Research Article

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Broad resistance to acetohydroxyacid-synthase-inhibiting herbicides in feral radish (*Raphanus sativus* L.) populations from Argentina

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Abstract

BACKGROUND: Soon after the commercial release of sunflower cultivars resistant to imidazolinone herbicides, several uncontrolled feral radish (*Raphanus sativus* L.) populations were found in south-eastern Buenos Aires, Argentina. These populations were studied in field, glasshouse and laboratory experiments aiming to characterise their resistance profile and to develop management tools.

RESULTS: Three feral radish accessions were highly resistant to ten active ingredients of five families of acetohydroxyacid synthase (AHAS)-inhibiting herbicides. Sequence analysis of the AHAS gene detected a Trp574Leu mutation in all resistant accessions. One accession with an intermediate level of resistance was heterozygous for this mutation, probably owing to gene exchange with a susceptible subpopulation located in the field margin. Herbicide-resistant and herbicide-susceptible radish could be controlled in sunflower by alternative herbicides.

CONCLUSION: This is the first report of feral radish with resistance to herbicides belonging to all the AHAS-inhibiting herbicide families, conferred by Trp574Leu mutation in the AHAS gene. An appropriate herbicide rotation with alternative herbicides such as fluorochloridone or aclonifen and an increase in the diversity of cropping systems are important for minimising the prevalence of these biotypes.

Keywords: *Raphanus sativus*; feral radish; herbicide resistance; AHAS; imazethapyr; metsulfuron-methyl

1 INTRODUCTION

Raphanus raphanistrum L. (wild radish) and *R. sativus* L. (radish) are cosmopolitan weeds in several crops owing to a high reproductive capacity and adaptation to a wide range of habitats.[1,2] Both species have been described as winter annuals or biennials, but *R. raphanistrum* might be able to germinate in the summer and thus is considered to be a facultative species.[1,3,4]

Raphanus sativus is an ancient crop, domesticated for its edible roots, that is not listed as a wild plant in any flora. It is suspected that weedy radish populations might have originated as escapes from cultivation.[4–6] Feral *R. sativus* is noxious in temperate zones of the Americas,[4–8] but it is also found in Europe and East Asia.[4,6,9]

Raphanus raphanistrum, a probable ancestor of radish, grows in natural habitats in Eurasia, where it is native, and it is a successful invader almost worldwide. In Australia and the southern United States, *R. raphanistrum* is considered to be one of the most troublesome weeds in winter crops.[1,10] High densities of wild radish (more than 60 plants m^{-2}) reduced wheat (*Triticum aestivum* L.) and canola (*Brassica napus* L.) yields by 50 and 91% respectively.[11,12]

In Argentina, *R. sativus* has been considered to be invasive species since the 1930s.[13] It is a weed of wheat and other winter cereals, maize, canola, flax, potato and forage crops. The seeds of *R. sativus* are usually considered to be impurities in cereal grains and oilseeds.[7] This species is widely distributed in the south-east of Buenos Aires Province,[14] and it is also an important weed in Paraguay, Chile and Brazil.[4,7,8] The interference of *R. sativus* in soybean [*Glycine max* (L.) Merr.] was evaluated in Brazil, and the presence of more than 40 plants m^{-2} of feral radish reduced the soybean yield by 15%.[15]

In the last decade, the number of herbicide-resistant biotypes of *Raphanus* spp. has increased, including those of *R. raphanistrum* with cross-resistance to acetohydroxyacid synthase (AHAS)-inhibiting herbicides from Australia, South Africa and Brazil and with multiple resistance across several modes of

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action from Western Australia.[16–21] A biotype of feral radish with herbicide resistance to AHAS-inhibiting herbicides was reported in Brazil,[8] Chile[22] and Argentina.[23]

AHAS, also referred to as acetolactate synthase (ALS), is the first enzyme in the biosynthesis of the branched-chain amino acids isoleucine, valine and leucine.[24,25] Inhibition of AHAS leads to the starvation of these amino acids in the plant and causes plant death. Five commercially available chemical families of herbicides share AHAS as their target site: sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinylbenzoates (PBs) and sulfonylaminocarbonyltriazolinones (SACTs).[24,26]

AHAS-inhibiting herbicides are widely used because of their low rate, low environmental impact, low mammalian toxicity, wide crop selectivity and high control efficacy.[24,25] The genetic resistance to these herbicide families is generally due to a reduction in the sensitivity of the target site conferred by one of several mutations within the AHAS gene.[27,28] To date, 26 amino acid substitutions at eight sites in the AHAS gene conferring herbicide resistance to field-selected weed biotypes have been identified. These consist of Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653 and Gly654 (amino acid numbering based on the *Arabidopsis thaliana* L. AHAS sequence); four of them (those underlined above) were identified in biotypes of *R. raphanistrum*.[27–31] At present there are more than 140 weed species that are resistant to AHAS-inhibiting herbicides, more than to any other herbicide group.[22,28]

