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Acetolactate Synthase Inhibitor–Resistant False Cleavers (*Galium spurium*) in Western Canada

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Cleavers species (false cleavers and catchweed bedstraw) are among the top 10 most abundant weeds across the prairie region of western Canada, and are increasing in relative abundance at the fastest rate since the 1970s. In 2008, two false cleavers populations from Tisdale and Choiceland, Saskatchewan, were suspected of acetolactate synthase (ALS) –inhibitor resistance. Dose-response experiments were conducted with the use of imazethapyr and florasulam, both ALS inhibitors, as well as fluroxypyr, a synthetic auxin. Additionally, a 1,954–base-pair region of the *ALS* gene including sites known to confer *ALS* resistance were sequenced. Both populations were highly resistant to imazethapyr (resistance factors greater than 100), one population (Tisdale) was highly resistant to florasulam (Choiceland population susceptible, although a second, larger screening of 200 individuals indicated low frequency [2%] florasulam resistance), and both populations were susceptible to fluroxypyr. All sequenced Tisdale individuals screened with imazethapyr posessed the $Trp_{574}Leu$ mutation. In contrast, three point mutations were found for Choiceland individuals sequenced: $Ser_{653}Asn$, $Trp_{574}Leu$, and $Asp_{376}Glu$. These *ALS* target-site mutations have not been documented previously in this species.
Nomenclature: Florasulam; fluroxypyr; imazethapyr; catchweed bedstraw, *Galium aparine* L.; false cleavers, *Galium spurium* L. GALSP.
Key words: ALS gene sequencing, ALS-inhibitor resistance, herbicide resistance, target-site mutation.

Las especies *Galium spurium* y *Galium aparine* están entre las diez malezas más abundantes a lo largo de la región Pradera del occidente de Canadá y están incrementando en abundancia relativa a la tasa más rápida desde los años setenta. En 2008, dos poblaciones de *G. spurium* de Tisdale y Choiceland, Saskatchewan, fueron sospechosas de ser resistentes a los inhibidores de acetolactate synthase (ALS). Se realizaron experimentos de respuesta a dosis usando imazethapyr y florasulam, ambos inhibidores de ALS, así como también fluroxypyr, una auxina sintética. Adicionalmente, fue secuenciada una región 1954-pb del gen ALS que incluye sitios que se sabe confieren resistencia ALS. Ambas poblaciones fueron altamente resistentes a imazethapyr (factores de resistencia mayores que 100); una población (Tisdale) fue altamente resistente a florasulam. La población Choiceland resultó ser susceptible, aunque una segunda y mayor selección de 200 individuos indicó baja frecuencia de resistencia a florasulam (2%), y ambas poblaciones fueron susceptibles a fluroxypyr. Todos los individuos secuenciados de Tisdale, tratados con imazethapyr, exhibieron la mutación $Trp_{574}Leu$. En contraste, tres puntos de mutación se encontraron en los individuos secuenciados de Choiceland: $Ser_{653}Asn$, $Trp_{574}Leu$ y $Asp_{376}Glu$. Estas mutaciones ALS en el sitio-objetivo no habían sido documentadas previamente en estas especies.

Cleavers (hereafter referring to false cleavers and catchweed bedstraw), members of the Rubiaceae (madder family), are common annual or winter annual weeds in many agricultural regions of North America (Malik and Vanden Born 1988). In the Canadian Prairies of western Canada (provinces of Alberta, Saskatchewan, and Manitoba), cleavers are the ninth most abundant weed species and are increasing in relative abundance at the fastest rate since the 1970s (Leeson et al. 2005). In Alberta, cleavers are the third most abundant weed species (Leeson and Neeser 2010). Cleavers are more abundant in the more humid Parkland region than semiarid Grassland region of the Prairies.

Cleavers can cause significant crop yield loss (Malik and Vanden Born 1987). Moreover, the semiprostrate or climbing stems measuring as long as 120 cm and possessing thornlike spines can cause crop lodging and interfere with combine operations (Malik and Vanden Born 1988). Acetolactate synthase [ALS; also referred to as acetohydroxyacid synthase (AHAS)] –inhibitor herbicides are relied upon in pulse crops such as field pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.) to control cleavers (Saskatchewan Ministry of Agriculture 2011). In cereal crops, fluroxypyr, a synthetic auxin, is commonly used to control cleavers.

