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ORIGINAL PAPER

Multiple allelic forms of acetohydroxyacid synthase are responsible for herbicide resistance in *Setaria viridis*

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Abstract In weed species, resistance to herbicides inhibiting acetohydroxyacid synthase (AHAS) is often conferred by genetic mutations at one of six codons in the *AHAS* gene. These mutations provide plants with various levels of resistance to different chemical classes of AHAS inhibitors. Five green foxtail [*Setaria viridis* (L.) Beauv.] populations were reported in Ontario with potential resistance to the AHAS-inhibiting herbicide imazethapyr. The objectives of this study were to confirm resistance, establish the resistance spectrum for each of the five populations, and determine its genetic basis. Dose response curves were generated for whole plant growth and enzyme activity, and the *AHAS* gene was sequenced. Resistance was confirmed by determining the resistance factor to imazethapyr in the five resistant green foxtail populations for whole plant dose response experiments (21- to 182-fold) and enzyme assays (15- to 260-fold). All five imazethapyr-resistant populations showed cross-resistance to nicosulfuron and flucarbazone while only three populations had cross-resistance to pyrithiobac. Sequence analyses revealed single base-pair mutations in the resistant populations of green foxtail. These mutations were coded for Thr, Asn, or Ile substitution at Ser_{653}. In addition, a new mutation was found in one population that coded for an Asp substitution at Gly_{654}. There is an agreement between the spectra of resistance observed and the type of resistance known to be conferred by these substitutions. Moreover, it indicates that, under similar selection pressure (imazethapyr), a variety of mutations can be selected for different populations, making the resistance pattern difficult to predict from herbicide exposure history.

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Introduction

The enzyme acetohydroxyacid synthase (AHAS, EC 2.2.1.6; also known as acetolactate synthase, ALS) is a key to the synthesis of the branched-chain amino acids valine, leucine, and isoleucine (Saari et al. 1994). It catalyzes two parallel reactions: the conjugation of a pyruvate with an α-ketobutyrate to form acetohydroxybutyrate and the conjugation of two pyruvate molecules to form acetolactate (Chipman et al. 1998). AHAS is the target site for five herbicide chemical classes currently commercialized in agriculture: sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinyl-oxybenzoates (POBs), and sulfonylamino-carbonyl-triazolinones (SCTs) (Saari et al. 1994; Santel et al. 1999). Following herbicide application, AHAS inhibition leads to plant death due to branched chain amino acids' starvation (Shaner and Singh 1997).

The repeated use of AHAS inhibitors has selected for resistance in many weed species. Currently, there are 97 species worldwide with confirmed resistance to AHAS-inhibiting herbicides (Heap 2009). In the majority of cases where it has been determined, resistance is caused by an insensitive AHAS enzyme due to point mutations in the *AHAS* gene coding for single amino acid substitutions. Six conserved mutation points have been identified in AHAS in weed species that are linked to resistance (Tranel and Wright 2002; Whaley et al. 2007). Some mutations confer plants with resistance to one or a few classes of AHAS

inhibitors while others give broad-spectrum protection to all classes (Saari et al. 1994; Boutsalis et al. 1999; Park and Mallory-Smith 2004). Specifically, mutations coding for $Ala_{205}Val$, $Asp_{376}Glu$, and $Trp_{574}Leu$ confer resistance to most or all of the five classes of inhibitors. $Ala_{122}Thr$, $Ser_{653}Thr$, and $Ser_{653}Asn$ are associated with IMI resistance with some cross-resistance to POBs. Finally, eight documented substitutions at Pro_{197} all confer resistance to SUs and in some cases to TPs and IMIs (Tranel and Wright 2002).

In 2001, a population of green foxtail from Lindsay, ON, Canada, was reported as having survived field application of the IMI herbicide imazethapyr at a dose that is normally lethal (75–100 g ai ha^{-1}). Subsequently, four more populations were reported from three other farms in Arthur (two separate fields), Brodhagen, and Alvinston, ON, Canada (Table 1). All these populations survived field applications of imazethapyr.

The primary objective of this study was to confirm resistance to the selective agent imazethapyr (IMI) and investigate any cross-resistance to the grass active herbicides nicosulfuron (SU), pyrithiobac (POB), and flucarbazone (SCT) at the whole plant and target site levels. The secondary objective was to sequence the AHAS gene from R and S populations and document allelic changes in the target site that could explain resistance.

Materials and methods

Plant material and growth conditions

Resistant green foxtail seeds were collected in different areas in Ontario: Lindsay (SETVI 01, November 2001), Arthur (SETVI 15 and SETVI 16, October 2003), Brodhagen (SETVI 17, October 2003), and Alvinston (SETVI 19, November 2003). Seeds from a susceptible population were collected from Cambridge (SETVI 04), ON, and used as a reference (Table 1). The seed heads were collected from 15 to 50 plants from a single patch. All seeds were stored in dry conditions at 5°C.

