Multiple Resistance to Acetohydroxyacid Synthase–Inhibiting and Auxinic Herbicides in a Population of Oriental Mustard (*Sisymbrium orientale*)

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A population of oriental mustard from Port Broughton in South Australia was reported as not being controlled by 2,4-D. Dose response experiments determined this population was resistant to both 2,4-D and MCPA, requiring greater than 20 times more herbicide for equivalent control compared to a known susceptible population (from Roseworthy, South Australia) and a population resistant only to the acetohydroxyacid synthase (AHAS)-inhibiting herbicides (from Tumby Bay, South Australia). The Port Broughton population was also found to be resistant to three chemical groups that inhibit AHAS; however, the level of resistance was lower than the known acetolactate synthase–resistant population from Tumby Bay. Herbicides from other modes of action were able to control the Port Broughton population. Assays of isolated AHAS from the Port Broughton population showed high levels of resistance to the sulfonylurea and sulfonamide herbicide groups, but not to the imidazolinone herbicides. A single nucleotide change in the AHAS gene that predicted a Pro to Ser substitution at position 197 in the protein was identified in the Port Broughton population. This population of oriental mustard has evolved multiple resistance to AHAS-inhibiting herbicides (AHAS inhibitors) and auxinic herbicides, through a mutation in AHAS and a second nontarget-site mechanism. Whether the same mechanism provides resistance to both AHAS inhibitors and auxinic herbicides remains to be determined. Multiple resistance to auxinic herbicides and AHAS inhibitors in the Port Broughton population will make control of this population more difficult.

Nomenclature: 2,4-D; MCPA; oriental mustard, *Sisymbrium orientale* Torn.

Key words: ALS, Multiple herbicide resistance, target site mutation.

Over the past 50 yr world agriculture has moved from a system where weeds were primarily controlled by tillage to one where herbicides are the main control tactic. The widespread and intensive use of herbicides for weed control has provided substantial benefits in terms of easier and less expensive weed control, reduced soil degradation, and more timely sowing of crops resulting in higher yields; however, this has not occurred without negative impacts. A major negative impact from herbicide-based weed control has been the evolution of herbicide-resistant weeds. To date, 209 weed species have evolved herbicide resistance, worldwide (Heap 2012).

Herbicide resistance does not evolve to all herbicide modes of action at the same rate. The auxinic herbicides were introduced in the 1940s and have been widely used to control broadleaf weeds (Grossmann 2010). Despite this, resistance has been slow to evolve to this herbicide mode of action. To date, there are 30 species with resistance to the auxinic herbicides (Heap 2012). The mechanism of resistance to these herbicides has been identified in only a few weed species (Mithila et al. 2011). In these species enhanced herbicide degradation (Coupland et al. 1990; Weinberg et al. 2006) and decreased herbicide translocation (Weinberg et al. 2006) have been identified as resistance mechanisms.

AHAS inhibitors were first introduced in the 1980s. As a result of their low use rates, excellent crop selectivity, and wide spectrum of weed species controlled, they were rapidly and widely adopted (Saari et al. 1994; Tranel and Wright 2002). Shortly after their introduction, resistance evolved to the AHAS inhibitors and currently 126 species have confirmed resistant populations to this mode of action (Heap 2012). Two different mechanisms of resistance to the AHAS-inhibiting herbicides have been identified (Christopher et al.

1991; Saari et al. 1990). In the majority of cases, a mutation within the enzyme targeted by the herbicide results in resistance (Powles and Yu 2010; Tranel and Wright 2002). There are currently seven known sites within the AHAS enzyme where amino acid substitutions confer resistance (Powles and Yu 2010). The other known mechanism is enhanced herbicide detoxification, which is mainly observed in grass weed species (Preston 2004). Target-site mutations within AHAS occur at high frequencies in weed populations (Preston and Powles 2002), which is why resistance is so common to herbicides inhibiting this enzyme.

Resistance to AHAS-inhibiting herbicides is common in Australia, occurring in both grass and broadleaf weeds (Boutsalis et al. 2012; Owen et al. 2007; Walsh et al. 2007). It is particularly widespread in Western Australia and South Australia where these herbicides have been widely used. In contrast, auxinic herbicide resistance is rare, only occurring in a few populations of wild radish (*Raphanus raphanistrum* L.) in Western Australia (Walsh et al. 2004, 2007). Auxinic herbicides are often the first strategy adopted for the control of AHAS-inhibiting herbicide–resistant weeds in Australia, because they are effective and inexpensive. If resistance to both auxinic and AHAS inhibitors became common, the options for broadleaf weed control in cereal crops would be greatly reduced and significantly more expensive.

Oriental mustard is a widespread Brassicaceae weed occurring in most Australian states. It is particularly common on alkaline soils and is one of the most common broadleaf weeds in South Australia (Chauhan et al. 2006). Resistance to the AHAS inhibitors was first reported in 1992 in South Australia (Boutsalis and Powles 1995). AHAS-inhibitor resistance is now widespread in many cropping areas of South Australia and also occurs in Western Australia and Victoria. Where the resistance mechanism has been examined, a mutation within AHAS has been identified (Boutsalis et al. 1999). To date, control of AHAS inhibitor–resistant oriental mustard has not been a major concern due to the effectiveness of the auxinic herbicides 2,4-D and MCPA in cereals. Other