ALS-inhibitor–resistant false cleavers was first documented worldwide in 1996 in Alberta, Canada (Hall et al. 1998; Horsman and Devine 2000). This population was characterized by high-level resistance to sulfonylurea and triazolopyrimidine class herbicides and moderate-level resistance to imidazolinones. Resistance was conferred by the target-site mutation $Pro_{197}Leu$ (Horsman and Devine 2000). The target-site mutation $Trp_{574}Gly$ was recently reported for a Chinese ALS-inhibitor–resistant population of catchweed bedstraw (Sun et al. 2011).

A random survey of 300 fields was conducted in Alberta in 2007 for herbicide-resistant weeds (Beckie et al. 2009). Of cleavers populations sampled in 30 fields from across the province, herbicide resistance screening results indicated that five populations (17%) were ALS-inhibitor resistant.

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Of cleavers samples submitted by Prairie growers to our lab since 2002, over 20 cases have been confirmed as ALS-inhibitor resistant (Beckie, unpublished data).

In 2008, bulk seed samples of two false cleavers populations near Tisdale and Choiceland, Saskatchewan, Canada, that were suspected of ALS-inhibitor resistance were submitted for resistance testing. Herein, we describe the ALS-inhibitor herbicide dose-response results for these two false cleavers populations and molecular basis for resistance. In addition, the response of both putative ALS-inhibitor–resistant populations to increasing doses of fluroxypyr, a synthetic auxin herbicide, was investigated as an alternative mode-of-action herbicide.

Materials and Methods

Herbicide Dose-Response Experiments. The herbicide dose-response experiments involving the putatively resistant Tisdale and Choiceland populations were conducted in the greenhouse in the fall of 2010 at Saskatoon, Saskatchewan and repeated once. The experiments were arranged in a completely randomized design with four replications (one pot per replicate) per treatment. The dose response of each of the two populations to each of the three herbicides (imazethapyr, florasulam, fluroxypyr) were separate experiments. Because of limited seed, a false cleavers population from Vermilion, Alberta served as the susceptible control for the Tisdale population dose-response experiments, whereas a population from Minburn, Alberta was the susceptible control for the Choiceland population dose-response experiments.

Four seeds were planted 1 cm deep in 10-cm-square pots containing a mixture of soil, peat, vermiculite, and sand (3:2:2:2 by volume) plus a controlled-release fertilizer (Scotts Osmocote PLUS, Scotts-Sierra Horticultural Products Co., Mississauga, Ontario) (15–9–12; 150 g 75 L^{-1}). Experiments were conducted under a 20/16 C day/night temperature regime with a 16-h photoperiod supplemented with 230 μmol m^{-2} s^{-1} illumination. Pots were watered daily to field capacity.

Seedlings (F_1 progeny) were treated with imazethapyr (Pursuit®, BASF Canada, Mississauga, Ontario), florasulam (Frontline A®, Dow AgroSciences, Calgary, Alberta), or fluroxypyr (Attain A®, Dow) at the two- to three-leaf stage. Herbicides were applied with the use of a moving-nozzle cabinet sprayer equipped with a flat-fan nozzle tip (TeeJet 8002VS, Spraying Systems Co., Wheaton, IL) calibrated to deliver 200 L ha^{-1} of spray solution at 275 kPa in a single pass over the foliage. Each herbicide was applied at eight doses (0.0625 to eight times the recommended dose), plus a nontreated control was included. The recommended dose in western Canada of imazethapyr, florasulam, and fluroxypyr is 50, 5, and 80 g ai ha^{-1}, respectively. Commercial formulations of the herbicides were used. A nonionic surfactant (Agral 90®, Norac Concepts Inc., Ottawa, Ontario) at 0.25% v/v was added to the imazethapyr spray solution. Two weeks after treatment, shoots were cut at soil level. Harvested biomass was dried at 65 C for 6 d, and weighed.

Because of perceived discrepancies between the cross-resistance patterns observed in the dose-response experiments and the results of the molecular analyses, an additional 200 seedlings of the Choiceland population were screened with florasulam at 5 g ai ha^{-1}. Methodology and greenhouse conditions were similar to those described previously. Plants that survived treatment were tissue-sampled 2 wk after herbicide application, and subjected to molecular analyses.