Table 1 Locations in Ontario from which the different green foxtail populations were collected

Location	Latitude/longitude
Alvinston	42°49′34N/81°51′50W
Arthur	43°49′60N/80°31′60W
Brodhagen	43°33′37N/81°12′34W
Cambridge	43°22′60N/80°19′0W
Lindsay	44°21′0N/78°43′60W

In order to increase germination rate, seeds were soaked in a 2% (w/v) potassium nitrate solution for 24 h at room temperature, rinsed in distilled water and air dried overnight. Seeds were germinated in Petri dishes on a solidified medium of water containing 0.6% (w/v) agar. Incubation occurred in a controlled environment: a 16 h day period at 25°C and an 8 h night period at 18°C. Photosynthetically active radiation was 200–300 μmol m^{-2} s^{-1}. After 3–4 days of germination, seedlings were transplanted into 15-cm diameter pots containing potting soil (Promix BX, Premier Horticulture Inc., Red Hill PA).

Determination of whole plant dose response

Plants were grown in growth room under the same conditions as described above until needed for experiments. Post-emergence herbicides were applied 6 days after transplanting when plants were at the two- to four-leaf stage of development. Imazethapyr (Pursuit, 240 g L^{-1}, solution) was applied at rates ranging from 0.39 to 3,200 g ai ha^{-1}; nicosulfuron (Accent, 75% by weight, dry flowable) at rates ranging from 0.098 to 800 g ai ha^{-1}; pyrithiobac (Staple, 75% by weight, soluble powder) at rates ranging from 0.059 to 15.05 g ai ha^{-1}; and flucarbazone (Everest, 70% by weight, wettable dry granular) at rates ranging from 0.059 to 15.05 g ai ha^{-1}. All herbicides were sprayed with 0.2% non-ionic surfactant (Agral 90, Norac Concepts Inc., Orleans, Canada, K1C 7H8). Imazethapyr was also applied with 2 L ha^{-1} of urea–ammonium nitrate (28% N, w/v) fertilizer. Herbicides were applied with a laboratory sprayer equipped with a Teejet 8002ES (Spraying Systems Co., Wheaton, IL 60189) nozzle delivering 210 L ha^{-1} at 276 kPa. Plants were returned to the growth room after herbicide treatments. Plants were sampled by cutting them at soil level 10 days after treatment and drying at 80°C for 48 h before taking dry biomass measurements.

The experiment was set up as a completely randomized design with four replications. Each experimental unit was comprised four plants. The entire experiment was repeated in time and the results were combined.

AHAS extraction and assay

Plants were grown as described above, collected at the four-leaf stage of development, and frozen at −86°C prior to assays. AHAS enzyme was extracted using procedures modified from Ray (1984).

Two grams of frozen leaf tissue was ground with a pestle in a chilled mortar with 0.5 g insoluble polyvinylpyrrolidone (PVP), sand (0.5–1.0 g), and 6 ml of 2× reaction buffer [50 mM potassium phosphate buffer at pH 7.5, 1,000 mM sodium pyruvate, 100 mM magnesium

chloride, 10 mM thiamine pyrophosphate (TPP), and 100 μM flavine adenine dinucleotide (FAD)]. The homogenate was centrifuged at 10,000*g* for 30 min at 4°C and the supernatant was filtered through a 25 mm syringe filter (0.45 μM). Assays were performed in 400 μl 96 well microplates. Crude enzyme fraction (30 μl) was mixed with 30 μl of herbicide at 2× final concentration and incubated at 37°C for 30 min. Blank reactions received 12 μl of 6 N sulfuric acid at the onset so as to determine non-enzymatic acetolactate production. The enzymatic reaction was terminated by the addition of 12 μl of 6 N sulfuric acid followed by 15 min incubation at 60°C to facilitate decarboxylation of acetolactate to form acetoin. Acetoin was detected as a colored complex ($A_{530\ nm}$) formed after adding 57 μl of 0.55% (w/v) creatine and 57 μl of 5.5% (w/v) α-naphthol (in 5 N sodium hydroxide) followed by dark incubation at 60°C for 15 min.

Imazethapyr and nicosulfuron were tested at concentrations ranging from 0.1 to 1,000 μM, pyrithiobac at rates ranging from 0.001 to 500 μM, and flucarbazone at rates ranging from 0.001 to 750 μM. The experimental design consisted of six replications for each herbicide. The whole experiment was repeated in time and then the results were combined.

Statistical analysis

SAS version 8.02 (SAS Institute Inc., Cary NC) was used to perform statistical analyses. The type 1 error rate (α) was set at 0.05 for all null hypotheses. Test of assumptions was performed for regression and variance analyses using PROC GLM, PROC UNIVARIATE, and PROC PLOT.

