.3	<0.001**
Tralkoxydim	S1	4.7 (0.6)	4.7 (0.9)	0	1.000
	S2	3.6 (1.8)	4.6 (1.0)	−30.6	1.000
	R3	88.8 (12.1)	107.5 (14.1)	−21.1	0.782
	R4	118 (11.7)	82.1 (12.6)	30.4	0.211
Difenzoquat	S1	24.7 (5.9)	24.0 (5.1)	2.8	1.000
	S2	24.6 (3.7)	27.2 (5.1)	−11.0	1.000
	R3	66.7 (4.2)	60.8 (4.9)	8.8	0.991
	R4	78.6 (7.5)	55.5 (4.4)	29.5	0.096*
Pinoxaden	S1	15.2 (1.5)	13.2 (1.7)	13.3	0.954
	S2	14.6 (1.5)	12.4 (1.6)	15.3	0.927
	R3	24.7 (2.9)	14.8 (1.9)	40.1	0.003**
	R4	19.7 (1.6)	15.0 (2.0)	23.9	0.341
Fenoxaprop-P-ethyl	S1	67.3 (10.3)	72.6 (9.8)	−7.9	0.999
	S2	51.7 (11.8)	78.0 (11.7)	−50.9	0.217
	R3	82.9 (10.5)	85.0 (10.9)	−2.5	0.999
	R4	80.9 (9.2)	90.4 (11.1)	−11.7	0.998
Triallate	S1	88.9 (3.6)	83.0 (1.9)	6.7	0.792
	S2	86.1 (3.5)	81.1 (3.4)	5.8	0.906
	R3	89.7 (4.2)	87.2 (1.7)	−0.6	1.000
	R4	92.2 (2.2)	85.4 (2.7)	7.3	0.660

†Plant biomass or shoot length (triallate) measured 21 or 7 days, respectively, after treatment with either malathion (3 h prior to herbicide treatment) plus herbicide or herbicide alone at the dosages listed in Material and Methods.
‡Susceptibility increase as a result of malathion pre-treatment ([percentage biomass without malathion – percentage biomass with malathion]/percentage biomass without malathion) × 100.
§Probability (**$P \leq 0.05$; *$P \leq 0.10$) of significant difference between percentage biomass values after herbicide alone and malathion + herbicide treatments according to Tukey's honestly significant difference test.

of its introduction in Montana indicated that previous selection pressure by other ACCase inhibitor herbicides may have led to resistance to this chemically unrelated compound. Specific annual herbicide use records for the fields where R3 and R4 originated are not available, but producers in this cropping region have used all of the above herbicides, except paraquat, for *A. fatua* control.

Triallate resistance levels of the R populations are similar to the level we reported 18 years ago for the triallate- and difenzoquat-resistant FG93R22 *A. fatua* population, which was collected in the same general area in Teton County MT (Kern *et al.*, 1996a). We showed that FG93R22 was deficient in triallate sulfoxidase activity, a pro-herbicide activation step required for toxicity (Kern *et al.*, 1996b, 1997), and that triallate resistance was controlled by two recessive nuclear genes (Kern *et al.*, 2002). Further, efflux studies of FG93R22 cell cultures showed that difenzoquat was excluded from the cytoplasm and irreversibly bound to cell wall components (Kern & Dyer, 1998). As FG93R22 and the R populations were collected from the same area, it is possible that the R triallate and difenzoquat resistance mechanism(s) are the same or similar to those we reported for FG93R22, an idea that is currently being investigated.

Malathion pre-treatment was used as a screen for P450-mediated enhanced herbicide metabolism rates. While the results must be considered preliminary, they indicate that metabolism may be involved in resistance to flucarbazone (both R populations), imazamethabenz (R4), difenzoquat (R4) and pinoxaden (R3), but not to tralkoxydim, fenoxaprop-P-ethyl or triallate. Beckie *et al.* (2012) reported a similar increase in the efficacy of flucarbazone and pinoxaden after malathion pre-treatment of five Canadian ACCase and ALS

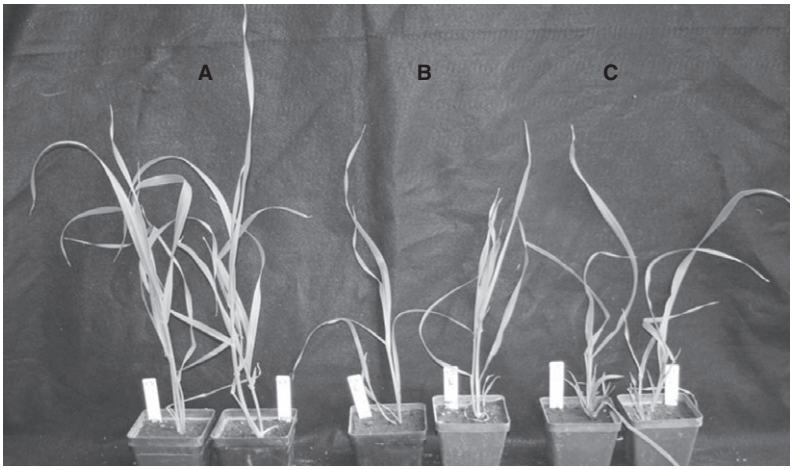

Fig. 2 Herbicide resistant (R3) plants 18 days after (A) no herbicide treatment, (B) treatment with 20 g a.i. ha^{-1} flucarbazone or (C) treatment with 1000 g a.i. ha^{-1} malathion followed 3 h later with 20 g a.i. ha^{-1} flucarbazone.