Herbicide active ingredient	Product name and loading	Manufacturer	Rates used
			——————— g ae or ai ha^{-1} ———————
2,4-D	Amicide 625 (625 g L^{-1})	Nufarm Australia, Laverton North, VIC Australia	87.5, 175, 350, 700, 1,400, 2,800, 5,600
MCPA	Agritone 750 (750 g L^{-1})	Nufarm Australia, Laverton North, VIC Australia	43.75, 87.5, 175, 250, 350, 700, 1,400, 2,800
Metsulfuron-methyl	Ally (600 g kg^{-1})	DuPont (Australia), Macquarie Park, NSW Australia	0.09, 0.19, 0.38, 0.75, 1.5, 3, 6, 12
Chlorsulfuron	Glean (750 g kg^{-1})	DuPont (Australia), Macquarie Park, NSW Australia	0.47, 0.94, 1.88, 3.75, 30, 60, 120, 240
Metosulam	Eclipse (100 g L^{-1})	Bayer CropScience Australia, Hawthorn, VIC Australia	0.63, 1.25, 2.5, 5, 10, 20, 40, 80
Florasulam	None[a] (50 g L^{-1})	Dow AgroSciences Australia, Frenchs Forest, NSW Australia	0.16, 0.32, 0.63, 1.25, 5, 10, 20
Imazathapyr	Spinnaker 700 (700 g L^{-1})	Nufarm Australia, Laverton North, VIC Australia	6.13, 12.3, 24.5, 49, 98, 196, 392
Imazamox	Raptor (700 g kg^{-1})	Crop Care Australasia, Murarrie, QLD Australia	4.22, 8.44, 16.9, 33.8, 67.5, 135, 270
Diflufenican	Brodal Options (500 g L-1)	Bayer CropScience Australia, Hawthorn, VIC Australia	12.5, 25, 50, 100
Picolinofen	Sniper (750 g kg^{-1})	Nufarm Australia, Laverton North, VIC Australia	62.5, 100
Bromoxynil	Bromicide 200 (200 g L^{-1})	Nufarm Australia, Laverton North, VIC Australia	100, 400
Dicamba	Cadence (700 g kg^{-1})	Syngenta Crop Protection, Macquarie park, NSW, Australia	115

[a] Experimental formulation from Dow AgroSciences.

chemistry is available for noncereal crops, such as diflufenican in pulses or atrazine in triazine-tolerant canola.

In 2005, a failure of 2,4-D plus metsulfuron-methyl to control oriental mustard was reported from a wheat crop near Port Broughton, South Australia. The objectives of the current research were to (1) confirm the resistance to AHAS inhibitors and auxinic herbicides in the same biotype, (2) determine the efficacy of other herbicide modes of action, and (3) identify the mechanism of resistance to AHAS inhibitors.

Materials and Methods

Plant Materials. The putative resistant oriental mustard population was collected from a wheat field near Port Broughton, South Australia, where 2,4-D and metsulfuron-methyl had failed to control this weed. Seeds were germinated and the seedlings treated with 700 g ae ha^{-1} 2,4-D, the survivors grown to maturity, and seed collected. This seed was used for all experiments. The susceptible population was obtained from a field near Roseworthy, South Australia, used for long-term organic farming trials and known to be susceptible to all herbicides registered for the control of oriental mustard in Australia. A known AHAS inhibitor–resistant population was used as a positive control. This population came from a wheat field near Tumby Bay, South Australia.

Dose Response Experiments. For the dose response experiments, seeds of each population were mixed with fine sand, 1 : 10 seed : sand by weight (1 : 1 for the Roseworthy population because of its lower germination rate). A scoop that measured out approximately 0.01 g of the seed–sand mix was constructed. One scoop of seed–sand mix was spread over the surface of potting mix (cocoa peat potting mix produced by mixing 540 L of cocoa peat, 220 L of water, and 60 L of sand prior to steaming for 1 hr). The following additives were then mixed to the pasteurized mix: 180 g dolomite lime, 600 g agricultural lime, 240 g hydrated lime, 180 g gypsum, 180 g superphosphate, 450 g iron sulphate, 30 g iron chelate, 180 g Micromax trace elements, 450 g calcium nitrate, and 1800 g of Osmocote mini 3–4M (16–3–9 plus trace elements) in 10-cm by 10-cm pots and watered. This procedure resulted in 15 to 25 seedlings germinating in each pot. There were four replicate pots for each herbicide treatment and each dose response experiment and single-rate herbicide experiment was

repeated. Plants were grown outdoors during winter, the normal growing season for this species in southern Australia, and watered as required.

When seedlings were at the two- to four-true-leaf stage they were treated with herbicides using a track sprayer. This equipment delivered 109 L ha^{-1} at 250 kPa and 1 m s^{-1} through flat-fan nozzles (Hardi ISO F-110-01 Standard Flat Fan, Hardi, Adelaide, Australia). Commercial herbicide formulations were used and are listed with rates in Table 1. Adjuvants were added as recommended by manufacturers: 0.2% v/v nonionic surfactant (BS1000, Crop Care Australasia, Murarrie, Queensland, Australia) was added to metsulfuron-methyl, chlorsulfuron, imazethapyr, imazamox, and dicamba, and 1% v/v crop oil (Hasten, Victorian Chemicals, Coolaroo, Victoria, Australia) was added to metosulam and florasulam. Different herbicide rates were applied to susceptible and resistant populations where the responses of the populations to the herbicides were widely different. A control treatment where only adjuvants were applied was included for each herbicide dose response experiment. Seedlings in each pot were counted before herbicide application. Mortality was assessed 28 d after herbicide application. Dose response experiments were analyzed by probit analysis (Pri Probit; Sakuma 1998). Data were transformed back to percentage of survival for display. Single-rate herbicide treatments were compared by Kruskal-Wallis test using GraphPad Prism (GraphPad Software, Inc. San Diego, CA) because the large number of zeros precluded a Gaussian distribution of these data. Means were separated by Dunn's multiple comparison test ($P = 0.05$).