For each population, dry weight and AHAS activity were expressed as a percentage of untreated. Dose response curves were obtained by a non-linear log-logistic regression model using PROC NLIN (Seefeldt et al. 1995) according to the following equation:

$$y = C + \frac{D - C}{1 + (x/I_{50})^b} \quad (1)$$

where y is the percent AHAS activity or percent dry weight, x is the herbicide concentration (rate), C and D are the lower and the upper limits of the curve, respectively, b is the slope at the inflexion point, and I_{50} (or GR_{50}) is the herbicide dose rate required to reduce AHAS activity (or dry weight) by 50%.

Parallel and nonparallel regression models were compared to deduce the regression model using the lack-of-fit F test (Seefeldt et al. 1995). The equation was:

$$F = \left[\left(SS_e^{II} - SS_e^{I}\right)/\left(DF_e^{II} - DF_e^{I}\right)\right]/\left(SS_e^{I}/DF_e^{I}\right) \quad (2)$$

where SS_e^{II} and DF_e^{II} are the sums of squares and degrees of freedom for the modified model, while SS_e^{I} and DF_e^{I} are the sums of squares and degrees of freedom for the original model.

For each herbicide tested, the resistance factor (RF) was obtained by dividing I_{50} or GR_{50} of each resistant population by that of the susceptible population.

Molecular basis of resistance

DNA extraction

Shoots and leaves of two individual plants per population were harvested at the four-leaf stage and stored at −86°C. Genomic DNA was extracted according to the protocol of the FastDNA kit (BIO 101, Vista, CA). The extracted DNA was measured using a DU-64 Spectrophotometer (Molecular Devices v. 007) and diluted with distilled deionized water to a concentration of 10 ng μl^{-1}.

Primer selection

No sequence information was available in any international database for the *AHAS* gene of green foxtail. Consequently, AHAS sequences of corn (*Zea mays* L.), rice (*Oryza sativa* L.), downy brome (*Bromus tectorum* L.), bread wheat (*Triticum aestivum* L.), and Italian ryegrass (*Lolium multiflorum* Lan.) (GenBank accessions X63553, AY885674.1, AF488771.1, AY210407.1, and AF310684.1, respectively) were aligned using ClustalW Multiple Sequence Alignment Tool (http://www.ebi.ac.uk/clustalw) to find conserved areas. A series of primers were designed using GeneRunner (http://www.genomes.com) or Primer3 Input (primer3_www.cgi v 0.2) to target homologous regions for optimal PCR amplifications. Primers were manufactured by Laboratory Services Division (University of Guelph, Guelph, ON, Canada) (Table 2).

PCR amplification

Polymerase chain reaction (PCR) mixture was performed in a 25 μl volume reaction that contained 50 ng of total DNA, 1× of PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, and 1.15 units of Platinum *Taq* DNA polymerase (Invitrogen Canada Inc., Burlington, ON, Canada). Inclusion of 5% dimethyl sulfoxide (DMSO) in the PCR reaction improved amplification. Since the 5′ end was GC rich, AccuPrime GC-Rich DNA polymerase (Invitrogen Canada Inc.) was used for this gene section. This PCR reaction was performed in a 25 μl volume that contained 50 ng of total DNA, 1× buffer I, 2 mM $MgSO_4$, 0.2 μM of each primer, and 1 unit of AccuPrime GC-Rich DNA polymerase. All PCR reactions were performed using a 96-well RoboCycler (Stratagene, La Jolla, CA, USA) with an initial denaturation at 94°C for 2 min; 35 cycles of

Table 2 Primer sets used for *AHAS* gene amplification

Set	Sequence 5′–3′		Melting temp. (°C)	Nucleotide coverage[a]	Targeted mutations[b]
1	F	TCGACGTCTTCGCCTAC	54	336–1,193	A_{122}, P_{197}, A_{205}, D_{376}
	R	TGGATCAATGTCAATGTGC			
2	F	GAGTTGTGCCGCTTTGTGGAG	57	946–1,507	D_{376}
	R	GCCTTGGCCGCTTGTAAGTG			
3	F	CTCGGGTTTCCCAAGAATGTG	51	1,319–1,775	W_{574}
	R	TTCGGCTCATGGCATGATG			
4	F	CGGATCAATACACAGTCCTG	57	1,650–2,017	W_{574}, S_{653}
	R	GATCCGTATTGAGAACCTCC			

F forward primer, *R* reverse primer

[a] Numbering of nucleotides as per *Arabidopsis thaliana AHAS* sequence

[b] Numbering of amino acids as per *Arabidopsis thaliana* AHAS sequence

45 s at 94°C, 1 min at variable annealing temperature (Table 2), 1.5 min at 72°C; and a final extension for 7 min at 72°C.

PCR product specificity was verified using electrophoresis on 1% (w/v) agarose gels. DNA bands were visualized under UV light by staining with ethidium bromide (10 μM) in a concentration of 5 μl per 100 ml gel solution. The desired PCR products were purified from solution or from gel using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, GE Healthcare Bio-Sciences Inc., Baie D'Urfé, Québec, Canada). DNA sequencing was performed by Laboratory Services Division, University of Guelph, ON, Canada.