Dose-Response Data Analysis. Results of each experiment were combined across runs upon confirmation of homogeneity of variances (Steel and Torrie 1980). The six experiments (three herbicides by two putatively resistant populations) were analyzed separately. Aboveground biomass (Y; percentage of nontreated control) was regressed against herbicide dose (x, g ha^{-1}) with the use of the quadratic model (Equation 1) for the herbicide-resistant (HR) populations and the double exponential decay model (Equation 2) for the herbicide-susceptible (HS) populations:

$$Y = ax^2 + bx + c, \quad [1]$$

$$Y = a\,e^{-bx} + c\,e^{-dx}, \quad [2]$$

where c is the intercept (% of nontreated control), b is the linear coefficient, and a is the curvilinear coefficient (Equation 1); or $a + c$ is the intercept (% of nontreated control) and b,d quantify the slope (Equation 2; Yoshimura et al. 2006). Data were fitted to the model with the use of a derivative-free nonlinear regression procedure, provided with PROC NLIN (SAS 1999). Regression analyses were performed on treatment means averaged over replications, as recommended by Gomez and Gomez (1984). The resistance factor was calculated as GR_{50} of the HR population divided by GR_{50} of the HS population, where GR_{50} is the dose resulting in a 50% reduction in aboveground biomass relative to the nontreated control.

***ALS* Gene Sequencing.** Following assessment of herbicide resistance, leaf tissue was sampled from HR F_1 progeny: Choiceland (CHO) population, 20 individuals (16 imazethapyr-treated, CHO-1 to CHO-16; four florasulam-treated, CHO-17 to CHO-20); Tisdale (TIS) population, 16 imazethapyr-treated individuals, TIS-1 to TIS-16. The following HS individuals were tissue sampled: Vermilion (VER), six untreated individuals, VER-1 to VER-6; and Minburn (MIN), six untreated individuals, MIN-1 to MIN-6. Samples were freeze-dried. DNA was extracted from freeze-dried leaf tissue (10–20 mg) with the use of Fast DNA SPIN kit (QBioGen, MP Biomedicals, 29525 Fountain Parkway, Solon, OH 44139), according to the manufacturer's instructions. Primers (Table 1) were designed based on two *Galium aparine ALS* sequences available in GenBank: HM006705 (HR) and GU377313 (HS) [complete gene with 2,312 base pairs (bp); Sun et al. 2011], and P3 Primer website (Rozen and Skaletsky 2000). The GenBank HR *G. aparine ALS* sequence has the amino acid substitution of Trp_{574} (TGG) to Gly (GGG).

Polymerase chain reaction (PCR) amplifications were performed with the use of Ready-To-Go (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK) PCR beads with approximately 25 ng of genomic DNA and 400 nM of each primer in a total of 25 μl. PCR was

Table 1. Primers designed in this study for amplifying and sequencing the *ALS* gene.[a]

Primer	5′-3′ sequence
Galium P3_26_F	CACACAGCCTACCACACACC
Galium P3_706_F	ACGCCGATTGTTGAGGTAAC
Galium P3_1352_F	ACTCCATGCTGGAAACAAGG
Galium P3_779_R	CGCGGAATATCCTCAACATT
Galium P3_1371_R	CCTTGTTTCCAGCATGGAGT
Galium P3_2078_R	TCTCCCTCGGTAATCACGTC

[a] Amino acids and nucleotide positions are numbered based on the amino acid sequence of *ALS* from *Arabidopsis* (Sathasivan et al. 1990).

performed in a Mastercycler epGradient under the following conditions: 2 min incubation at 94 C, 40 cycles of 30 s at 94 C, 45 s at 60 C, 1.5 min at 72 C, then 5 min at 72 C. PCR fragments were excised from a 1.2% agarose gel and purified with the use of the QIAquick Gel Extraction kit (QIAGEN Sciences, Germantown, MD) and sequenced directly. Sequencing reactions used 50 ng of purified PCR product, 160 nM of primer, and 0.5 µl of ABI BigDye™ Terminator v. 3.1 (PE Corporation, PE Biosystems, Foster City, CA) in a final volume of 10 µl, and were run on a ABI 3130 DNA Sequencer (Applied Biosystems, Foster City, CA). Primers used for sequencing were the same as those used for PCR amplification. Sequences were determined and compared with the use of Sequencher software v. 4.7 (Gene Codes Corporation, Ann Arbor, MI). Polymorphism or nucleotide heterozygosity was based on the appearance of two peaks at a single nucleotide position on the chromatograms. Amino acids and nucleotide positions were numbered based on the amino acid sequence of *ALS* from mouseear cress [*Arabidopsis thaliana* (L.) Heynh] (Sathasivan et al. 1990).

