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Mechanism of Resistance to ACCase-Inhibiting Herbicides in Wild Oat (*Avena fatua*) from Latin America

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ABSTRACT: Whole-plant response of two suspected resistant *Avena fatua* biotypes from Chile and Mexico to ACCase-inhibiting herbicides [aryloxyphenoxypropionate (APP), cyclohexanedione (CHD), and pinoxaden (PPZ)] and the mechanism behind their resistance were studied. Both dose−response and ACCase enzyme activity assays revealed cross-resistance to the three herbicide families in the biotype from Chile. On the other hand, the wild oat biotype from Mexico exhibited resistance to the APP herbicides and cross-resistance to the CHD herbicides, but no resistance to PPZ. Differences in susceptibility between the two biotypes were unrelated to absorption, translocation, and metabolism of the herbicides. PCR generated fragments of the ACCase CT domain spanning the potential mutations sited in the resistant and susceptible biotypes were sequenced and compared. A point mutation was detected in the aspartic acid triplet at the amino acid position 2078 in the Chilean biotype and in isoleucine at the amino acid position 2041 in the Mexican wild oat biotype, which resulted in a glycine triplet and an asparagine triplet, respectively. On the basis of in vitro assays, the target enzyme (ACCase) in these resistant biotypes contains a herbicide-insensitive form. This is the first reported evidence of resistance to pinoxaden in *A. fatua*.

KEYWORDS: resistance, ACCase inhibitors, mechanisms, mutation

INTRODUCTION

Wild oat (*Avena fatua* L.) is a major annual grass weed growing in cereal crops (wheat, barley, and oat) in Latin America. Herbicide resistance in *A. fatua* was first recorded in Chile in 1997 and, by 1998, in Mexico. Three cases of resistance have so far been confirmed in Mexico[1] and five in Chile.[2] However, the specific mechanisms behind the resistance of the Mexican and Chilean populations of this species remain unknown. The most widespread instances of herbicide resistance in *Avena* worldwide have been to the aryloxyphenoxypropionate (APP) and cyclohexanedione (CHD) herbicides.

APP and CHD herbicides have been widely used for the highly successful control of grass weed species such as wild oat since their inception in the 1970s and 1980s, respectively.[3] APP and CHD herbicides are two chemically dissimilar classes of herbicides, both of which inhibit chloroplastic acetyl-CoA carboxylase (ACCase) and cause plant death.[4] Pinoxaden (PPZ), a phenylpyrazoline herbicide introduced in 2006, is another ACCase inhibitor. Although PPZ targets ACCase, its chemistry differs from that of APPs and CHDs. Thus, pinoxaden is also applied post-emergence at rates from 30 to 60 g/ha and is effective in controlling a wide range of grass species including wild oat in wheat and other cereal crops.[5]

Resistance to ACCase inhibitors was first reported in 1982.[6] A total of 39 weed species have so far been identified with resistance to ACCase inhibitors, mostly in North America, Australia, and Chile.[6] Repeated use of APP or CHD herbicides has been found to elicit resistance to ACCase inhibitors in a number of grass weed species including *Alopecurus myosuroides*,[7] *Lolium multiflorum*,[8] and *Avena fatua*.[9] The first case of ACCase inhibitor resistance in wild oat was reported in Western Australia in 1985.

The biochemical basis for resistance to ACCase inhibitors in *Avena* spp. has been investigated in Australian,[10] Canadian,[11] and North American populations.[12] Most studies have implicated insensitive ACCase as the agent conferring resistance.[12] Although potential re-establishment of a normal membrane potential after perturbation by herbicides has been investigated,[13] whether this membrane recovery response provides a resistance mechanism or simply has a secondary effect is uncertain.[14] The role of enhanced metabolism in conferring resistance to ACCase inhibitors has only been demonstrated in one population of *Avena* spp., which exhibited resistance to APP diclofop-methyl.[10] The enhanced metabolism of fenoxaprop-*p*-ethyl, another commonly used APP, was investigated, but the herbicide was not found to confer resistance to biotypes of resistant *Avena* spp.[12] Seefeldt et al.[12] found resistance in two wild oat biotypes to originate from ACCase alteration rather than from differential absorption, translocation, or metabolism of diclofop.