Non-target-site resistance to AHAS-inhibiting herbicides has also been found in weed biotypes. For non-target-site resistance, the amount of herbicide reaching AHAS is reduced below the lethal level, allowing plant survival.[28,32] This mechanism has generally resulted in a low magnitude (less than tenfold) of cross-resistance to herbicides with different modes of action.[27] Very few cases of non-target-site resistance to AHAS-inhibiting herbicides have been identified and studied, especially in dicotyledonous weed species, but it is very widespread in Australian *Lolium rigidum* Gaudin populations. There has been only one well-characterised case in the dicotyledonous weed *Sinapis arvensis* L.[28,32,33]

Sunflower (*Helianthus annuus* L.) is a traditional oil crop in Argentina, with around 2 million ha planted over the last decade, and is an important crop for world trade.[34,35] Weeds are one of the most important limitations in sunflower production, and the availability of selective herbicides is limited, especially under no-tillage systems. The available post-emergence sunflower herbicides in sunflower crops are mainly graminicides for grass weed control. The development of sunflower hybrids with resistance to IMI herbicides has enabled the control of a broad spectrum of weeds, including several dicotyledonous species.[36,37]

Imidazolinone-resistant sunflower varieties were first commercialised as Clearfield sunflower in Argentina in 2003,[36] and this technology is currently used in more than 45% of the planted area (BASF 2014, http://www.agro.basf.com.ar/Prensa_Detalle.aspx?id = 56&origen = prensa). Almost 10 years after the commercial release of these varieties, some fields were seriously invaded by feral radish. The failure of IMI herbicides to control these feral radish biotypes was reported in at least five sunflower crops in fields throughout south-eastern Buenos Aires Province. In 2013, the Argentine No-Till Farmers Association (AAPRESID) gathered information about the presence of resistant *R. sativus* reported by farmers and agronomists in 26 districts in the south of Buenos Aires Province. This covers an area of more than 12.5 million ha, 41.5% of the total area of the province (AAPRESID 2013, http://www.aapresid.org.ar/rem/mapa-de-malezas-resistentes/).

In this region there had been intensive use of metsulfuron-methyl, an SU applied during fallow and the early growth stages of wheat. SU herbicides became commonly used in Argentina in the 1980s, when they largely replaced auxin herbicides. Currently, metsulfuron-methyl is the most commonly applied herbicide for controlling dicotyledonous weeds in wheat and barley in Argentina.[14] Other AHAS-inhibiting herbicides, such as imazaquin, imazethapyr and chlorimuron, have been widely used in soybean. The high adoption of AHAS-inhibiting herbicides was also promoted by the adoption of other crops with IMI resistance, such as corn, wheat and canola.[36]

The repeated applications of herbicides with the same target site and the persistence of their active residues in the soil may have resulted in the selection of herbicide-resistant biotypes. Sulfonylurea herbicides show a wide range of persistence in both laboratory and field conditions, depending on the soil pH, temperature and soil moisture. Metsulfuron-methyl soil half-life ranges between 20 and 80 days.[38–40] If the feral radish biotypes with resistance to IMI were originated by this selection mechanism, the weeds should be resistant to other families of herbicides with the same mode of action.

The objective of this study was to characterise the herbicide resistance profile of four of these uncontrolled feral radish populations in IMI-resistant crops in Argentina. A range of AHAS-inhibiting herbicides and other possible herbicides that could be used in sunflower were evaluated.

2 MATERIALS AND METHODS

2.1 Plant material

During the summer of 2010, the mature pods of three feral *R. sativus* accessions (RSBA8, RSBA9 and RSBA10) were collected from a minimum of 15 plants. These plants survived imazapyr application at three different farms in IMI-resistant sunflower in the south-east of Buenos Aires Province, and were reported to technical personnel of BASF Co. by the farmers. The RSBA3 accession was collected in the spring of 2008, in an IMI-resistant canola field treated with imazethapyr.[23]

Pods of feral radish plants growing in the field margins were also collected on the farms where RSBA3 and RSBA10 populations were found (accessions RSBA3f and RSBA10f).

The herbicide-susceptible accessions RSBA1 and RSLP1 were collected from populations in south-western Buenos Aires and La Pampa provinces, in areas without any herbicide application. Two varieties of canola were used for comparison purposes, as negative and positive controls: conventional N1700 (Nexera 1700) and IMI-resistant N8450 (Nexera 8450), both from Dow Agrosciences Co.