Sequence analysis

To minimize sequencing errors, each amplified product was sequenced in both the forward and reverse directions. Sequences of both resistant and susceptible biotypes were aligned and nucleotide changes compared using ClustalW Multiple Sequence Alignment Tool (http://www.ebi.ac.uk/clustalw). Amino acid sequence was deduced using ExPASy Translation Tool (http://www.expasy.org). Furthermore, amino acid sequence comparison with Basic Local Alignment Search Tool protein–protein (http://www.ncbi.nlm.nih.gov/BLAST/) was used to show similarities with other grass species.

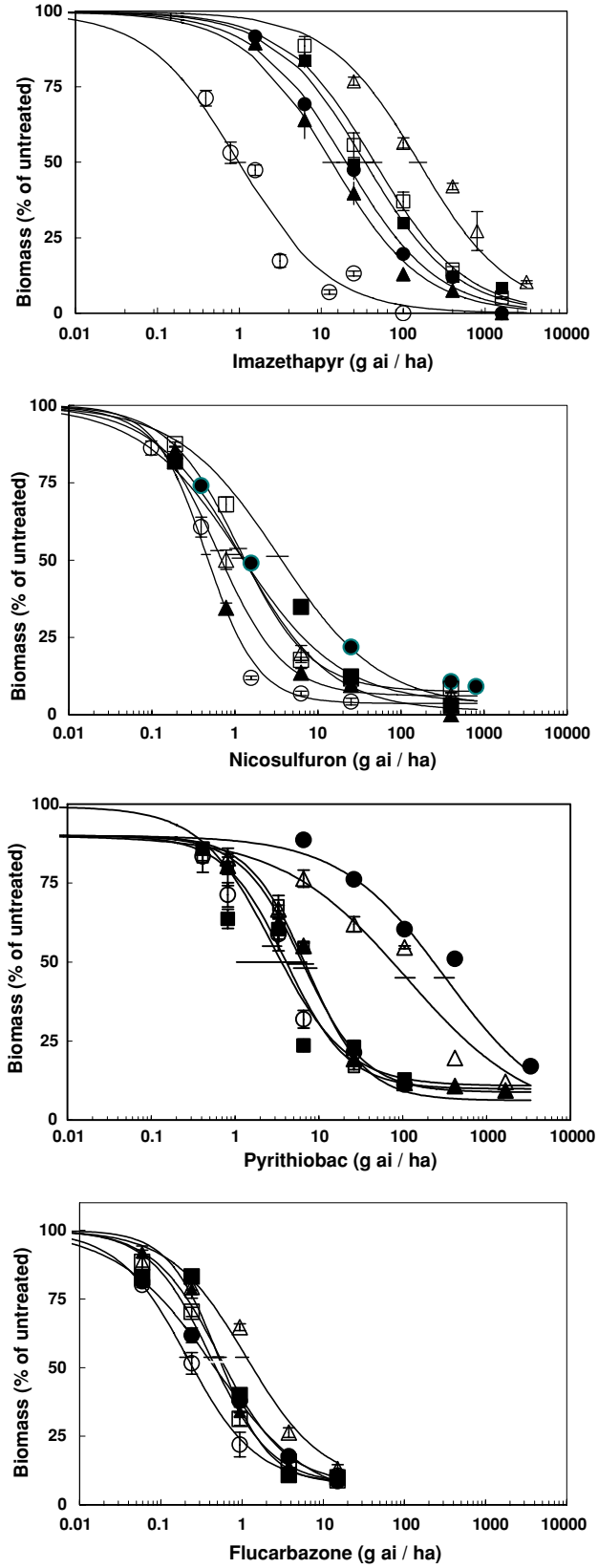
Results

Whole plant dose response

Dose response analyses confirmed resistance to imazethapyr in all five suspected populations (Fig. 1). Furthermore, cross-resistance to nicosulfuron, pyrithiobac, and flucarbazone was also observed in some of the populations (Fig. 1). Based on comparison of imazethapyr GR_{50} levels (Table 3), resistant populations could be assigned into three distinct groups. Population Arthur 2 showed the highest RF with a value of 158 (Table 3). Populations Arthur 1, Alvinston and Lindsay had moderate resistance (RF ranging from 20 to 46) while population Brodhagen had the lowest resistance level (RF = 14). RF values for nicosulfuron were much lower than for imazethapyr and the population grouping was different. Population Arthur 1, which had moderate resistance to imazethapyr, was significantly more resistant to nicosulfuron (RF = 7.5) compared to the other populations. The remaining populations had very low resistance to nicosulfuron (RF ranging from 1.4 to 2.5). Resistance to pyrithiobac was confirmed in populations Arthur 1 and Arthur 2 while the three remaining populations were not significantly different from the susceptible reference population (Table 3). In Arthur 1, resistance to pyrithiobac was very high with a 113-fold RF. Resistance was lower in Arthur 2 at 39-fold compared to the reference population (Cambridge). Resistance to flucarbazone was less compared to the other herbicides: all populations had 2- to 5.4-fold resistance compared with the reference population.