Recent research has revealed that certain point mutations in the gene encoding the CT domain of plastidic ACCase are responsible for the enzyme insensitivity to inhibiting herbicides in various grass weeds.[15] Also, a point mutation in the region of the

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Table 1. Resistance-Endowing Plastid ACCase CT Domain Amino Acid Substitutions in Field-Evolved Resistant Grass Weed Species[15]

	resistance spectrum[a]			
amino acid substitution	APP	CHD	PPZ	ref
Ile-1781-Leu	R	R	r	17
Trp-1999-Cys	R	S	—	18
Trp-2027-Cys	R/r	r	—	17
Ile-2041-Asn	R	r	—	17
Ile-2041-Val	S/R	S	—	15
Asp-2078-Gly	R	R	—	17
Cys-2088-Arg	R	R	R	20
Gly-2096-Ala	R	r/S	S	16

[a] R, resistant; S, susceptible; r, low resistance.

plastid ACCase gene encoding the carboxyl transferase (CT) domain has been identified and found to result in Ile-1781-Leu substitution in resistant biotypes of *Alopecurus myosuroides*,[16] *Avena fatua*,[17] *Avena sterilis*,[18] *Lolium multiflorum* [19] and *Lolium rigidum*.[20] Other mutations causing resistance-endowing amino acid substitutions have also been identified including amino acids at 1999 in *Avena sterilis*;[18] 2027 in *Alopecurus myosuroides*,[16] *L. multiflorum*,[21] *L. rigidum*,[20] *Avena sterilis*,[18] and *Phalaris paradoxa*;[22] 2078 in *Alopecurus myosuroides*,[16] *A. sterilis*,[18] and *P. paradoxa*;[22] 2088 in *L. rigidum*;[20] and 2096 in *Alopecurus myosoroides*.[16] In all instances, substitution rendered ACCase in resistant grass weeds insensitive to inhibiting herbicides. Table 1 shows the main mutations found in various weeds and the resistance pattern for APP and CHD herbicides and PPZ.

In 1997, wild oat with resistance to diclofop and clodinafop-propargyl was found on two farms in Quino and Lautauro (IX region, Chile). Since then, the number of diclofop-resistant wild oat species in wheat and barley fields has increased in this country.[23] In 1998, wild oat was reported to be resistant to fenoxaprop-*p*-ethyl and clodinafop-propargyl in wheat fields from a county of Guanajuato, Mexico.[24,25] The underlying resistance mechanisms were not elucidated.

The primary aims of this study were (1) to confirm resistance to ACCase inhibitors (APP, CHD, and PPZ) in two wild oat biotypes from Chile and Mexico; (2) to elucidate the mechanism behind their resistance from penetration, metabolic, and ACCase assays; and (3) to determine the molecular basis for resistance to ACCase inhibitors in both biotypes.

MATERIALS AND METHODS

Chemicals. The herbicides and reagents used included technical grade fenoxaprop acid, 2-[4-[(6-chloro-1,3-benzoxazol)-2-yloxy]phenoxy]-propionic acid (*R*), cyhalofop acid, 2-[4-(4-cyano-2-fluorophenoxy)phenoxy]propanoic acid (*R*), propaquizafop, diclofop-methyl (*R,S*) 2-(4-(2,4-dichlorophenoxy)phenoxy)methyl propionate, and sethoxydim [(±)2-(1-ethoxyiminobutyl) 5-(2-ethylthiopropyl) 3-hydroxycyclohex-2-one]. The commercial herbicide formulation Puma Super (5.5% w/v fenoxaprop-*p*-ethyl EC; Clincher, 20% w/v cyhalofop-butyl EC; Iloxan, 36% w/v diclofop-methyl EC; Agil, 10% w/v propaquizafop EC, Focus Ultra, 10% w/v cycloxydim EC; Centurion Plus, 12% w/v clethodim w/v; Splendor SC, 25% w/v tralkoxydim) was used for dose−response assays. All other reagents were of analyticalgrade.

Plant Material. Seeds of the resistant (R) biotypes of *A. fatua* (wild oat) were collected in winter wheat fields of El Bajío, Mexico, in 2003. The R biotype had exhibited resistance to fenoxaprop-*p*-ethyl, clodinafop-propargyl, and diclofop-methyl; and the susceptible (S) populations originated from fields in Pénjamo, Guanajuato Mexico, which had never been treated with herbicides. Seeds from the Chilean biotype were collected in winter wheat crops from Temuco. The R biotype had exhibited resistance to diclofop-methyl and clodinafop-propargyl, and the susceptible (S) populations originated from Lautaro (IX Region).