Results and Discussion

Herbicide Dose-Response Experiments. For the dose-response experiments involving the Tisdale HR false cleavers population, the GR_{50} values for the Vermilion HS population were 4 g ai ha^{-1} (imazethapyr), 0.30 g ai ha^{-1} (florasulam), and 3 g ai ha^{-1} (fluroxypyr) (Figures 1–3). For the dose-response experiments involving the Choiceland HR false cleavers population, the GR_{50} values for the Minburn HS population were 3 g ai ha^{-1} (imazethapyr), 0.33 g ai ha^{-1} (florasulam), and 2.5 g ai ha^{-1} (fluroxypyr) (Figures 4–6). Therefore, both HS populations responded similarly to increasing doses of the three herbicides.

The Tisdale false cleavers population was relatively insensitive to increasing doses of imazethapyr or florasulam. When imazethapyr or florasulam were applied at eight times the field-recommended dose, only a small decline in aboveground biomass was observed. The GR_{50} value for the imazethapyr dose response was greater than 400 g ai ha^{-1} (Figure 1), and greater than 40 g ai ha^{-1} for the florasulam dose response (Figure 2). However, the Tisdale population was susceptible to fluroxypyr, a synthetic auxin herbicide. Thus, the resistant factor for imazethapyr was greater than 100, 130 for florasulam, and 1.3 for fluroxypyr.

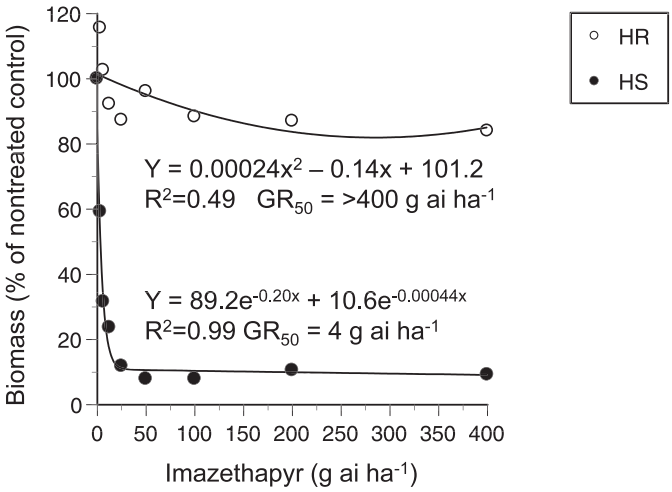

Figure 1. Aboveground biomass response of ALS-inhibitor–resistant (HR: Tisdale) and –susceptible (HS: Vermilion) false cleavers populations to increasing doses of imazethapyr.

For the Choiceland false cleavers population, the GR_{50} value for the imazethapyr dose response was also greater than 400 g ai ha^{-1} (Figure 4), but only 0.32 g ai ha^{-1} for the florasulam dose response (Figure 5). Therefore, in contrast to the Tisdale population, the Choiceland population was susceptible to florasulam. As expected, the Choiceland population was also susceptible to fluroxypyr (Figure 6). For this population, the resistant factor for imazethapyr was greater than 130, 0.97 for florasulam, and 1.0 for fluroxypyr. The Tisdale and Choiceland populations were homogenous resistant to imazethapyr (i.e., 100% of individuals evaluated).