AHAS activity and inhibition

Acetohydroxyacid synthase assay results confirmed that an altered target site was the resistance mechanism in all populations. Enzyme inhibition curves showed different responses for the susceptible and resistant populations to the four AHAS inhibitors tested (Fig. 2). All five resistant green foxtail populations had AHAS activity that was significantly less sensitive to inhibition by herbicides compared to the susceptible (Table 4). The same population groupings observed at the whole plant level were seen at the enzyme level. For the herbicide imazethapyr, population Arthur 2 showed the highest resistance (RF = 182);



◀ **Fig. 1** Percentage dry weight of *Setaria viridis* Cambridge (*open circle*) (wildtype), Arthur 1 (*filled circle*), Arthur 2 (*open triangle*), Brodhagen (*filled triangle*), Alvinston (*filled square*), and Lindsay (*open square*) after treatment with (**a**) imazethapyr, (**b**) nicosulfuron, (**c**) pyrithiobac, and (**d**) flucarbazone. Dry weights were measured 10 days after treatment and results were expressed as the percentage of the untreated control for each population. Dose response curves were generated by non-linear log-logistic regression. *Horizontal error bars* represent the 95% confidence interval of the GR_{50} values. Each point is the mean of the mean of two runs each containing four seedlings, plotted with standard error of the mean ($n = 8$)

populations Arthur 1, Alvinston and Lindsay had moderate resistance (RF from 32 to 49); and population Brodhagen showed the lowest resistance (RF = 22) (Table 4). Resistance to nicosulfuron was confirmed in all resistant populations. Population Arthur 1 had the highest resistance factor (RF = 38) while the remaining four populations had moderate resistance (RF from 4.4 to 7.4). Resistance to pyrithiobac was observed in populations Arthur 1, Arthur 2 and Brodhagen (RF from 5 to 191). RFs of populations Alvinston and Lindsay were not significantly different from the susceptible population. Although RF values were low (≤4), resistance to flucarbazone in all populations was significantly higher than in the susceptible population.

Sequence analysis

Following PCR amplification of the *AHAS* gene, 1,684 base pairs were sequenced. The deduced amino acid sequence comprised 561 residues. Amino acid sequence comparison with BLASTP (NCBI) showed high level of similarities with other grass species (% sequence identity and GenBank number): *Zea mays* L. (95%, CAA45116.1), *Triticum aestivum* L. (92%, AAO53548.1), *Hordeum vulgare* L. (92%, AAC14572.1), *Bromus tectorum* L. (91%, AAM03119.1), *Lolium multiflorum* Lam. (91%, AAG30931), and *Oryza sativa* L. (84%, AAX14282.1).

Amplification of *AHAS* with primer sets 1–4 encompassed the region coding for the conserved amino acids Ala_{122}, Pro_{197}, Ala_{205}, Asn_{376}, Ala_{205}, Trp_{574}, and Ser_{653} [numbering standardized according to *Arabidopsis thaliana* (L). Heynh. sequence] implicated in herbicide resistance in weeds (Tranel and Wright 2002; Whaley et al. 2007). Sequences analyzed by comparing the susceptible population to all the resistant populations uncovered the presence of four distinct substitutions (Table 5). Arthur 1 demonstrated a single-point mutation from guanine to thymine at nucleotide 1,624, conferring a $Ser_{653}Ile$ substitution. A guanine to adenine substitution at nucleotide 1,624 encoded a $Ser_{653}Asn$ substitution in population Arthur 2. Populations from Alvinston and Lindsay had a guanine for

Table 3 GR_{50} values and resistance factors (RF) of green foxtail populations for imazethapyr (Ima), nicosulfuron (Nic), pyrithiobac (Pyr), and flucarbazone (Flu)

Population	GR_{50} (g ai ha^{-1})				RF[a]			
	Ima	Nic	Pyr	Flu	Ima	Nic	Pyr	Flu
Cambridge	0.98 a	0.45 a	2.9 a	0.21 a	1	1	1	1
Alvinston	34.3 c	1.1 c	4.1 a	0.53 b	35*	2.5*	1.4	2.5*
Arthur 1	20.0 c	3.4 d	324 c	0.44 b	20*	7.5*	113*	2.1*
Arthur 2	156.0 d	1.2 c	111 b	1.1 c	158*	2.5*	39*	5.4*
Brodhagen	13.5 b	0.6 b	6.5 a	0.52 b	14*	1.4*	2.3	2.5*
Lindsay	44.9 c	1.1 c	7.2 a	0.41 b	46*	2.5*	2.5	2.0*

Values within the same column followed by the same letter are not significantly different ($P = 0.05$). Groupings according to 95% confidence intervals of GR50 values