Growth Assays. Seeds of the R and S biotypes of *Avena fatua* were dehulled by hand and germinated according to ref 1. Seedlings were planted in 8 × 8 × 10 cm pots (five plants per pot) that were filled with a peat−sandy loam potting mixture in a 1:2 w/w ratio and placed in a growth chamber (28/18 °C day/night, 16/8 h day/night cycle, light at a photosynthetic photon-flux density of 850 μmol m^{-2} s^{-1}, and 80% relative humidity).

Dose−Response Assays. Specimens of the R and S biotypes at the 3−4-leaf growth stage were sprayed by means of a laboratory track sprayer equipped with a Tee Jet 8002E-VS flat-fan nozzle delivering a spray volume of 300 L ha^{-1} at 200 kPa.

Herbicides were applied at the following rates: fenoxaprop-*p*-ethyl at 5−60 g ai ha^{-1} to biotype S and at 80−180 g ai ha^{-1} to biotype R; clethodim at 40−200 g ai ha^{-1} to biotype S and at 80−400 g ai ha^{-1} to biotype R; cyhalofop-butyl at 20−70 g ai ha^{-1} to biotype S and at 100−900 g ai ha^{-1} to biotype R; tralkoxydim at 200−500 g ai ha^{-1} to biotype S and at 300−600 g ai ha^{-1} to biotype R; cycloxydim at 20−100 g ai ha^{-1} to biotype S and at 50−400 g ai ha^{-1} to biotype R; diclofop-methyl at 72−360 g ai ha^{-1} to biotype S and at 1080−1800 g ai ha^{-1} to biotype R; and propaquizafop at 20−100 g ai ha^{-1} to biotype S and at 300−500 g ai ha^{-1} to biotype R. Above-ground fresh weight per pot was determined 21 days after treatment (DAT), and the results were expressed as percentages of the value for the untreated control. The herbicide rates needed to inhibit plant growth by 50% with respect to the untreated control (ED_{50}) were determined for each biotype, and the resistant factor (RF) was computed as $ED_{50}(R)/ED_{50}(S)$.[26] Treatments were conducted in triplicate and arranged in a completely randomized design with four replications per rate. Data were pooled and fitted to a nonlinear, log−logistic regression model defined by

$$Y = c + \{(d - c)/[1 + (x/g)^b]\}$$

where Y is the fresh above-ground weight, expressed as a percentage of the untreated control; c and d are the coefficients corresponding to the lower and upper asymptote, respectively; b is the slope of the curve; g is the herbicide rate at the point of inflection halfway between the upper and lower asymptote; and x (the independent variable) is the herbicide rate. The regression analysis was conducted by using the statistical software Sigmaplot 10.0.

[^{14}C]DM Penetration and Translocation. [2,4-*dichlorophenoxy*-U-^{14}C]DM was mixed with commercially formulated DM to prepare an emulsion with a specific activity of 37.9 Bq mg^{-1} and a DM concentration of 6.6 g L^{-1} (corresponding to 1.0 kg ha^{-1} DM at 150 L ha^{-1}). This formulation of labeled herbicide was applied to the adaxial surface of the second leaf in each plant in four 0.5 μL droplets by means of a Hamilton PB-600 microapplicator. A total of 833.33 Bq was applied to each plant.

Plants were harvested in batches of three after variable lengths of time (0, 3, 6, 12, 24, and 48 h) following application of the herbicide and split into treated leaves and the remainder of the shoots. Roots were discarded because herbicide translocation from leaves to roots had previously been found to be undetectable in wheat and *L. rigidum*.[27,28] Unabsorbed [^{14}C]DM was removed from the leaf surface by washing the treated area with 1.5 mL of acetone. Washes from each batch were pooled and analyzed by liquid scintillation spectrometry (LSS) on a Beckman LS 6000 TA instrument. Plant tissue was dried at 60 °C for 48 h and combusted in a sample oxidizer (Packard 307). Evolved $^{14}CO_2$ was

trapped and counted in 10 mL of Carbosob/Permafluor E^+ (3:7 v/v) (Packard Instruments Co.). Radioactivity was quantified by LSS and expressed as a percentage of recovered radioactivity, using the following expression: % absorption = [^{14}C in combusted tissue/(^{14}C in combusted tissue + ^{14}C in leaf washes)] × 100. The experiment was repeated three times. For translocation tests, [^{14}C]DM was applied to the second leaf as described above. At intervals of 6, 12, 24, and 48 h after herbicide application, the treated (second) leaf and untreated (first and third) leaves were harvested separately. The treated leaf was rinsed and unabsorbed radiolabel quantified by LSS as described above.