2.2 Screening test

The response of the feral radish accessions RSBA1, RSBA3, RSBA8, RSBA9 and RSBA10 to six herbicides was determined. The applied herbicides were two IMIs (imazethapyr and imazamox), two SUs (metsulfuron-methyl and iodosulfuron), glyphosate and 2,4-D (Table 1).

Pods were crushed by hand or using a mortar to extract seeds with minimum damage. The seeds were cleaned and stored at room temperature until use. Plants were established by sowing ten seeds in 15 cm diameter plastic pots containing 75% soil and 25% potting mix (Grow Mix Terrafertil, with composted bark, peat moss, vermiculite, calcite and dolomite). Plants were grown in the

Table 1. Herbicides used to evaluate the resistance profile of feral radish (*Raphanus sativus*) populations

Chemical family[26]	Active ingredient	Trade name and loading	Field rate (g AI ha^{-1})
Imidazolinone	Imazethapyr	Pivot (700 g kg^{-1})	100.0
	Imazamox	Sweeper (700 g kg^{-1})	49.0
	Imazapyr	Clearsol (304 g kg^{-1})	80.0
Sulfonylurea	Metsulfuron-methyl	Generic (600 g kg^{-1})	6.0
	Iodosulfuron	Hussar (53 g kg^{-1})	3.2
	Chlorimuron	Backup (250 g kg^{-1})	15.0
Triazolopyrimidine	Diclosulam	Spider (840 g kg^{-1})	33.6
	Flumetsulam	Preside (120 g L^{-1})	36.0
Sulfonylaminocarbonyltriazolinone	Flucarbazone-sodium	Everest (700 g kg^{-1})	49.0
Pyrimidinylthiobenzoate	Bispyribac-sodium	Nominee (400 g L^{-1})	40.0
Glycine	Glyphosate	Roundup (480 g L^{-1})	1440.0
Phenoxy	2,4-D	Generic (602 g L^{-1})	602.0
Chloroacetamide	Acetochlor	Harness (900 g L^{-1})	1125.0
	S-Metolachlor	Dual Gold (960 g L^{-1})	1200.0
Triazine	Prometryn	Gasagard 50 (500 g L^{-1})	1000.0
Triazinone	Sulfentrazone	Authority (500 g L^{-1})	200.0
Pyridinecarboxamide	Diflufenican	Brodal (500 g L^{-1})	125.0
Pyridazinone	Fluorochloridone	Defender (250 g L^{-1})	312.5
Triazole	Aclonifen	Prodigio (600 g L^{-1})	480.0
Benzothiazole	Benazolin-ethyl	Dasen (500 g L^{-1})	300.0

greenhouse at 20 ± 5 °C, watered twice daily and fertilised with a liquid fertiliser (Chase LI, grade 5-3-3).

Herbicides were applied at double the recommended rate (2×) 53 days after emergence (DAE) at the 1.3 to 1.4 growth stage,[41] using a sprayer equipped with flat spray tips (TeeJet 8004 EVB), at 4 km h^{-1} and calibrated to deliver 188 L ha^{-1}. Alkylaryl polyglycol ether adjuvant (Canaplus 1050, 500 g AI L^{-1}; Canamex Argentina S.A., Buenos Aires, Argentina) was added at the recommended dose (0.25% by vol.).

RSBA3 and RSBA10 accessions and their counterparts that had originated in the field margins (RSBA3f and RSBA10f) were also characterised for their response to five different AHAS-inhibiting herbicides: chlorsulfuron (SU), diclosulam (TP), imazapyr (IMI), flucarbazone (SACT) and bispyribac-sodium (PTB) (Table 1). The RSBA1 susceptible accession was used as a control.

The plants were grown under the same conditions as described above, and the herbicides were applied in a similar way, using a conveyor belt carrying the plants under a stationary sprayer equipped with flat spray tips (TeeJet 8001 EVB), at 1.45 km h^{-1} and calibrated to deliver 202 L ha^{-1}. Adjuvants were added at the recommended dose for imazapyr and bispyribac-sodium.

Plant response was evaluated 35 days after treatment (DAT) in both assays. Plants were classified as herbicide survivors if the growing point remained alive.[19] Survival was assessed using a visual scale that classified individual damage in the following categories: 1 = no damage, 2 = ≤25% leaf damage, 3 = 26–75% leaf damage, 4 = >75% leaf damage, 5 = dead plant. Biotypes were considered to be resistant if 20% of the individuals survived the recommended herbicide rate for field application.[42]

The experiments were arranged as a completely randomised design, with four replications. Data was transformed by

$$y = \arcsin\,(x + 0.5)^{1/2} \qquad (1)$$

ANOVA analysis and a mean comparison Tukey test were conducted with R3.0.2 statistical software.[43]

The resistance to flumetsulam was evaluated in an independent assay under the same experimental conditions, except RSLP1 was used as the susceptible control.