***ALS* Gene Sequencing.** A 1,954-bp region of the *ALS* gene including sites known to confer ALS-inhibitor resistance were sequenced in this study for all individuals listed in Table 2. *ALS* gene sequences were found to be very conserved, with only four base differences observed among the four false

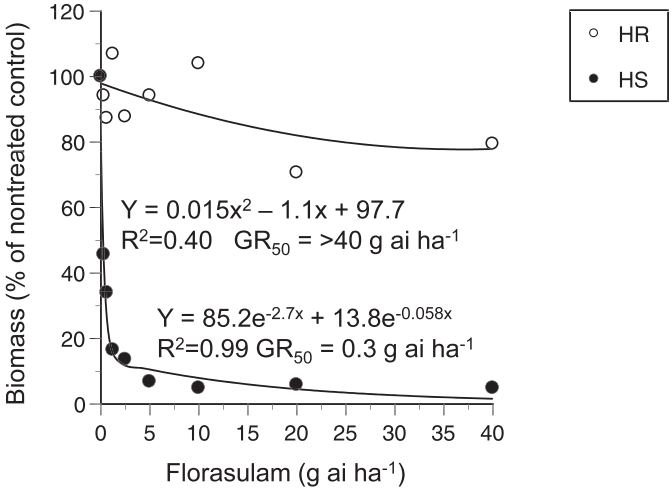

Figure 2. Aboveground biomass response of ALS-inhibitor–resistant (HR: Tisdale) and –susceptible (HS: Vermilion) false cleavers populations to increasing doses of florasulam.

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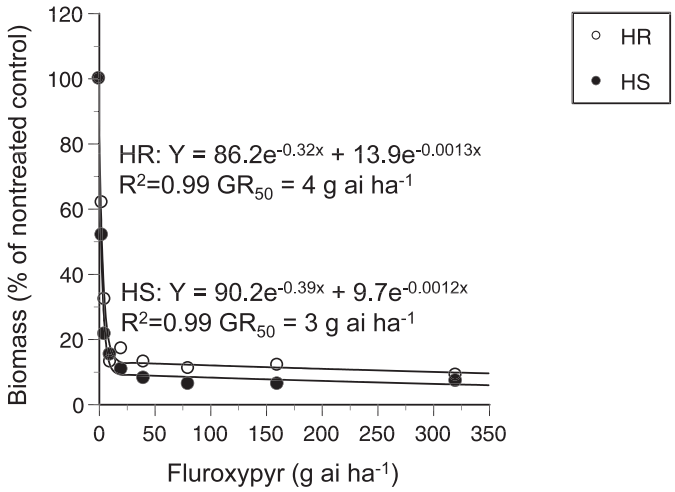

Figure 3. Aboveground biomass response of ALS-inhibitor–resistant (HR: Tisdale) and –susceptible (HS: Vermilion) false cleavers populations to increasing doses of fluroxypyr.

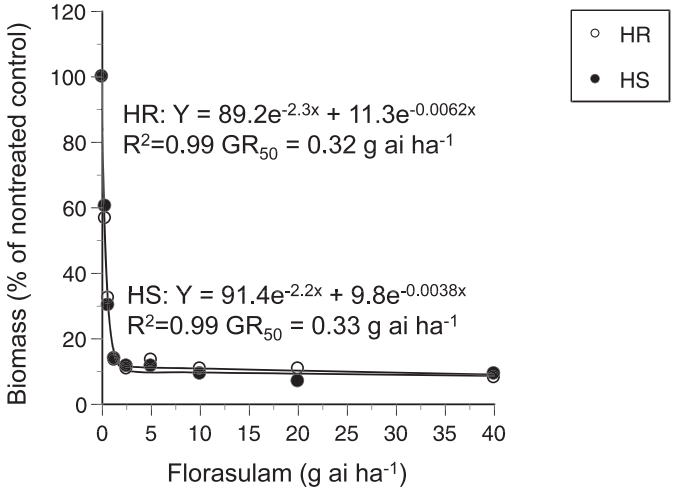

Figure 5. Aboveground biomass response of ALS-inhibitor–resistant (HR: Choiceland) and –susceptible (HS: Minburn) false cleavers populations to increasing doses of florasulam.