AHAS Enzyme Assays and Gene Sequencing. Plants for AHAS enzyme assays were sown in pots as described above and grown in a growth room set at 12 h, 20 C, 400 μmol m^{-2} s^{-1} light period and a 12 h, 15 C dark period. Seedlings were watered as required and a commercial fertilizer applied once a week. Seedlings were thinned to four per pot after germination. At the ten- to twelve-leaf stage, young fully expanded leaves were harvested for chloroplast extraction. Intact chloroplasts were extracted using the method described by Preston et al. (2006). The chloroplasts were lysed and the lysate, containing AHAS and other stromal enzymes, was used directly for the AHAS assay. AHAS assays were conducted as described by Preston et al. (2006), except the herbicides used were metsulfuron-methyl, chlorsulfuron,
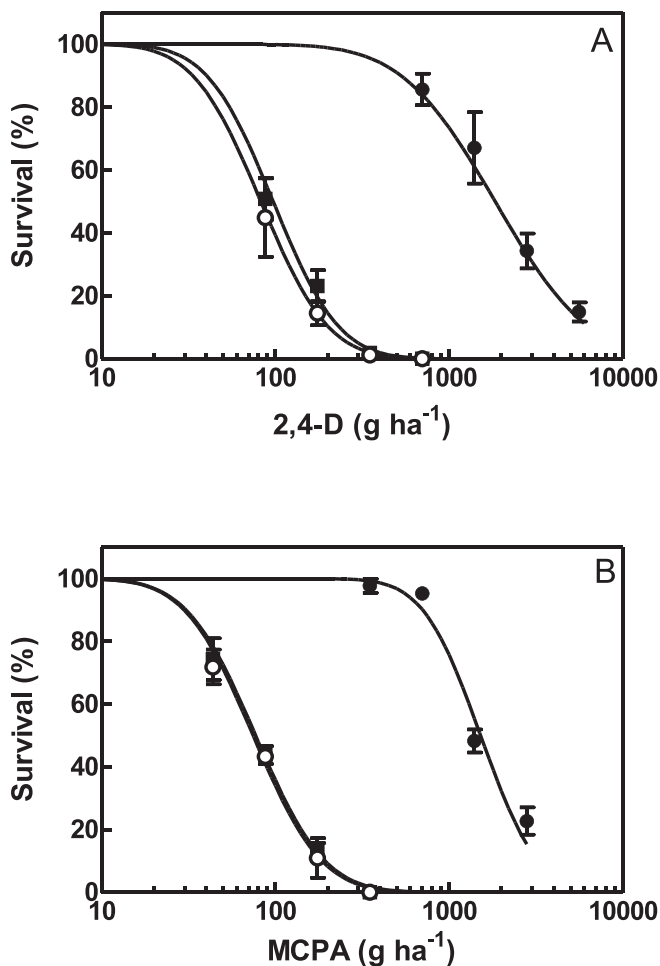
Figure 1. Response of susceptible and resistant oriental mustard populations to the auxinic herbicides (A) 2,4-D and (B) MCPA. Populations are Roseworthy (○), Port Broughton (●), and Tumby Bay (■). Curves are fitted probit curves: (A) $Y = 11.27 - 3.27 \times \log(x)$, $12.72 - 2.36 \times \log(x)$, and $11.67 - 3.36 \times \log(x)$ for Roseworthy, Port Broughton, and Tumby Bay populations respectively; (B) $Y = 11.05 - 3.23 \times \log(x)$, $17.22 - 3.85 \times \log(x)$, and $11.04 - 3.20 \times \log(x)$ for Roseworthy, Port Broughton, and Tumby Bay respectively, where x is herbicide dose, with probits back-transformed to percentage of survival.

metosulam, florasulam, and imazethapyr. Enzyme activity was calculated as a percentage of activity in the absence of inhibitor and I_{50} concentrations (the concentration of herbicide required to inhibit enzyme activity by 50%) for herbicides were calculated following log-sigmoidal transformation of these data using GraphPad Prism.

Table 2. Concentrations of auxinic herbicides required to produce 50% mortality of populations of oriental mustard and the ratio of LD_{50} for the resistant compared to the susceptible populations (R/S).

Herbicide	RW[a] LD_{50}	PB LD_{50}	R/S	TB LD_{50}	R/S
	— g ae ha^{-1} —	— g ae ha^{-1} —		— g ae ha^{-1} —	
2,4-D	83	1,843	22	97	1.2
MCPA	75	1,519	20	77	1