The treated leaf and untreated leaves were oven-dried at 60 °C for 2 days, combusted in a sample oxidizer as described above, and analyzed for radioactivity by LSS. Percent diclofop translocation was expressed as (dpm in shoot tissue outside the treated leaf/dpm in rinsed, treated leaf + dpm in shoot tissue outside the treated leaf) × 100.

Metabolic Tests with [^{14}C]DM. [^{14}C]DM metabolism was studied in leaf tissue from two R biotypes and S plants at the two-leaf stage as in the penetration studies. Labeled herbicide was applied to the adaxial surface of the second leaf in 10 0.5 μL droplets by using a microapplicator. A total of 5000 Bq was used on each plant. Plants of the R biotypes, and of S, were sampled 6, 12, 24, and 48 h after treatment (HAT). Treated leaves were washed as described above. An aliquot of leaf wash solution was assayed for radioactivity, and the remaining was solution stored at −20 °C until analysis. Treated plants were split into roots and shoots. The shoots from each plant were powdered in liquid nitrogen by using a mortar and pestle. The powder was extracted with 4 mL of 80% methanol at 4 °C and the homogenate centrifuged at 20000*g* for 20 min. The resulting pellet was washed with 80% methanol until no further ^{14}C was extracted. The pellets were oven-dried and combusted as described above. The supernatants were combined, evaporated to dryness at 40 °C under a stream of N_2 at 10 kPa and redissolved in 500 μL of 80% methanol. DM and its metabolites in the supernatant were identified by thin-layer chromatography on 20 cm × 20 cm, 250 μm silica gel plates (Merck; silica gel 60), using a 150:7:7 v/v/v toluene/ethanol/acetic acid mixture as mobile phase. Radioactive zones were detected with a radiochromatogram scanner (Berthold LB 2821), and their chemical nature was identified by comparing their R_f values with those for standards (DM, 0.70; diclofop acid, 0.44; hydroxy-diclofop, 0.34; polar conjugates, 0.00). The experiment was repeated three times.

Enzyme Extraction. The enzyme ACCase was isolated by using the protocol of ref 28. Leaves (6 g fresh weight) from the R and S biotypes of *A. fatua* were harvested from plants at the 3−4-leaf stage, ground in liquid N_2 in a mortar, and supplied with 24 mL of extraction buffer (0.1 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid−KOH, pH 7.5, 0.5 M glycerol, 2 mM EDTA, and 0.32 mM PMSF). The homogenate was mixed with a magnetic stirrer for 3 min and filtered sequentially through four layers of cheesecloth and two layers of Miracloth. The crude extract was centrifuged at 24000*g* at 4 °C for 30 min. The supernatant was fractionated with $(NH_4)_2SO_4$ and centrifuged at 12000*g* at 4 °C for 10 min. All material precipitating between 35 and 45% $(NH_4)_2SO_4$ saturation was resuspended in 1 mL of S400 buffer [0.1 M Tricine−KOH (pH 8.3), 0.5 M glycerol, 0.05 M KCl, 2 mM EDTA, and 0.5 mM DTT]. The clarified supernatant was applied to a desalting column previously equilibrated with S400 buffer, and ACCase was subsequently eluted from the column with 2 mL of the same buffer.

ACCase Assays. Enzyme activity was determined by measuring ATP-dependent incorporation of NaH[^{14}C]O_3 into an acid-stable ^{14}C-labeled product. The reaction product had previously been identified as [^{14}C]-malonyl-CoA.[29] Assays were conducted in 7 mL scintillation vials containing 0.1 M Tricine−KOH (pH 8.3), 0.5 M glycerol, 0.05 M KCl, 2 mM EDTA, 0.5 mM DTT, 1.5 mM ATP, 5 mM $MgCl_2$, 15 mM NaH[^{14}C]O_3 (1.22 MBq μmol^{-1}), 50 μL of the enzyme fraction, and 5 mM acetyl-CoA in a final volume of 0.2 mL. Activity was assayed at 34 °C for 5 min, and the reaction stopped by adding 30 μL of 4 N HCl.