cleavers populations sequenced. The first, an A/G synonymous transition, was found at nucleotide position 1458 (Table 2): "A" was observed in HR individuals CHO-1 and CHO-4 to CHO-16, HS individuals VER-1 and VER-2, and HS individuals MIN-1 to MIN-6, whereas "G" was found in HR individuals CHO-2, CHO-3, CHO-17 to CHO-20, HR individuals TIS-1 to TIS-16, and HS individuals VER-3 to VER-6. The (A/G) transition is in the third position, GC (A/G), and codes for Ala_{494} in all populations, and therefore does not confer resistance. Three ALS target-site mutations were observed in the HR Choiceland population (Table 2). The most frequent, a $Ser_{653}Asn$ mutation (AGT-to-AAT substitution), was found in individuals CHO-1 and CHO-4 to CHO-16. A $Trp_{574}Leu$ mutation (TGG-to-TTG substitution) was observed for CHO-2, CHO-3, CHO-17 to CHO-19 (latter a heterozygote and codes for both Trp and Leu), whereas an $Asp_{376}Glu$ mutation (GAT-to-GAA substitution) was discovered in CHO-20. For all 16 individuals from the Tisdale-HR population, a TGG-to-TTG substitution resulted in a $Trp_{574}Leu$ mutation (Table 2). The *ALS* gene sequences of the HS populations Vermilion and Minburn did not exhibit these mutations or amino acid changes (Table 2).

In summary, three previously described mutations known to confer ALS-inhibitor resistance were found within the *ALS* sequences of individuals from the Choiceland-HR population. Mutations $Trp_{574}Leu$ (two individuals screened with imazethapyr, three individuals screened with florasulam), $Ser_{653}Asn$ (14 individuals screened with imazethapyr), and $Asp_{376}Glu$ (one individual screened with florasulam) confer resistance in the Choiceland population, but were not found within one plant. However, the $Trp_{574}Leu$ mutation confers resistance in all 16 individuals of the Tisdale-HR population. In contrast, the $Pro_{197}Leu$ mutation was found in the Alberta

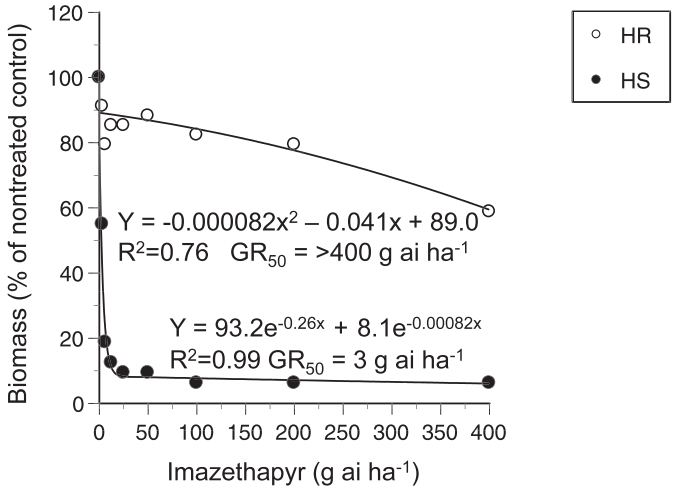

Figure 4. Aboveground biomass response of ALS-inhibitor–resistant (HR: Choiceland) and –susceptible (HS: Minburn) false cleavers populations to increasing doses of imazethapyr.

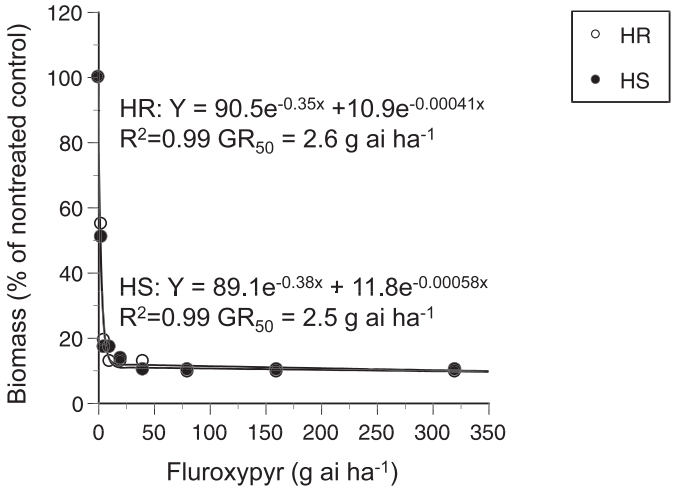

Figure 6. Aboveground biomass response of ALS-inhibitor–resistant (HR: Choiceland) and –susceptible (HS: Minburn) false cleavers populations to increasing doses of fluroxypyr.