[a] Abbreviations: RW, Roseworthy population susceptible to all herbicides; PB, Port Broughton population; TB, Tumby Bay population susceptible to auxinic herbicides and resistant to AHAS inhibitors; R/S, ratio of LD_{50} for the resistant compared to the susceptible populations.
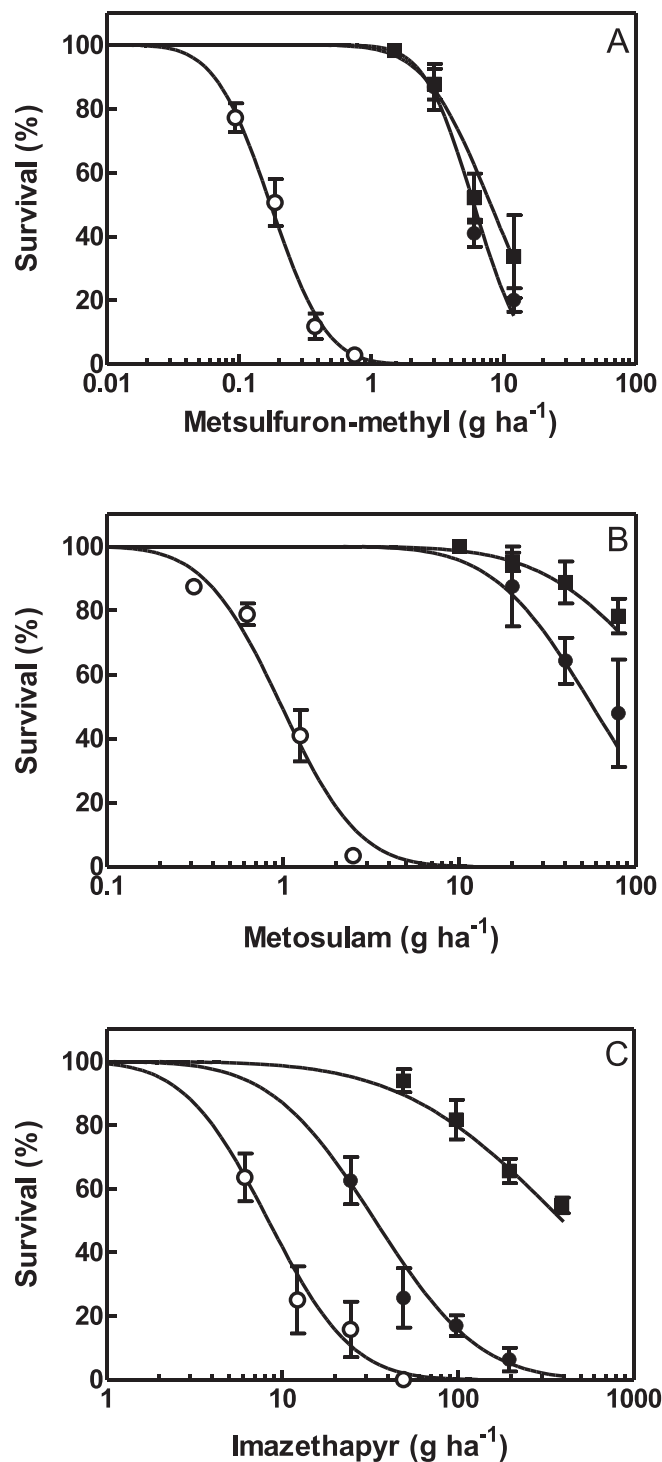

Figure 2. Response of susceptible and resistant oriental mustard populations to the acetohydroxyacid synthase inhibitors (A) metsulfuron-methyl, (B) metosulam, and (C) imazethapyr. Populations are Roseworthy (○), Port Broughton (●), and Tumby Bay (■). Curves are fitted probit curves with probits back-transformed to percentage survival: (A) $Y = 2.72 - 3.0 \times \log(x)$, $7.66 - 3.42 \times \log(x)$ and $7.29 - 2.53 \times \log(x)$ for Roseworthy, Port Broughton, and Tumby Bay; (B) $Y = 4.98 - 2.89 \times \log(x)$, $8.97 - 2.26 \times \log(x)$, and $8.95 - 1.75 \times \log(x)$ for Roseworthy, Port Broughton, and Tumby Bay populations respectively; (C) $Y = 7.45 - 2.66 \times \log(x)$, $8.27 - 2.14 \times \log(x)$, and $8.60 - 1.39 \times \log(x)$ for Roseworthy, Port Broughton, and Tumby Bay respectively, where x is herbicide dose, with probits back-transformed to percentage of survival.

Table 3. Concentrations of acetohydroxyacid synthase inhibitors required to produce 50% mortality of populations of oriental mustard and the ratio of LD_{50} for the resistant compared to the susceptible populations (R/S).

Herbicide	RW[a]	PB		TB	
	LD_{50}	LD_{50}	R/S	LD_{50}	R/S
	———— g ae ha^{-1} ————	———— g ae ha^{-1} ————		———— g ae ha^{-1} ————	
Metsulfuron-methyl	0.17	6.0	35	8.0	47
Chlorsulfuron	0.46	507	1,102	585	1,271
Metosulam	1.0	57	57	182	182
Florasulam	0.50	6.3	13	45	90
Imazethapyr	8.4	34	4	394	47
Imazamox	2.4	13	5.4	272	113

[a] Abbreviations: RW, Roseworthy population susceptible to all herbicides; PB, Port Broughton population; TB, Tumby Bay population susceptible to auxinic herbicides and resistant to AHAS inhibitors; R/S, ratio of LD_{50} for the resistant compared to the susceptible populations.

Plants used for DNA extraction were sown in pots and grown in a growth room as described above. Individual leaves were obtained from two plants from each population, snap frozen in liquid nitrogen, and stored at -80 C. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Australia) according to the manufacturer's instructions. The concentration of nucleic acids was determined spectrophotometrically on a NanoDrop ND-1000 (Thermo Scientific, USA) at 260 nm. PCR amplification was carried out in an automated DNA thermal cycler (Eppendorf Mastercycler® Gradient, Germany) with PCR conditions as follows: 2 min denaturing at 94 C; 35 cycles of 30 s denaturation at 94 C, 30 s annealing at 60 C, and 105 s elongation at 68 C; with a final extension of 7 min at 68 C. PCR reactions of 20 μl contained 20 ng DNA, 1× PCR reaction buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl], 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.2 M of primers ALSFwd5 [5′ ATC CTS GTS GAA GCC CTS GAG CGT CA 3′] and ALSRev4 [5′ GAA RGT GCC RCC ACT WGG GAT 3′] and 1 unit high-fidelity Taq DNA polymerase (Invitrogen, Australia).