2.3 Dose–response assay to imazethapyr and metsulfuron-methyl

A dose–response experiment of herbicide-resistant accessions was conducted with imazethapyr and metsulfuron-methyl at 0, 1/25, 1/10, 1/5, 1/2, 1, 2, 5, 10, 30 and 50 times the commercial field rate (100 g ha^{-1} for imazethapyr and 6 g ha^{-1} for metsulfuron-methyl). The susceptible accession was treated with 0, 1/200, 1/100, 1/50, 1/25, 1/10, 1/5, 1/2, 1, 2, 5 and 10 times the commercial field rate. Dose–response experiments were repeated in two different years.

Plants were grown under the same conditions as described for the screening test. Herbicides were applied 29 DAE at the 1.4 to 1.6 growth stage, using an experimental sprayer equipped with extended range flat spray tips (TeeJet XR8004 VB), at 4.5 km h^{-1} and calibrated to deliver 184 L ha^{-1}. Alkylaryl polyglycol ether adjuvant was added at the recommended dose (0.25% by vol.).

Plant survival was evaluated at 35 DAT. Data were fitted to a non-linear log-logistic regression model with three parameters, to a Weibull type 1 model with three parameters or to a Weibull type 2 model with three parameters, depending on which one fitted better. Dose–response curves were made using the drc package of the R3.0.2 statistical software. The effective rate required for 50% plant injury (LD_{50}) was estimated. This value was used to calculate the resistance factor (RF), defined as the ratio between LD_{50} of the resistant and susceptible biotypes ($ED_{50\,R}/ED_{50\,S}$).

The log-logistic model equation used was

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

The Weibull type 1 model equation used was

$$Y = d\,(\exp\{-\exp[b(\log\,(x) - \log\,(e)]\}) \qquad (3)$$

Table 2. Plant survival, expressed as a percentage of the untreated control (mean ± standard error) of feral radish (*Raphanus sativus*) accessions and two canola (*Brassica napus*) cultivars (conventional N1700 and IMI-resistant N8450 CL), to herbicides at double the commercial rate (2×)[a]

	Accession						
	RSBA1	RSBA3	RSBA8	RSBA9	RSBA10	N1700	N8450
Herbicide	Plant survival (%)						
Imazethapyr	0.0 ± 0.0 a	66.9 ± 4.3 b	98.8 ± 0.7 c	99.4 ± 0.6 c	100.0 ± 0.0 c	0.0 ± 0.0 a	100.0 ± 0.0 c
Imazamox	0.0 ± 0.0 a	86.9 ± 4.3 b	99.4 ± 0.6 c	100.0 ± 0.0 c	100.0 ± 0.0 c	0.0 ± 0.0 a	100.0 ± 0.0 c
Metsulfuron-methyl	0.0 ± 0.0 a	56.9 ± 11.2 b	46.9 ± 3.7 b	70.6 ± 8.1 b	59.4 ± 12.0 b	0.0 ± 0.0 a	7.5 ± 7.5 a
Iodosulfuron	0.0 ± 0.0 a	87.5 ± 4.8 b	98.1 ± 1.2 c	98.8 ± 0.7 c	100.0 ± 0.0 c	0.0 ± 0.0 a	97.5 ± 1.0 c
2,4-D	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Glyphosate	3.8 ± 2.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 2.5	0.0 ± 0.0	0.0 ± 0.0

[a] In each row, different letters indicate significant differences according to Tukey's test ($P < 0.05$).

The Weibull type 2 model equation used was

$$Y = d\left[1 - \exp\left(-\exp\left\{b\left[\log(x) - \log(e)\right]\right\}\right)\right] \quad (4)$$

where Y is the percentage survival, e is the inflection point of the curve, d is the coefficient corresponding to the upper curve limit, b is the response line slope around e and x (independent variable) is the herbicide rate. The lower limit was fixed at 0 (three parameters), assuming that at high herbicide concentrations all plants die.[44]

2.4 Gene sequencing

Plants of resistant accessions RSBA3, RSBA8, RSBA9 and RSBA10 were grown in the greenhouse as described above. The susceptible accessions RSBA1 and RSLP1 were included as controls. Individual leaves were obtained from ten plants from each accession and dried with silica gel in plastic bags. DNA was extracted using the DNA Landmarks in-house microextraction protocol. The DNA concentrations of the samples were measured by Hoescht dye fluorescence, and the quality of the samples was assessed by agarose gel electrophoresis. The primers WR122F and W653R[31] were used to amplify the regions containing all potential resistance-endowing AHAS gene known mutation sites. The ~1750 bps PCR product obtained with these two primers covers >90% of the *A. thaliana* AHAS coding sequences and 100% of the conserved coding sequence across plant species. The ~1750 bps PCR product amplified lines were run on agarose gels, and the bands of interest were purified using the Zymoclean kit and eluted in 2× 8 µL of H_2O. Purified PCR products were analysed by Sanger sequencing on ABI3730xl. Raw sequence data were assembled using Staden Package Sequence analysis software, and sequence alignments were performed using BioEdit software. The *A. thaliana* AHAS gene (AT3G48560; AY042819) used as an alignment reference and the *R. raphanistrum* AHAS (AJ344986) were both downloaded from NCBI.