Table 2. Primers Used for Regions A and B

name	sequence (5′ to 3′)	position
UpACCase-n591	AAGGATGGGCGAAGACAGTAGTTA	5993−6016
LowACCase-n591	CTCCATCAGATAGGCTTCCATTT	6583−6561
UpACCase-n600	GCGTGCTGCTGGGCTGAAT	5058−5076
LowACCase-n600	CCGGTCAAAATAATGGGCTGGTC	5657−5653

A piece of filter paper was added to the reaction vial and the sample dried at 40 °C under an air stream. After the sample was dried, 0.5 mL of 1:1 v/v ethanol/water mixture was added to the vial, followed by 5 mL of scintillation cocktail. Radioactivity was determined by LSS. Background radioactivity, measured as acid-stable counts (dpm) in the absence of acetyl-CoA, was subtracted from the result for each treatment. One unit of ACCase activity was defined as 1 μmol of malonyl CoA formed per minute. Herbicide concentrations resulting in a 50% inhibition of enzyme activity (I_{50} values) were determined in crude extracts for fenoxaprop, diclofop, clethodim, cycloxydim, and pinoxaden. Data were pooled and fitted to the above-described log−logistic model. All tests were repeated three times.

Molecular Analysis. *DNA Extraction and PCR Amplification.* Seeds of the R and S biotypes of *Avena fatua* were germinated and grown as described above. Seedlings at the 3−4-leaf stage were treated with fenoxaprop-*p*-ethyl at a rate of 200 g ha^{-1}. Shoot material of individual plants from the resistant and susceptible populations was taken before treatment for use in the molecular analysis. This rate killed all plants in the susceptible biotype within 21 DAT.

Genomic DNA was extracted from shoot tissues by using the Qiagen DNA Extraction Kit. In all cases, DNA was quantified with NanoDrop and immediately used for PCR reaction or stored at −20 °C until use.

Primers were designed to amplify regions in the CT domain known to be involved in the sensitivity to ACCase-inhibiting herbicides. DNA fragments encoding part of the CT domain in plastid ACCase were designated on the basis of DNA sequences of *Avena fatua* (GenBank accession no. AF231334, AF231335, and AF231337). Two sets of primers spanning all five known mutation sites in regions A (1781) and B (2027, 2041, 2078, and 2096), designated with GenBank accession no. AJ310767, were used (Table 2). The 591 and 600 bp fragments, which contained the information in all relevant alleles responsible for target-site resistance, were amplified with the primer pairs Up/LowACCase-n600 and Up/LowACCase-n591. The 600 bp fragment contained the information for the Leu_{1781} mutation and the 591 bp fragment the information for the other four potential mutations, at positions 2027, 2041, 2078, and 2096 (designated with EMBL accession no. AJ310767). PCR products were purified directly or from agarose gel with Wizard SV gel and PCR Clean-up system (Promega) for sequencing at the central facilities of the University of Córdoba.

RESULTS

Dose−Response Assays. The ED_{50} values and resistance factors (RFs) in each plant group are listed in Table 3. The APP herbicides (fenoxaprop, diclofop, and propaquizafop) failed to control the resistant biotypes at their maximum rate. The whole-plant dose−response assays showed that the cross-resistance levels and patterns for ACCase inhibitors differed between the wild oat biotypes (Table 3). Thus, the biotype from Chile was resistant to cyhalofop, fenoxaprop, propaquizafop, and diclofop; their RFs were 4, 5.1, 5.1, and 8.7, respectively. On the other hand, the biotype from Mexico was resistant to propaquizafop, cyhalofop, diclofop, and fenoxaprop, with RF values of 7.8, 8, 9.3, and 11.6, respectively. The biotype from Mexico was resistant to CHD herbicides (Table 3), with RFs of 2.1, 7.9, and 8.1 for

Table 3. Parameters of the Log−Logistic Equation[a] Used To Calculate the Herbicide Rate Required for a 50% Reduction of Fresh Weight (ED_{50}) in the R and S Biotypes of *A. fatua*