The PCR products were visualized on a 0.8% agarose gel prestained with ethidium bromide (1 g ml^{-1}). Samples were electrophoresed in 1× TAE Buffer (40 mM Trizma base, 1 mM Na$_2$EDTA, pH to 8 with glacial acetic acid) at 100 V and photographed under ultraviolet light (λ = 302 nm). DNA fragment sizes were estimated by comparing their mobility to bands of known sizes in a low-mass molecular weight marker (Invitrogen). PCR products of the correct size ($\sim$ 1,665 bp) were extracted from the gel and sequenced (Australian Genome Research Facility, Australia) using primers ALSFwd1 (5′ GCA TGT CTA GAA CGT CCT TCC YCG TCA CGA ACA 3′) and ALSRev2 (5′ CGT GGA TCC TMG TTA CCT CAA CAA 3′) for region 1 and ALSFwd3 (5′ GTT GTT GAC ATT GAY GGY GAT GG 3′) and ALSRev4 for region 2 to obtain both forward and reverse sequence data.

Results and Discussion

Dose Response Experiments. Dose response experiments confirmed that the putative resistant population from Port Broughton was resistant to both AHAS-inhibiting and auxin-mimic herbicides. The susceptible population was easily controlled by 2,4-D, with no survival at 350 g ha^{-1}, which is half the normal field use rate of 700 g ha^{-1} in Australia (Figure 1A). In contrast, the Port Broughton population had less than 20% mortality at the normal use rate for this herbicide. The Tumby Bay population was not resistant to 2,4-D and had a dose response similar to the susceptible population (Figure 1A). The LD_{50} for the Roseworthy population was estimated at 83 g ha^{-1} and that of the Tumby Bay population at 97 g ha^{-1}. The LD_{50} for the Port Broughton population was estimated at 1843 g ha^{-1}, making this population 22-fold more resistant to 2,4-D than the susceptible population.

The Port Broughton population was also resistant to MCPA. The susceptible population and the Tumby Bay population were both controlled by the normal use rate of 350 g ha^{-1} MCPA (Figure 1B). In contrast, there was less than 5% mortality for the Port Broughton population at this herbicide rate. The LD_{50} for MCPA for the susceptible population was 75 g ha^{-1} and for the resistant population LD_{50} was estimated at 1,519 g ha^{-1}. Therefore, the Port Broughton population is approximately 20-fold more resistant

Table 4. Effect of alternative herbicides for the control of oriental mustard populations resistant to acetohydroxyacid synthase inhibitors and auxinic herbicides.

Herbicide(s)	Mode(s) of action	Rate(s)	RW[a]	PB	TB	P
				Survival[b]		
		——— g ai or ae ha^{-1} ———	——————————— % ———————————			
Bromoxynil	PS II	400	1.3 a	3.6 a	0 a	0.31
Diflufenican	PDS	100	0	0	0	—
Picolinofen	PDS	37.5	0 a	2.2 a	0 a	0.12
Diflufenican + MCPA	PDS + auxin mimic	62.5 + 250	0 a	1.9 a	0 a	0.37
Picolinofen + MCPA	PDS + auxin mimic	25 + 250	0 a	2.5 a	0 a	0.37
Florasulam + MCPA	AHAS + auxin mimic	5 + 250	0 a	42.2 b	5.5 a	< 0.0001
Florasulam + Bromoxynil	AHAS + PS II	5 + 100	0 a	33.3 b	38.7 b	0.0008
Dicamba + Metsulfuron-methyl	Auxin mimic + AHAS	115 + 3	0 a	45 c	17.5 b	< 0.0001

[a] Abbreviations: RW, Roseworthy population susceptible to all herbicides; PB, Port Broughton population; TB, Tumby Bay population susceptible to auxinic herbicides and resistant to AHAS inhibitors; PS II, photosystem II; PD, phytoene desaturase; AHAS, acetohydroxyacid synthase.
[b] For any herbicide, survival values followed by different letters are significantly different at p=0.05.

than the susceptible population to MCPA as well as 2,4-D (Table 2).

Resistance to the auxinic herbicides has occurred in 30 species worldwide (Heap 2012). This is a relatively rare type of herbicide resistance given that auxinic herbicides have been used for more than 60 yr (Grossmann 2010). The level of resistance to auxinic herbicides is typically modest. For example, resistant biotypes of kochia [*Kochia scoparia* (L.) Schrad.] have 30-fold greater resistance to dicamba (Preston et al. 2009), prickly lettuce (*Lactuca serriola* L.) has 25-fold greater resistance to 2,4-D (Burke et al. 2009), yellow starthistle (*Centaurea solstitialis* L.) has threefold greater resistance to picloram and 100-fold greater resistance to 2,4-D (Fuerst et al. 1996), common hempnettle (*Galeopsis tetrahit* L.) has threefold greater resistance to MCPA and eightfold greater resistance to fluroxypyr (Weinberg et al. 2006), and wild radish has twofold greater resistance to 2,4-D (Walsh et al. 2004). The 2,4-D–resistant oriental mustard is 20-fold more resistant than susceptible populations to 2,4-D and MCPA, within the range reported for other species.