A CAPS marker for the Trp574Leu mutation[31] was tested on three susceptible (RSBA1) and three resistant samples (RSBA3). The primers used were WA574F and WA653R. A 1 µL aliquot of the PCR product from each sample was first checked on 1% agarose gel. The PCR products from the samples were then digested with MfeI restriction enzyme, and the digested products were analysed on 2% agarose gel. The 504 bps PCR products, amplified using primers WR574F and WR653R, were sequenced to confirm the Trp574Leu CAPS results. The amplified PCR products were analysed by Sanger sequencing. The obtained sequences were aligned together using Staden Package Sequence analysis.

2.5 Alternative herbicides

The response of AHAS-resistant radish accessions to nine alternative herbicides for sunflower crop was evaluated (Table 1). Plants were grown in the greenhouse under the same conditions as described for the screening and dose–response tests. Herbicides were applied at the recommended rate (×) using a sprayer equipped with flat spray tips (TeeJet 8001 VB), at 1.54 km h^{-1} and calibrated to deliver 185 L ha^{-1}.

Pre-emergence herbicides were applied immediately after sowing 25 seeds in 15 cm diameter plastic pots containing soil from the field where the accession RSBA10 had originated. Post-emergence herbicides were applied 21 DAE. Plants for post-emergence application were grown as described for the screening test. Plant response was evaluated 35 DAT.

The experiment was arranged as a completely randomised design, with four replications. The data were transformed by arcsin function [Eqn (1)]. ANOVA analysis and a mean comparison Tukey test were conducted with R3.0.2 statistical software.

3 RESULTS

3.1 Screening test

Almost all plants of feral radish accessions RSBA8, RSBA9 and RSBA10 survived treatment with a double rate of imazethapyr and imazamox, differing ($P < 0.05$) from RSBA1 and N1700 which were completely killed (Table 2). These three feral radish accessions and the IMI-resistant canola N8450 were similar in their ability to survive such a high herbicide rate. RSBA3 had an intermediate response that was different ($P < 0.05$) from both the susceptible and the other resistant radish accessions.

The survival of RSBA3, RSBA8, RSBA9 and RSBA10 accessions to metsulfuron-methyl at 2× was between 46 and 70%, differing from RSBA1 and both canola varieties which were all nearly killed (Table 2). Survival to iodosulfuron was close to 100% in RSBA8, RSBA9 and RSBA10 and canola N8450, but RSBA3 had a lower survival (87%), differing from the susceptible and other resistant accessions. Glyphosate and 2,4-D caused almost complete mortality of all accessions (Table 2).

The wide screening test confirmed that RSBA3 and RSBA10 accessions were resistant to representative active ingredients of the five AHAS-inhibiting herbicide families, evaluated at 2× rates (Table 3). The plant survival of RSBA10 was greater than 83% in all the treatments. RSBA3 had a lower plant survival, but it was only statistically different to RSBA10 with chlorimuron. The accession originating from the field margin where RSBA10 was

Table 3. Plant survival, expressed as a percentage of the untreated control (mean ± standard error) of two herbicide-resistant feral radish (*Raphanus sativus*) accessions (RSBA3 and RSBA10) and their counterparts from the field margin (RSBA3f and RSBA10f), to six herbicides of five AHAS-inhibiting families at double the commercial rate (2×)[a]

	Accession				
Herbicide	Susceptible[b]	RSBA3	RSBA3f	RSBA10	RSBA10f
Imazapyr	0.0 ± 0.0 a	82.5 ± 4.8 b	0.0 ± 0.0 a	90.0 ± 10.0 b	67.5 ± 8.5 b
Chlorimuron	8.8 ± 3.0 a	45.6 ± 7.0 b	6.3 ± 2.2 a	83.8 ± 11.4 c	79.4 ± 7.8 c
Diclosulam	6.9 ± 1.2 a	76.8 ± 11.5 b	5.0 ± 2.3 a	87.2 ± 6.6 b	57.5 ± 9.2 b
Flumetsulam	2.8 ± 1.8 a	n.d.	n.d.	100.0 ± 0.0 b	n.d.
Flucarbazone-sodium	0.0 ± 0.0 a	79.4 ± 7.4 b	0.0 ± 0.0 a	89.8 ± 4.7 b	74.4 ± 8.7 b
Bispyribac-sodium	0.0 ± 0.0 a	80.6 ± 10.2 b	0.0 ± 0.0 a	87.5 ± 1.8 b	65.0 ± 9.3 b

[a] In each row, different letters indicate significant differences according to Tukey's test ($P < 0.05$).
[b] The susceptible accession was RSBA1, except for flumetsulam, where RSLP1 was used.