herbicide	biotype	*d*	*c*	*b*	ED_{50} (g ai ha^{-1})	RF
fenoxaprop-*p*-ethyl	CHS	100.00	0.37	1.21	38.82	–
	CHR	98.91	0.16	3.11	201.79	5.19
	MXS	100.00	0.61	1.01	15.68	–
	MXR	99.96	5.16	4.9	182.25	11.62
cyhalofop-butyl	CHS	99.74	13.25	1.54	48.21	–
	CHR	100.00	8.29	1.57	194.75	4.03
	MXS	99.82	3.95	1.46	72.77	–
	MXR	100.00	3.68	1.25	586.55	8.06
diclofop-methyl	CHS	98.82	2.37	1.52	137.76	–
	CHR	100.00	4.15	1.44	1208.14	8.76
	MXS	100.00	1.21	1.15	121.23	–
	MXR	99.98	1.82	1.19	1138.20	9.38
propaquizafop	CHS	100.00	4.28	4.36	38.62	–
	CHR	99.81	7.94	1.71	200.16	5.18
	MXS	100.00	4.03	1.31	11.39	–
	MXR	99.98	8.01	2.76	89.43	7.85
clethodim	CHS	100.00	3.46	2.72	38.78	–
	CHR	99.19	2.71	3.27	201.24	5.18
	MXS	100.00	1.03	2.31	11.62	–
	MXR	100.00	1.24	2.87	92.19	7.93
cycloxydim	CHS	100.00	1.05	2.63	38.72	–
	CHR	98.79	3.38	1.98	201.18	5.19
	MXS	99.97	3.18	2.52	11.72	–
	MXR	100.00	1.53	1.44	90.3	2.11
tralkoxydim	CHS	99.97	1.58	2.98	80.64	–
	CHR	100.00	7.83	1.58	418.91	5.19
	MXS	98.97	0.16	3.27	42.73	–
	MXR	99.00	3.44	1.71	350.26	8.19
pinoxaden	CHS	100.00	0.32	1.73	11.03	–
	CHR	99.73	0.19	3.09	29.64	2.68
	MXS	99.17	1.17	2.04	10.87	–
	MXR	98.60	0.36	3.18	10.93	1.00

[a] $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where Y is the fresh above-ground weight, expressed as a percentage of the value for the untreated control, x (independent variable) is the herbicide rate, c and d are the coefficients corresponding to the lower and upper asymptotes, respectively, b is the slope of the curve, and g is the herbicide rate at the point of inflection halfway between the upper and lower asymptotes. At $c = 0$ and $d = 100$, g equals ED_{50}. Data were pooled and fitted to a nonlinear regression model.

cycloxydim, clethodim, and tralkoxydim, respectively. The resistant biotype from Chile exhibited an RF of around 5.1 for the three CHDs and an RF of 2.68 for PPZ.

[^{14}C]DM Penetration and Translocation. There were no significant differences in [^{14}C]DM penetration between the R and S biotypes of *Avena fatua* (results not shown). About 75% of

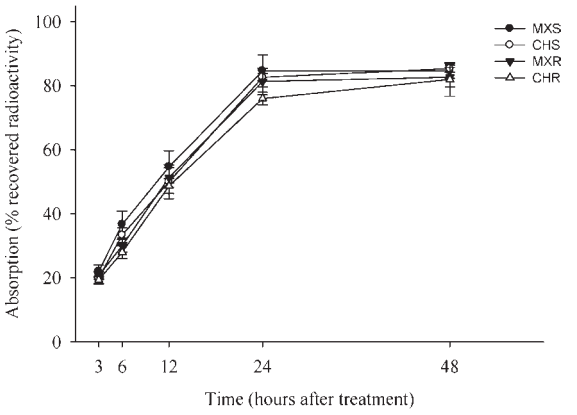

Figure 1. Absorption of DM in different *Avena fatua* biotypes: susceptible biotypes from Mexico (MXS) and Chile (CHS) and resistant biotypes from Mexico (MXR) and Chile (CHR).

Table 4. Radiolabel Translocation from the Treated Leaf in Resistant and Susceptible Biotypes of *A. fatua* 48 HAT with [^{14}C]DM[a]

		translocation (% of absorbed)		
biotype	% absorption	treated leaf	rest of plant	roots
CHS	86.3 ± 3.6	90.4 ± 2.3a	6.4 ± 1.2b	3.2 ± 0.8c
CHR	88.2 ± 4.8	91.8 ± 3.1a	7.1 ± 3.2b	1.1 ± 0.3c
MXS	85.6 ± 3.2	88.6 ± 4.8a	10.3 ± 4.2b	1.1 ± 0.6c
MXR	84.8 ± 5.2	90.8 ± 6.4a	8.7 ± 2.1b	0.5 ± 0.2c

[a] The experiment was conducted in triplicate. Means (n = 8) ± SE. Means in a column followed by the same letter were not significantly different at the 5% probability level as per Tukey's test.

all recovered radioactivity had penetrated into leaf tissue in the R and S wild oat biotypes 24 HAT. Maximum penetration was recorded 24 HAT, after which no significant accumulation was observed (Figure 1).