[a] RF is calculated by dividing the GR_{50} of R populations by that of the GR_{50} of the wildtype Cambridge population

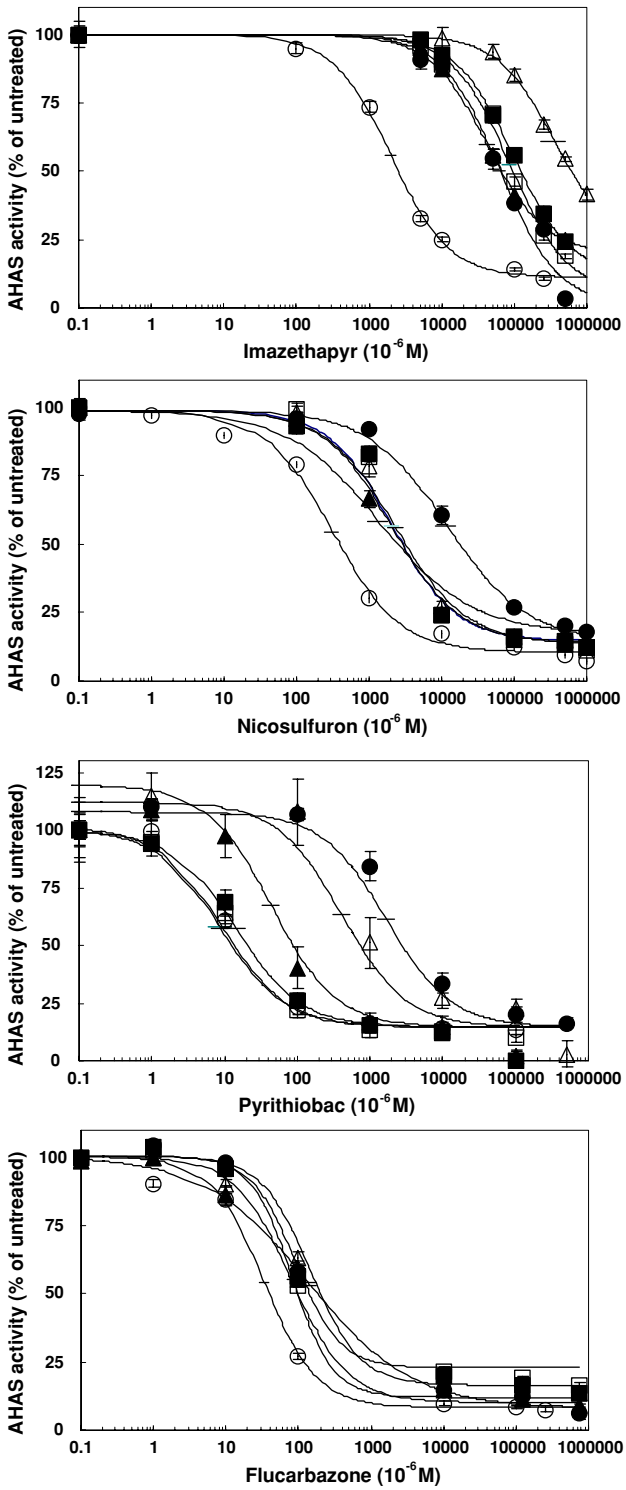
* denotes RF value derived from GR_{50} value significantly different from that of wildtype Cambridge population

cytosine at nucleotide 1,624 change that conferred a Ser_{653}Thr substitution. Finally, population Brodhagen had a mutation one codon downstream from the other resistant populations. This novel mutation exchanged a guanine for thymine at nucleotide 1,627, which coded for a Gly_{654}Asp substitution. No other polymorphisms were observed either within individuals of a population or among the different populations.

Discussion

Resistance to imazethapyr was confirmed in five green foxtail populations with RFs ranging from 20 to 158. Resistance levels differed among populations, but each was sufficient for the survival under field rate conditions and applications of the herbicides. Furthermore, cross-resistance to nicosulfuron, pyrithiobac, and flucarbazone was also found. Resistance to nicosulfuron was seen in all resistant populations (RF ranging from 1.4 to 7.5). Only two populations were resistant to the herbicide pyrithiobac with RFs of 39 and 113. Compared to the susceptible, all resistant populations showed significant resistance to the herbicide flucarbazone (RF from 2 to 5.4). Enzyme inhibition curves showed different responses for the susceptible population and the resistant populations to the four AHAS inhibitors tested indicating that a modified enzyme is responsible for resistance. In addition, the concordance between the AHAS assays and whole plant dose responses proves the hypothesis of target site resistance in resistant populations. Many studies with AHAS-resistant plants have shown target site modification as the mechanism of resistance (Saari et al. 1994; Boutsalis et al. 1999; Park and Mallory-Smith 2004; Heap 2009). Indeed, altered AHAS has been documented in other grass species such as *Alopecurus myosuroides* Huds., *Lolium rigidum* Gaudin, and *Hordeum leporinum* Link (Marshall and Moss 2008; Yu et al. 2007; Yu et al. 2008) However, rapid herbicide metabolic inactivation due to increased herbicide metabolism is also possible and has been documented in a restricted number of species such as *L. rigidum, Sinapis arvensis* L., *A. myosuroides*., and *Digitaria sanguinalis* (L.) Scop. (Saari et al. 1994; Hidayat and Preston 1997; Veldhuis et al. 2000). Other mechanisms have not been investigated in this study, such as absorption, translocation, uptake, and metabolism. While it is possible that other mechanism of resistance could confer resistance in the foxtail populations, the overall agreement between the resistance patterns observed at the whole plant and enzymic levels strongly suggests altered AHAS as the principal resistance mechanism.