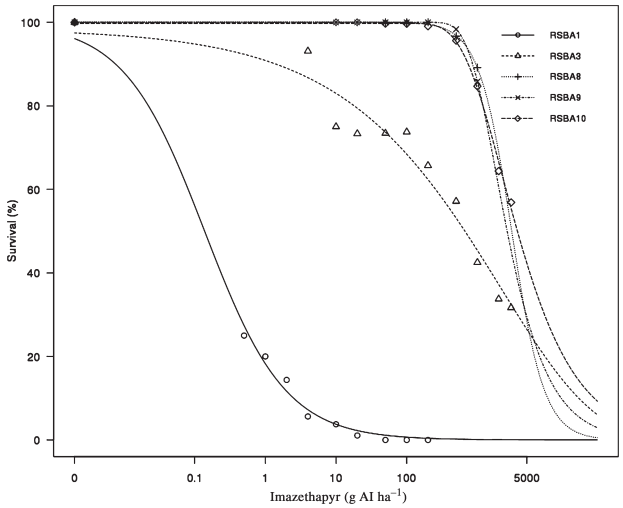

Figure 1. Response of the susceptible RSBA1 (○) and the resistant RSBA3 (△), RSBA8 (+) RSBA9 (×) and RSBA10 (◇) feral radish (*Raphanus sativus*) accessions to the application of imazethapyr, expressed as percentage survival.

found (RSBA10f) was similar to RSBA10, being resistant to all the treatments. In contrast, RSBA3f was statistically different to RSBA3, being susceptibile to all herbicides.

3.2 Dose–response assay to imazethapyr and metsulfuron-methyl

RSBA3, RSBA8, RSBA9 and RSBA10 were highly resistant to imazethapyr (Fig. 1), with more than 60% plant survival at 5 times the commercial rate (500.0 g AI ha^{-1}). In contrast, the susceptible accession RSBA1 was totally killed at half the commercial rate. The LD_{50} of RSBA1 was 0.14 g AI ha^{-1}, whereas RSBA3, RSBA8, RSBA9 and RSBA10 LD_{50} values were over 700.0 g AI ha^{-1}. The resistance factor of these accessions ranged between 5000 and 26400 (Table 4).

The RSBA8, RSBA9 and RSBA10 accessions were intermediately resistant to metsulfuron-methyl (Fig. 2), but survival was greater than 20% even at the 5× rate (30.0 g AI ha^{-1}). In contrast, the survival of RSBA1 accession was reduced by more than 99% with only 1/5 of the commercial field rate (1.2 g AI ha^{-1}). The RSBA3 plant survival at the 5× rate was 12%, an intermediate value between the highly resistant accessions and the susceptible control. The RSBA3, RSBA8, RSBA9 and RSBA10 LD_{50} values were 3.32, 5.51, 7.49 and 17.97 g AI ha^{-1} respectively, indicating an

Table 4. Estimated parameters[a] for non-linear regression equations comparing survival from resistant (RSBA3, RSBA8, RSBA9 and RSBA10) and susceptible (RSBA1) feral radish (*Raphanus sativus*) accesions to AHAS-inhibiting herbicides. Comparative levels of resistance to each herbicide were also estimated

Herbicide	Accession	*b*	*d*	*e*	LD_{50}	RF
Imazethapyr	RSBA1	0.76	99.98	0.14	0.14	
	RSBA3	0.33	98.47	2179.20	712.94	5092.4
	RSBA8	1.88	100.11	3055.06	3055.06	21821.9
	RSBA9	−1.07	100.00	1870.80	2634.30	18816.4
	RSBA10	−0.75	99.77	2273.29	3696.80	26405.7
Metsulfuron-methyl	RSBA1	1.86	100.98	0.13	0.13	
	RSBA3	0.75	99.96	3.32	3.32	25.5
	RSBA8	−0.74	100.40	3.35	5.51	42.4
	RSBA9	−0.79	100.10	4.72	7.49	57.6
	RSBA10	−0.83	97.54	11.54	17.97	138.2

[a] *b* = slope of the curves around *e*; *d* = upper limit of curves; *e* = inflection point of the curves; LD_{50} = effective rate required for 50% reduction in plant survival; RF = resistance factor ($LD_{50\,R}/LD_{50\,S}$).

increased resistance (25–138-fold) over RSBA1, the LD_{50} of which was 0.13 g AI ha^{-1} (Table 4).