Both resistant and susceptible biotypes exhibited little translocation of [^{14}C]DM from treated leaves 48 HAT, most of the absorbed herbicide (>80%, Table 4) remaining intact on the leaves in all biotypes. Therefore, differential herbicide translocation is unlikely to contribute to resistance to DM in the resistant *Avena fatua* populations from Chile and Mexico.

Metabolic Studies Using [^{14}C]DM. The pattern of DM metabolism was qualitatively similar in the resistant and susceptible biotypes of *A. fatua*. DM is de-esterified by an esterase enzyme to diclofop acid, which is then further metabolized to other compounds that are more polar than the parent substance.[28] Such compounds are mainly sugar ester conjugates of diclofop acid and sugar conjugates of hydroxy-diclofop. These metabolites (DM, diclofop acid, and polar conjugates) were detected in all biotype assays (results not shown). The absence of significant differences in DM absorption, translocation, and metabolism between the R and S biotypes suggests that these processes by themselves cannot account for herbicide resistance in this *Avena fatua*.

Enzyme Activity. ACCase in the R biotype from Mexico was about 3 times more resistant to fenoxaprop, diclofop, clethodim, and cycloxydim than in the susceptible biotype (Table 5). The resistant factors for ACCase preparations of the R biotype from

Chile ranged from 2 (clethodim) to 5 (fenoxaprop). On the basis of these results, resistance to these herbicides in the R biotypes might be related to a mutation in the ACCase gene diminishing the enzyme sensitivity to the herbicides. The RF value for the new herbicide pinoxaden of the R biotype from Mexico was 1.3, whereas that of the R biotype from Chile was 16.4, the difference possibly being the result of one of their resistance mechanisms.

Molecular Analysis. The nucleotide sequences of the B region for the resistant population from Mexico differed from that of the S population in a single nucleotide substitution (G/C), namely that of Ile 2041 with Asn (Figure 2). The resulting sequences also revealed that the plants in the resistant population from Chile included Asp 2078 to Gly substitution caused by a G/A transition. No other mutations were found at positions 1781 and 2096.

Table 5. Parameters of the Log−logistic Equation[a] Used To Calculate the Herbicide Concentration Required for a 50% Reduction of ACCase activity (I_{50}) in the R and S Biotypes of *Avena fatua*

herbicide	biotype	d	c	b	I_{50} (μM)	RF
fenoxaprop	CHS	100.00	12.17	0.91	0.73	
	CHR	100.00	11.82	0.93	3.94	5.3
	MXS	100.00	1.17	2.17	0.90	
	MXR	100.00	2.81	3.64	2.71	3
diclofop	CHS	100.00	12.64	1.18	31.18	
	CHR	99.92	4.27	1.04	117.23	3.7
	MXS	97.29	2.04	2.04	35.19	
	MXR	100.00	1.69	1.93	108.21	3
clethodim	CHS	98.67	6.27	1.36	2.14	
	CHR	100.00	10.46	0.92	4.87	2.3
	MXS	100.00	2.19	2.78	3.81	
	MXR	98.37	3.01	0.95	8.69	2.2
cycloxydim	CHS	100.00	4.18	1.73	4.43	
	CHR	100.00	3.29	0.88	16.84	3.8
	MXS	100.00	8.23	1.16	7.83	
	MXR	100.00	8.51	2.09	19.76	2.5
pinoxaden	CHS	100.00	1.02	1.16	0.28	
	CHR	100.00	2.57	1.18	4.6	16.4
	MXS	100.00	2.98	2.16	0.33	
	MXR	100.00	0.91	0.98	0.44	1.3

[a] $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where Y is the fresh above-ground weight, expressed as a percentage of the value for the untreated control; x (independent variable) is the herbicide rate; c and d are the coefficients corresponding to the lower and upper asymptote, respectively; b is the slope of the curve; and g is the herbicide rate at the point of inflection halfway between the upper and lower asymptotes. At $c = 0$ and $d = 100$, g equals I_{50}. Data were pooled and fitted to a nonlinear regression model

DISCUSSION

This study examined in detail the mechanism behind the resistance of *Avena fatua* biotypes from Chile and Mexico to ACCase-inhibiting herbicides with emphasis on foliar uptake, translocation, and metabolism, ACCase enzyme activity, and detection of ACCase mutations at DNA level.

An *A. fatua* biotype from Chile and another from Mexico were assayed for resistance to APP, CHD, and PPZ herbicides at the whole-plant level. Dose−response assays (Table 3) confirmed that the two biotypes were resistant to all herbicides; by exception, the biotype from Mexico was not resistant to the new herbicide pinoxaden (PPZ). The *Avena fatua* biotype from Mexico was highly resistant to the APP and CHD herbicides, but was susceptible to pinoxaden, its I_{50} value being similar to that for the susceptible biotype.