The mode of action of auxinic herbicides is not well understood (Grossmann 2010). For most weed species in which resistance has evolved to the auxinic herbicides, the mechanism of resistance is unknown (Mithila et al. 2011). Resistance to mecoprop in common chickweed [*Stellaria media* (L.) Vill.] (Coupland et al. 1990) and to MCPA in common hempnettle (Weinberg et al. 2006) has been determined to be the result of increased herbicide detoxification. Resistance to MCPA in common hempnettle is also the result of decreased translocation of the herbicide in the roots (Weinberg et al. 2006). Resistance to 2,4-D in wild mustard [*Brassica kaber* (DC.) L.C. Wheeler] is suspected of being the result of decreased herbicide binding to auxin receptors (Zheng and Hall 2001). The level of cross-resistance to other auxinic herbicides can be highly variable; however, this oriental mustard population was equally resistant to 2,4-D and MCPA (Fuerst et al. 1996; Weinberg et al. 2006). This indicates the resistance mechanism involved provides equal resistance to both herbicides.

The sulfonylurea herbicide metsulfuron-methyl totally controlled the susceptible population at 1 g ha^{-1} (Figure 2A), well below the normal use rate in Australia of 3 g ha^{-1}. In contrast, the populations from Port Broughton and Tumby Bay were not controlled by the normal use rate of 3 g ha^{-1}. The LD$_{50}$ for metsulfuron-methyl for the susceptible population was 0.17 g ha^{-1}, whereas the LD$_{50}$ for the Port Broughton population was 6 g ha^{-1} and for the Tumby Bay population, 8 g ha^{-1}. The Port Broughton population was also resistant to another sulfonylurea herbicide, chlorsulfuron. The LD$_{50}$ for chlorsulfuron for the susceptible population was 0.46 g ha^{-1} compared with 507 g ha^{-1} for the Port Broughton population and 585 g ha^{-1} for the Tumby Bay population.

The oriental mustard populations from Port Broughton and Tumby Bay were also resistant to another group of AHAS inhibitors: the sulfonamide herbicides metosulam and florasulam. The susceptible population was controlled by 2.5 g ha^{-1} metosulam, below the normal use rate of 5 g ha^{-1}. In contrast, the field rate of 5 g ha^{-1} provided no control of either resistant population (Figure 2B). The LD$_{50}$ for metosulam for the susceptible population was 1 g ha^{-1} compared with 57 g ha^{-1} for the Port Broughton population and 182 g ha^{-1} for the Tumby Bay population (Table 2).
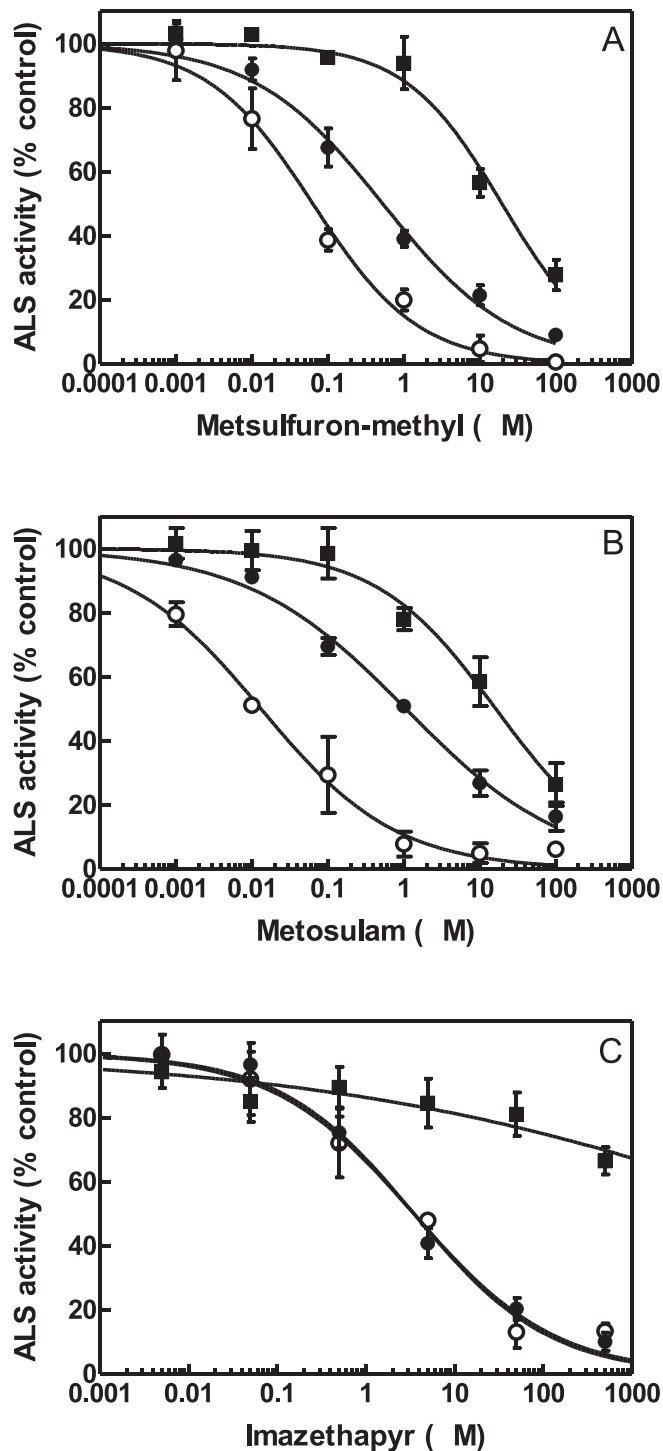