3.3 Gene sequencing

Compared with the sequence of the susceptible accession, the AHAS gene from plants of RSBA8, RSBA9 and RSBA10 accessions had a single nucleotide change at position 1720, from guanine (G) to thymine (T), resulting in a predicted amino acid change from Trp to Leu at position 574. This change was homozygous in all the samples, as was also observed in the sequence chromatograms. The sequence of the AHAS gene from plants of the RSBA3 accession had the same nucleotide change, but this was not observed for all the samples. The sequence was deposited in GeneBank (KP899558).

The Trp574Leu CAPS assay with primers W574F/WA635R was applied to three susceptible samples (RSBA1) and three suspected resistant samples (RSBA3). Digestion of the PCR products with Mfe1 restriction enzyme revealed that all three susceptible samples and one of the suspected resistant samples had the wild-type (WT) allele represented by the undigested fragment of 0.5 kb. The two other suspected samples were revealed to be heterozygous

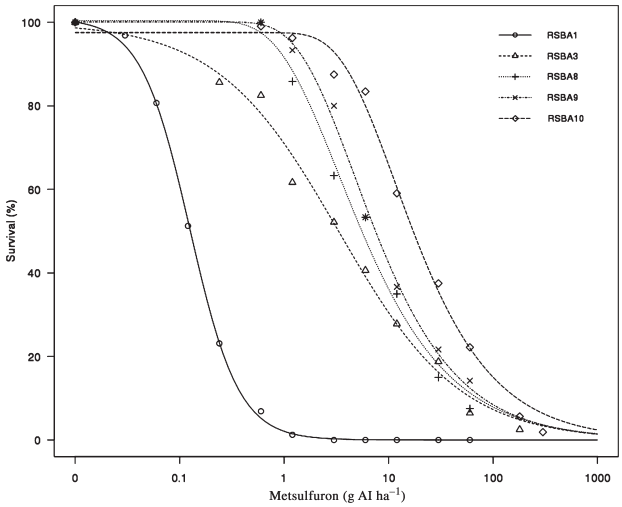

Figure 2. Response of the susceptible RSBA1 (○) and the resistant RSBA3 (△), RSBA8 (+) RSBA9 (×) and RSBA10 (◇) feral radish (*Raphanus sativus*) accessions to the application of metsulfuron-methyl, expressed as percentage survival.

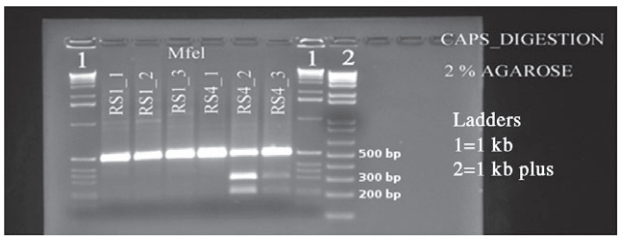

Figure 3. Gel photo showing the results of the Trp574Leu CAPS assay on three samples of RSBA1 (RS1-1, RS1-2 and RS1-3) and three samples of RSBA3 (RS4-1, RS4-2 and RS4-3) feral radish (*Raphanus sativus*) accesions. The wild-type allele is represented by the 500 bp fragment, and the Trp574Leu mutant allele is represented by the two digested fragments of 291 bp and 213 bp.

with both the WT allele of 0.5 kb and the mutant allele represented by the digested fragments of 0.29 and 0.23 kb (Fig. 3).

3.4 Alternative herbicides

The pre-emergence herbicide mixture of acetochlor plus fluorochloridone was the most effective treatment for controlling all feral radish accessions. Sulfentrazone, chloroacetamide herbicides (*S*-metolachlor and acetochlor), prometryn and diflufenican were not effective in controlling all the accessions. Fluorochloridone and acetochlor alone were also ineffective, but their mixture was synergistic and reduced plant survival of all accessions by more than 80%.

Both post-emergence herbicides, aclonifen and benazolin, were highly effective against radish accessions at their respective field rates.

4 DISCUSSION AND CONCLUSIONS

The screening test showed a broad herbicide resistance to active ingredients with the same target site in all the feral radish accessions collected from IMI-resistant crops. Dose–response assays showed a very high resistance to imazethapyr, but intermediate levels of resistance to metsulfuron-methyl. The RSBA10 accession was cross-resistant to ten active ingredients of the five chemical families of AHAS-inhibiting herbicides: imazapyr, imazetapyr, imazamox, metsulfuron-methyl, iodosulfuron, chlorimuron, diclosulam, flumetsulam, flucarbazone-sodium and bispyribac-sodium. The resistant biotypes were found in three different districts of south-eastern Buenos Aires Province, one of them situated almost 100 km apart from the others.