Grass weeds have frequently exhibited cross-resistance to APP and CHD herbicides.[30−32] By using a Petri dish bioassay, Bourgeois et al.[33] identified three major types of cross-resistance in *Avena* species, namely (A) resistance to APP herbicides and little or no resistance to CHD herbicides; (B) low resistance to all herbicides; and (C) comparatively high resistance to all herbicides. None of the 82 *Avena* lines they studied had high CHD resistance and low APP resistance. Our *Avena fatua* biotype from Chile is the first phenylpyrazolin-resistant biotype found to possess a high resistance to pinoxaden (Table 3).

Differences in absorption and translocation cannot by themselves explain those in herbicide field performance between the R biotypes of *Avena fatua*. This is another case when *Avena fatua* resistance is not a result of low DM penetration, as previously found in other grass weeds.[11,34−36]

Overall, differences in herbicide translocation between the R and S biotypes from Chile and Mexico were not significant. The acid form of APP is widely assumed to be the translocated form of the herbicide. Our results are consistent with previous studies indicating that grasses exhibit little translocation of diclofop.[35]

Diclofop-methyl metabolism was both quantitatively and qualitatively similar in all *Avena fatua* biotypes. Similar results were previously obtained by ref 28 in *L. rigidum* from Spain.

The resistance factors at the enzyme level for the biotypes from Mexico and Chile confirmed the reduced ACCase sensitivity in the R biotypes relative to the S biotypes. This suggests that a less sensitive form of the target enzyme, acetyl-CoA carboxylase, confers cross-resistance to ACCase-inhibiting herbicides in these *Avena fatua* biotypes. The two biotypes resistant to APP and CHD behaved similarly to *Lolium* spp., *Avena fatua*, and other grass weeds.[29]

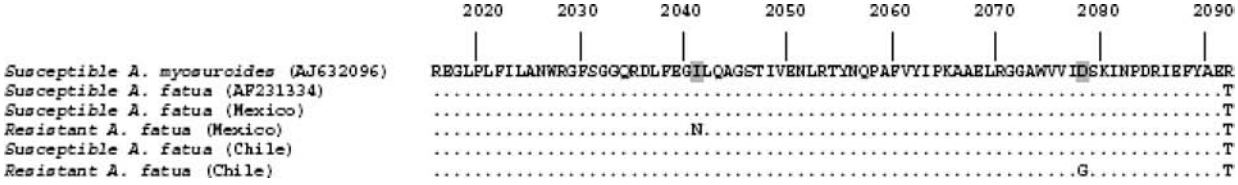

Figure 2. Alignment of partial amino acid sequences of chloroplastic homomeric ACCase from various grass species. The substitutions in the two resistant populations of *Avena fatua* from Mexico and Chile are shown in bold, underlined type.

These instances of grass resistance to APP and CHD herbicides have been related to a reduced sensitivity of the target enzyme. The nature and genetic profile of the ACCase mutation in *Avena fatua* are still to be elucidated.

Consistent with the previous results of ref 8, this paper confirms that wild oat is resistant to pinoxaden; these authors found *L. multiflorum* to exhibit 20% cross-resistance to pinoxaden. Further instances of wild oat resistance to this herbicide could be detected in the future given the widespread occurrence of APP-resistant wild oats in Chile.

An Asp-2078-Gly substitution in the R biotype from Chile conferred resistance to APP and CHD herbicides, as well as to pinoxaden (PPZ). An identical mutation was previously reported in *Alopecurus myosuroides*,[16] *L. multiflorum*,[21] *L. rigidum*,[20] and *P. paradoxa* [22] that induced resistance of ACCase in these weeds to all chemical groups in the previous herbicides. Therefore, one can expect plants of the R biotype to be resistant to PPZ as well as to APP and CHD herbicides. It has also been confirmed that the Ile-2041-Asn mutation in the R biotype from Mexico confers resistance to APP and CHD herbicides, but not to pinoxaden. An identical pattern (i.e., resistance to APP and CHD herbicides) was previously identified in other grass species including *Alopecurus myosuroides*,[16] *P. paradoxa*,[22] and *L. rigidum*,[20] and also in a population of *Avena sterilis*. Resistance to PPZ herbicides was not studied in any of these species.[15]

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