Figure 3. Response of acetohydroxyacid synthase (AHAS) isolated from susceptible and resistant oriental mustard populations and assayed in vitro in the presence of AHAS inhibitors: (A) metsulfuron-methyl, (B) metosulam, and (C) imazethapyr. Populations are Roseworthy (○), Port Broughton (●), and Tumby Bay (■). Curves are fitted sigmoidal inhibition curves to percentage of AHAS activity: (A) $Y = 100/(1 + 10^{((-1.195 - \log(x)) \times -0.628)})$, $Y = 100/(1 + 10^{((-0.276 - \log(x)) \times -0.510)})$ and $Y = 100/(1 + 10^{((1.292 - (\log(x)) \times -0.681)})$ for Roseworthy, Port Broughton, and Tumby Bay populations, respectively; (B) $Y = 100/(1 + 10^{((-1.877 - \log(x)) \times -0.488)})$, $Y = 100/(1 + 10^{((0.029 - \log(x)) \times -0.415)})$ and $Y = 100/(1 + 10^{((1.211 - (\log(x)) \times -0.553)})$ for Roseworthy, Port Broughton, and Tumby Bay populations, respectively; and (C) $Y = 100/(1 + 10^{((0.542 - \log(x)) \times -0.545)})$, $Y = 100/(1 + 10^{((0.544 - \log(x)) \times -0.57)})$ and $Y = 100/(1 + 10^{((4.961 - (\log(x)) \times -0.161)})$ for Roseworthy, Port Broughton, and Tumby Bay populations, respectively, where x is the herbicide concentration.

Table 5. Concentrations of acetohydroxyacid synthase (AHAS) inhibitors required to inhibit AHAS activity in vitro by 50% for AHAS extracted from populations of oriental mustard and the ratio of I_{50} for the resistant compared to the susceptible populations (R/S).

| | RW[a] | PB | | TB | |
| | I_{50}[b] | I_{50} | | I_{50} | |
Herbicide	μM	μM	R/S	μM	R/S
Metsulfuron-methyl	0.06	0.53	9	19.3	322
Chlorsulfuron	0.018	6.7	372	29.5	1,639
Metosulam	0.013	1.1	85	16.3	1,254
Florasulam	0.082	0.39	5	19.6	239
Imazethapyr	3.5	3.5	1	> 500	> 143

[a] Abbreviations: RW, Roseworthy population susceptible to all herbicides; PB, Port Broughton population; TB, Tumby Bay population susceptible to auxinic herbicides and resistant to AHAS inhibitors; R/S, ratio of LD_{50} for the resistant compared to the susceptible populations.
[b] I_{50} is the concentration of herbicide required to inhibit AHAS activity by 50%.

The resistant populations exhibited less resistance to florasulam compared with metosulam.

The oriental mustard populations from Port Broughton and Tumby Bay were also resistant to imidazolinone herbicides. The susceptible population was controlled by the normal field rate of 49 g ha^{-1} imazethapyr. Only a small percentage of the Tumby Bay population was controlled by this herbicide rate; however, 75% of the Port Broughton population was controlled. The LD_{50} for the susceptible population was 8.4 g ha^{-1}. The LD_{50} for the Port Broughton population was 34 g ha^{-1} and that of the Tumby Bay population was 394 g ha^{-1}. The Tumby Bay population was much more resistant to imazethapyr than was the Port Broughton population. Both resistant populations were also resistant to the imidazolinone herbicide imazamox (Table 3).

A number of alternative herbicides and herbicide mixtures that are registered for oriental mustard control were tested on the susceptible and resistant populations (Table 4). All herbicides and mixtures controlled the susceptible population. All alternative herbicides were effective on the resistant Tumby Bay population except florasulam plus bromoxynil and dicamba plus metsulfuron-methyl, which are mixtures containing AHAS inhibitors and sublethal rates of other herbicides. In contrast, the Port Broughton population was not controlled by any herbicide mixture containing an AHAS inhibitor or an auxinic herbicide, excepting the mixtures of diflufenican plus MCPA and picolinofen plus MCPA. Photosystem II (PS II) and phytoene desaturase (PDS) inhibitors were able to control this population. These results show that resistance to both AHAS inhibitors and auxinic herbicides has greatly reduced the range of herbicides available to control this species.

The Port Broughton oriental mustard population has resistance to both auxinic herbicides and to AHAS inhibitors, a phenomenon described as multiple resistance. This occurs where selection with different herbicides selects for resistance to each herbicide independently (Powles and Yu 2010). Resistance to AHAS inhibitors is common in weed species and the pattern of resistance among chemical groups varies depending on the specific mutation within AHAS (Powles and Yu 2010; Tranel and Wright 2002). Both resistant populations of oriental mustard had different patterns of resistance among the AHAS inhibitors. The Tumby Bay population exhibited high resistance to all chemical groups, whereas the Port Broughton population tended to have lower levels of resistance, particularly to florasulam and the imidazolinone herbicides. This suggests the mechanism of resistance in these two populations is not identical.

AHAS Enzyme Assays and Gene Sequencing. Resistance to AHAS inhibitors in broadleaf weeds is commonly caused by mutations in the AHAS gene resulting in an enzyme with reduced capacity to bind AHAS inhibitors (Tranel and Wright 2002). AHAS was extracted from plants of the three populations and tested in vitro with a sulfonylurea herbicide, a sulfonamide herbicide, and an imidazolinone herbicide. Metsulfuron-methyl inhibited AHAS activity at low concentrations in the susceptible population (Figure 3A); however, AHAS activity extracted from the Tumby Bay population was less sensitive to metsulfuron-methyl. AHAS activity extracted from the Port Broughton population was also less sensitive to metsulfuron-methyl than that of the susceptible population, but more sensitive than the Tumby Bay population. Comparing the ratio of I_{50} for the resistant to the susceptible populations (R/S value) of the AHAS from the Tumby Bay population was 322 compared to 9 for the Port Broughton population (Table 5). The AHAS activity of both populations was also less affected by chlorsulfuron compared with the susceptible population; with an R/S value of 1,639 for the Tumby Bay population and 372 for the Port Broughton population.