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Table 2. Target-site mutations in the *ALS* gene conferring herbicide resistance in two HR false cleavers biotypes from Saskatchewan, Canada.[a]

Population-individual[b]	Resistant phenotype	NP 1458	Amino acid mutations causing HR			
			$Asp_{376}Glu$ GA(T/A)	$Trp_{574}Leu$ T(G/T)G	$Ser_{653}Asn$ A(G/A)T	GenBank no.
CHO-1, CHO-4 to CHO-16	HR	A	GAT Asp_{376}	TGG Trp_{574}	AAT Asn_{653}	JN038046
CHO-2, CHO-3, CHO-17, CHO-18	HR	G	GAT Asp_{376}	TTG Leu_{574}	AGT Ser_{653}	JN038047
CHO-19	HR	G	GAT Asp_{376}	T(G/T)G Trp/Leu_{574}	AGT Ser_{653}	JN038048
CHO-20	HR	G	GAA Glu_{376}	TGG Trp_{574}	AGT Ser_{653}	JN038049
TIS-1 to TIS-16	HR	G	GAT Asp_{376}	TTG Leu_{574}	AGT Ser_{653}	JN038050
VER-1, VER-2	HS	A	GAT Asp_{376}	TGG Trp_{574}	AGT Ser_{653}	JN038051
VER-3 to VER-6	HS	G	GAT Asp_{376}	TGG Trp_{574}	AGT Ser_{653}	JN038052
MIN-1 to MIN-6	HS	A	GAT Asp_{376}	TGG Trp_{574}	AGT Ser_{653}	JN038053

[a] Abbreviations: HR, herbicide resistant; HS, herbicide susceptible; CHO, Choiceland population; MIN, Minburn population; NP, nucleotide position; TIS, Tisdale population; VER, Vermilion population.

[b] Individuals CHO-1 to CHO-16 and TIS-1 to TIS-16 were screened with imazethapyr, whereas individuals CHO-17 to CHO-20 were screened with florasulam; individuals VER-1 to VER-6 and MIN-1 to MIN-6 were untreated.

ALS inhibitor-resistant false cleavers population sequenced by Horsman and Devine (2000).

It is of interest that a different Trp_{574} mutation ($Trp_{574}Gly$) was reported in a Chinese ALS-inhibitor–resistant population of the related cleavers species, catchweed bedstraw (Sun et al. 2011). A comparison of the *ALS* gene sequences of Canadian HS and HR false cleavers populations and the Chinese HS and HR populations of catchweed bedstraw (data not shown) indicated 10 nucleotide differences, only 2 of which resulted in amino acid differences Thr_{73} vs. Ala_{73} and Thr_{383} vs. Ala_{383}, respectively.

Herbicide response in HR populations containing multiple target sites is more difficult to assess than single target sites. The $Trp_{574}Leu$ mutation is known to confer high-level resistance to imidazolinone and triazolopyrimidine class ALS inhibitors (cross-resistance patterns associated with *ALS* mutations reviewed in Beckie and Tardif 2011), which was observed in the dose-response experiments involving the Tisdale false cleavers population. In the 16 originally sequenced individuals screened with imazethapyr from the Choiceland population, 14/16 = 88% possessed the $Ser_{653}Asn$ mutation and 2/16 = 12% the $Trp_{574}Leu$ mutation. The former mutation confers high-level resistance to imidazolinones, but nil to moderate-level resistance to triazolopyrimidines (e.g., florasulam) (Beckie and Tardif 2011). The susceptibility of the Choiceland population observed for all doses in the florasulam trial (i.e., a total of 128 seedlings—eight herbicide doses times four replicates times four seedlings per replicate) would suggest predominance of the $Ser_{653}Asn$ mutation in those treated seedlings. Subsequent sequencing of four survivors of 200 Choiceland seedlings screened with florasulam at 5 g ai ha^{-1} (CHO-17 to CHO-20) indicated that three of them (CHO-17 to CHO-19) had the $Trp_{574}Leu$ mutation and one individual (CHO-20) had the $Asp_{376}Glu$ mutation, both of which confer high-level resistance to triazolopyrimidine class herbicides (i.e., florasulam). The presence of the $Trp_{574}Leu$ mutation in individuals of both HR false cleavers populations indicates that an alternative site-of-action herbicide, such as fluroxypyr in cereal crops, is required for their control.

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