Using PCR amplifications, a partial sequence of the *AHAS* gene was obtained for all resistant populations and for the susceptible population from Cambridge, ON, Canada. The coding sequences were not interrupted by introns and consisted of an open reading frame of 1,684 bp that encoded 561 amino acids. Comparative sequencing of *AHAS* gene in resistant and susceptible plants determined amino acid changes at position 653 or 654. Four different mutations in the *AHAS* gene were observed in the five different populations of green foxtail tested. Two new mutations, one of which has never been reported before for a field selected weed population, were identified: Ser_{653}Ile and Gly_{654}Asp. The Ser_{653}Ile substitution seen in population Arthur 1 has been identified in a spontaneous mutant of *Oryza sativa* L. subsp. *japonica*, but has not been documented in a weedy species (Kawai et al. 2007). To our knowledge, the Gly_{654}Asp substitution found in population Brodhagen has not been documented before in any plant species. Because no other mutations were found in the *AHAS* gene for population Brodhagen and as it is located near an imidazolinone resistance site, this new mutation is



◀ **Fig. 2** Percentage AHAS activity of *S. viridis* Cambridge (*open circle*) (wildtype), Arthur 1 (*filled circle*), Arthur 2 (*open triangle*), Brodhagen (*filled triangle*), Alvinston (*filled square*), and Lindsay (*open square*) after treatment with (**a**) imazethapyr, (**b**) nicosulfuron, (**c**) pyrithiobac, and (**d**) flucarbazone. AHAS activity was measured after completion of the enzyme assay and results were expressed as the percentage of the untreated control for each biotype. Inhibition curves were generated by non-linear log-logistic regression. *Horizontal error bars* represent the 95% confidence interval of the I_{50} values. Each point is the mean of three runs with four replicates, plotted with standard error of the mean ($n = 12$)

very likely the cause of resistance in this population. Moreover, a $Gly_{654}Glu$ substitution has been observed in imazethapyr resistant mutant rice confirming this amino acid residue as being involved in resistance (Croughan 2005). While the evidence is fairly strong, there is still a possibility that $Gly_{654}Asp$ would not be involved in resistance. Ultimate confirmation would come from segregation studies following crosses between population Brodhagen and wildtype populations.

The $Ser_{653}Thr$ mutation found in the Lindsay and Alvinston populations conferred a high resistance level to imazethapyr (IMI) with low resistance to nicosulfuron (SU). These results contradict some of the results previously reported in *Amaranthus powellii* S. Wats.: in this species, $Ser_{653}Thr$ provided resistance to imazethapyr (IMI) but not to thifensulfuron (SU) (Ferguson et al. 2001; McNaughton et al. 2005). However, in this study, the SU herbicide nicosulfuron was tested instead of thifensulfuron because of being primarily a grass herbicide. It is possible that binding to the AHAS enzyme is different for each SU herbicide, which leads to a variation in the response. Sibony et al. (2001) observed different RFs within SU herbicides in whole plant studies and at the enzyme level for *A. retroflexus* L. (RF ranged from 11 to 126). The testing of populations from Lindsay and Alvinston with other SU herbicides would further document the cross-resistance pattern to SUs. In addition, there are, among plant species, non-conserved areas in the gene that could slightly affect enzyme binding to herbicides causing variation in herbicide response.

In the Arthur 2 population, the $Ser_{653}Asn$ mutation conferred the highest level of resistance to imazethapyr (IMI) with moderate resistance to pyrithiobac (POB), flucarbazone (SCT), and nicosulfuron (SU). Other studies have reported resistance to IMIs and POBs, but not to SUs (Chang and Duggleby 1998; Sathasivan et al. 1991; Chong and Choi 2002; Zhu et al. 2000, and Patzoldt and Tranel 2007).