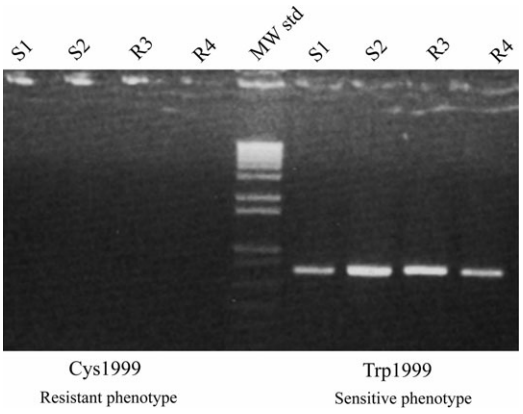

Fig. 3 Allele-specific PCR amplification from genomic DNA of herbicide sensitive (S) and herbicide resistant (R) *Avena fatua* plants of the *ACC1* locus at amino acid 1999 conferring herbicide sensitivity (Trp1999) or herbicide resistance (Cys1999).

inhibitor-resistant *A. fatua* populations. In contrast, the inconsistent malathion effect on imazamethabenz efficacy on R plants is the opposite of results from four of five Canadian *A. fatua* populations (Beckie *et al.*, 2012). The trends of herbicide antagonism we saw for some malathion/herbicide treatments of R plants, such as tralkoxydim and fenoxaprop-P-ethyl (Table 3), were unexpected but are not unprecedented. For example, Hidayat and Preston (2001) reported that malathion antagonised fluazifop activity in an imazethapyr- and fluazifop-resistant *Digitaria sanguinalis* (L.) Scop. population, perhaps as a result of malathion-induced enzyme activity of some P450s (Persans & Schuler, 1995). The lack of malathion effect for other herbicides may indicate the involvement of other resistance mechanisms that do not involve P450s.

We did not detect any of the nine known *ACC* mutations correlated with resistance in *Avena* spp. (Jang *et al.*, 2013) via cDNA and genomic sequencing or by allele-specific PCR assays of the R populations. These results indicate that: (i) R plants may contain other undocumented *ACC1* mutations that confer resistance and/or (ii) non-target site mechanisms are responsible for resistance. The significantly improved efficacy of pinoxaden on R3 plants after malathion pre-treatment indicates that enhanced metabolism may be involved in resistance to this herbicide. ACCase inhibitor resistance in other *A. fatua* populations and related *Avena* spp. has been shown to be conferred by target site mutations, enhanced metabolism or a combination of both (reviewed in Beckie & Tardif, 2012; Yu *et al.*, 2013). Beckie *et al.* (2012) recently surveyed 16 *A. fatua* populations from western Canada and determined that four of 12 ACCase inhibitor-resistant populations did not contain known resistance-conferring SNPs. For ALS, the lack of known *ALS* mutations in our R populations and the malathion results suggest that metabolism may be involved in resistance to flucarbazone and perhaps imazamethabenz. Nandula and Messersmith (2001) reported that ALS inhibitor resistance in the AR1 *A. fatua* population from North Dakota was not due to an insensitive enzyme. Both of these reports are from locations that are geographically adjacent to, and with similar cropping systems as, the origins of the Montana populations reported here.

Conclusion

Overall, the dose–response assays reported here and the data from Lehnhoff *et al.* (2013a) show that the R populations from Teton County, Montana, are resistant to multiple herbicides from multiple mode of action families. Malathion pre-treatment indicates that P450-mediated enhanced metabolism rates may be involved in resistance to flucarbazone (both R populations), imazamethabenz (R4), difenzoquat (R4) and pinoxaden (R3), but not for tralkoxydim, fenoxaprop-P-ethyl or triallate. DNA sequencing and PCR assays did not

detect known target site mutations conferring resistance to ACCase or ALS inhibitors. Further research is required to identify the mechanism(s) conferring resistance to these different modes of action.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Materials and methods, Results and Literature Cited.

Figure S1 CLUSTAL 2.1 multiple sequence alignment of the carboxyltransferase (CT) domains of the plastid acetyl-CoA carboxylase (*ACC1*) loci of herbicide sensitive (S1, S2) and resistant (R3, R4) *Avena fatua* populations compared with a consensus sequence from an S *A. fatua* population (Beckie *et al.*, 2012; GenBank JN175313).

Figure S2 CLUSTAL 2.1 multiple sequence alignment of partial acetolactate synthase (ALS) amino acid sequences from herbicide sensitive (S1, S2) and herbicide resistant (R3, R4) *Avena fatua* populations compared with a consensus sequence from an S *A. fatua* population (Beckie *et al.*, 2012; GenBank JN175292).