The five AHAS-resistant accessions were highly susceptible to glyphosate and 2,4-D at commercial rates. These herbicides could be used in fallow, RR soybeans or in cereal crops. The mixture of acetochlor and fluorochloridone, which caused the highest mortality, could be applied as pre-emergence herbicides in sunflower and maize crops. For a post-emergence application, aclonifen and benazolin could also be used to control feral radish biotypes in sunflower.

The sequencing of the AHAS-resistant accessions confirmed a single amino acid change from tryptophan to leucine at position 574 in the AHAS gene. This substitution is one of the commonly occurring mutations in AHAS-resistant weeds.[28,29] It has been shown that substitutions of Trp574 result in high levels of resistance to both IMI and SU herbicides (as well as TP and PTB herbicides) in several weed species, including *R. raphanistrum*.[28–31] The presence of this mutation in resistant biotypes associated with broad resistance to all the five chemical families of AHAS-inhibiting herbicides in feral radish (*R. sativus*) has not been reported previously.

The RSBA3 accession had a lower level of resistance, and in the assays segregation for resistance was clearly noticed. This biotype was first observed in a small population in a field with a long history of cropping and herbicide usage. This was attributed[23] to a recent mutational event. This situation was in accordance with a model proposed by Richter *et al.*,[45] which states that the build-up of target-site resistance in a field often occurs in a stepwise manner. Usually, resistant plants are not noticed by the farmer until about 30% of the plants in a field are resistant. The sequencing of this accession showed heterozygosis of the resistance genes and the presence of individuals with the wild-type, susceptible genotype.

Under farm conditions, the RSBA3 biotype did not increase in density, probably because of the use of alternative herbicides such as 2,4-D and glyphosate. Also, the presence of an extensive feral radish population along the field margin might have served as a refuge for the susceptible trait. The plants of this population proved to be susceptible to the same herbicides tested for the resistant biotype. In contrast, the few plants found in the field margins where the resistant accession RSBA10 was collected had a high herbicide resistance profile. This could be due to high selection pressure by the use of herbicides within the fields and also in the field margins.

Analogous to the refuge tactic in *Bt* crops, herbicide-susceptible weed refuges might be useful in delaying herbicide resistance evolution. Such plants could dilute the resistance allele frequency in a population by gene flow and slow down the evolution of resistance within a field or act as a barrier against the invasion of resistant plants. However, gene flow could also increase the resistance frequency in the refuges, reducing its efficiency in counteracting resistance evolution.[46–48]

Models of population genetics suggest that the spread of the resistance is always greatly delayed by a heterogeneous environment. The presence of an unfavourable habitat strongly contributes to selection against the spread of an advantageous allele such as resistance. The fate of a resistance allele may depend on the balance between herbicide treatment favouring the resistance

and the capacity to maintain the resistance gene in untreated areas or during years without treatment.[47,48] It is well established that field margins play an important role in the biological diversity of farmland. Field margins are refuges for many species and a key to the conservation of plant diversity. Thus, weeds have a role within agroecosystems in supporting biodiversity more generally.[49]

The magnitudes of resistance to different AHAS-inhibiting herbicides could vary widely, depending on the amino acid substitutions of the enzyme. In SU-resistant biotypes, resistance to one herbicide does not guarantee cross-resistance to all members of that chemical family.[27] This may explain the intermediate levels of metsulfuron-methyl resistance and the high levels of iodosulfuron and IMI herbicide resistance of the biotypes.

The soil dissipation of SU herbicides occurs via chemical hydrolysis and microbial degradation, with half-lives ranging from days to months. The process involves a rapid degradation during the initial 15 day period before entering a phase of slower first-order kinetics over time.[38–40] The resistant feral radish biotypes may have evolved through the selection of plants emerging during the spring and beginning of summer. The emergence of these plants would overlap with the dissipation of metsulfuron-methyl applied to winter cereals. An increase in the frequency of these selected resistant feral radish plants over several generations would become a serious limiting factor for summer IMI-resistant crops.

This is the first report of feral radish with resistance to herbicides belonging to all AHAS-inhibiting herbicide families. AHAS herbicide resistance alleles are present in Argentine radish biodiversity. The increased use of IMI-resistant crops and the intensification of the application of AHAS-inhibiting herbicides have resulted in a high selection pressure for resistant biotypes. An appropriate herbicide rotation and increased diversity of cropping systems are important for minimising the occurrence and prevalence of this resistant weed. The results of this research have helped to develop management strategies for controlling these biotypes in IMI-resistant sunflower crops, including the use of the two commonly known herbicides acetochlor and fluorochloridone for pre-emergence, and aclonifen for post-emergence.

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