Lee et al. (1999) have suggested that a change in coding for the amino acid side-chain size at position 653 affects herbicide binding and, therefore, resistance. They observed that a Ser to Ala substitution did not cause resistance while a Ser to Asn, Thr, or Phe substitutions did. Change in amino acid size is likely to displace herbicide on the imidazolinone side (McCourt et al. 2006). This observation is consistent with a study by Chong and Choi (2002) who observed no resistance for the Ser to Cys change. Serine, alanine, and cysteine have a smaller side chain than the other amino acid chains involved in resistance at the serine

Table 4 Herbicide dose rates required to reduce AHAS activity by 50% (I_{50}) and resistance factor (RF) in resistant populations and susceptible population of green foxtail for imazethapyr (Ima), nicosulfuron (Nic), pyrithiobac (Pyr), and flucarbazone (Flu)

Population	I_{50} (mM)				RF[a]			
	Ima	Nic	Pyr	Flu	Ima	Nic	Pyr	Flu
Cambridge	2.0 a	0.3 a	8.8 a	33.4 a	1	1	1	1
Alvinston	97.3 c	2.3 b	14.6 a	91.8 b	49*	7.4*	1.1	2.7*
Arthur 1	63.9 c	12.0 c	1680 d	147 c	32*	38*	191*	4.4*
Arthur 2	364.1 d	2.1 b	411 c	136 c	182*	6.6*	44*	4.1*
Brodhagen	42.7 b	1.4 b	44.3 b	79.4 b	22*	4.4*	5.0*	2.4*
Lindsay	87.7 c	2.1 b	9.3 a	81.4 b	44*	6.7*	1.7	2.4*

Values within the same column followed by the same letter are not significantly different ($P = 0.05$)

[a] RF is calculated by dividing the I_{50} of R populations by that of the I_{50} of the wildtype Cambridge population

* denotes RF value derived from GR_{50} value significantly different from that of wildtype Cambridge population

Table 5 DNA and amino acid polymorphisms associated with resistance in five populations of green foxtail

Population	Codon		Amino acid	
	1,623–1,625	1,626–1,628	653	654
Cambridge	AGC	GGT	Ser	Gly
Alvinston	A**C**C	GGT	Thr	Gly
Arthur 1	AT**C**	GGT	Ile	Gly
Arthur 2	A**A**C	GGT	Asn	Gly
Brodhagen	AGC	G**A**T	Ser	Asp
Lindsay	A**C**C	GGT	Thr	Gly

DNA sequence numbering identifies the nucleotide positions within of each codon. Corresponding amino acid residues are numbered according to the *Arabidopsis* numbering. Underlined nucleotides show differences from the wild type Cambridge population. Amino acids showed in bold are substituted from the wild type

residue. This could explain resistance observed for the $Gly_{654}Asp$ in population Brodhagen and for the $Gly_{654}Glu$ in mutant rice. Glycine has a small non-polar side chain and is weakly hydrophobic, whereas aspartic acid and glutamic acid have a larger side chain and are hydrophilic (Fristrom and Clegg 1989). Unlike the other highly conserved amino acids that have been linked to herbicide resistance, the Ser_{653} residue is not totally conserved across species: in *Xanthium strumarium* L. and *Amaranthus* species, an alanine is found at position 653 (Duggleby and Pang 2000). Serine and alanine are similar in size and in structure but serine differs from alanine in that a hydroxyl group replaces one of the methylenic hydrogens. Because of their similarity, substitution of Ser to Ala at 653 does not impact sensitivity to IMIs.

The mutations we observed are most likely to be the cause of resistance. Concordance between AHAS assays and whole plant dose responses proves the notion of target site resistance in these resistant populations. Most of the mutations found have been reported in previous studies as conferring resistance to AHAS-inhibitors (Tranel and Wright 2002; Kawai et al. 2007). In addition, there is an agreement between the spectrum of resistance observed here and the type of resistance known to be conferred by these substitutions. It is also interesting to note that two populations from the same commercial farm operation had two distinct mutations, which emphasizes the inherent local variability in response to AHAS inhibitors selection pressure. This species is autogamous and is not prone to spontaneous dispersal which reinforces the independence of distinct populations. The variability in possible mutations in the *AHAS* gene emphasizes the difficulty in predicting what mutation might be selected despite similar cropping systems. Similar results were observed with *Amaranthus* sp. whereby five different AHAS mutations were selected among numerous distinct populations (McNaughton et al. 2005).

There are 125 foxtail species found worldwide, which includes food crops and a number of weeds (Dekker 2003). Foxtail millet [*Setaria italica* (L.) Beauv.] is an important cereal in China, Japan, South Asia, and Eastern Europe. Evidence exists that foxtail millet is closely related to green foxtail (Darmency et al. 1987). Spontaneous hybridizations between the two species have produced the fertile hybrid *S. viridis* var. *major* (Darmency et al. 1987). Because foxtail millet has low genetic diversity, the possibilities for its improvement in a breeding program are limited. Therefore, AHAS-resistant green foxtail could be used as a gene source in foxtail millet breeding programs to improve foxtail millet performance. In the past, genes from a triazine-resistant green foxtail have been successfully introduced into foxtail millet through hybridization (Darmency and Pernès 1989). The latter approach seems particularly attractive from the standpoint of increased popularity of herbicide resistant crops.

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