AHAS activity extracted from the susceptible population was also susceptible to metosulam (Figure 3B). More herbicide was required to inhibit AHAS from the Tumby Bay population and Port Broughton populations compared with the susceptible population with R/S values of 1,254 for

Table 6. Variations in acetohydroxyacid synthase gene sequence between susceptible and resistant populations of oriental mustard and the predicted amino acid.

| | Position | | | | | | | | | | | |
Population	367, Codon	123, Amino acid	376, Codon	126, Amino acid	394, Codon	132, Amino acid	472, Codon	158, Amino acid	589, Codon	197, Amino acid	1720, Codon	574, Amino acid
RW[a]	TCC	Ser	ATT	Ile	CGT	Arg	TCC	Ser	CCT	Pro	TGG	Trp
PB	TCC	Ser	ATT	Ile	CGT	Arg	TCC	Ser	TCT	Ser	TGG	Trp
TB	TCA	Ser	ATC	Ile	CGC	Arg	TCT	Ser	CCT	Pro	TTG	Leu

[a] Abbreviations: RW, Roseworthy population susceptible to all herbicides; PB, Port Broughton population; TB, Tumby Bay population susceptible to auxinic herbicides and resistant to AHAS inhibitors.

the Tumby Bay population and 85 for the Port Broughton population (Table 5). The AHAS activity from the resistant populations was also less sensitive to the sulfonamide herbicide florasulam compared with the susceptible population, with R/S values of 239 for the Tumby Bay population and 5 for the Port Broughton population.

AHAS activity extracted from the susceptible population was inhibited by imazethapyr at relatively low concentrations (Figure 3C), with an I_{50} of 3.5 μM (Table 5). The AHAS activity extracted from the Port Broughton population was also susceptible to imazethapyr and had the same I_{50} as the susceptible population. In contrast, AHAS activity extracted from the Tumby Bay population was highly resistant to imazethapyr with an I_{50} of greater than 500 μM.

Two sections of the gene for AHAS were amplified and sequenced. These two sections composed 27% of the gene and contained four of the seven sites where mutations in AHAS are known to provide resistance to herbicides (Powles and Yu 2010). Compared with the sequence of the susceptible population, the sequence of the AHAS gene from the Port Broughton sample had a single nucleotide change at position 589, leading to a predicted amino acid change from Pro to Ser at position 197 (Table 6). The sequence of the AHAS gene from the Tumby Bay population had five nucleotide changes compared with the sequence of the susceptible population (Table 6). Four of these five nucleotide changes did not result in predicted amino acid changes in the protein. The other nucleotide change, at position 1722, predicted a substitution of Leu for Trp at position 574. Each of the resistant populations had a mutation within AHAS that would predict resistance to sulfonylurea herbicides.

The level of resistance in the extracted AHAS of the two resistant populations was different. It is known that the specific amino acid substitution present in the AHAS enzyme results in different levels of resistance to specific AHAS-inhibitor chemical groups (Powles and Yu 2010; Tranel and Wright 2002). Typically the Trp 574 to Leu substitution results in high levels of resistance across the AHAS inhibitors (Tranel and Wright 2002). In contrast, substitutions at Pro 197 result in high levels of resistance to the sulfonylurea and sulfonamide herbicides and little or no resistance to the imidazolinone herbicides (Tranel and Wright 2002). The mutations observed in this study matched these patterns of resistance, with the Port Broughton population possessing a Pro 197 to Ser substitution in AHAS, giving the enzyme resistance to the sulfonylurea and sulfonamide herbicides, but not to the imidazolinone herbicides. In contrast, the Tumby Bay population had a Trp 574 to Leu substitution in AHAS giving the enzyme resistance to all three classes of AHAS inhibitors.

The general patterns of resistance of the Tumby Bay population at the whole plant level and at the isolated enzyme level were similar in that high levels of resistance were detected across all AHAS inhibitors tested. This indicates the target-site mutation is responsible for resistance in this population. In contrast, there was a poor correlation between resistance at the whole plant level and isolated enzyme level for the Port Broughton population. This was most obvious for the imidazolinone herbicides, where the whole plants exhibited four- to fivefold resistance, whereas the AHAS was completely susceptible in vitro. Likewise, the Port Broughton population was nearly as resistant to metsulfuron-methyl as the Tumby Bay population, yet the AHAS of the Tumby Bay population was considerably more resistant to this herbicide in vitro. These results suggest that a target-site mutation alone is not responsible for resistance to the AHAS inhibitors and another mechanism is likely contributing to resistance. That mechanism is unknown; however, higher AHAS activity has been detected in resistant populations of oriental mustard (Boutsalis et al. 1999).

These experiments have demonstrated that a population of oriental mustard has evolved resistance to both the auxinic herbicides 2,4-D and MCPA, and to a broad range of AHAS inhibitors. This pattern of resistance severely limits the options for controlling this population; however, PS II and PDS-inhibiting herbicides are still effective. Multiple resistance across herbicide modes of action is rare in weeds species resistant to auxinic herbicides, only having previously been reported for wild radish (Walsh et al. 2004) and false cleavers (*Galium spurium* L.) (Van Eerd et al. 2004). The increased reliance on auxinic herbicides to control broadleaf weeds that evolve resistance to other modes of action, such as that of glyphosate, will likely see more cases of multiple resistance in broadleaf weeds. The mechanism of resistance to the auxinic herbicides in oriental mustard has not yet been determined. The resistant population contains a target-site modification in AHAS, which explains some of the resistance to AHAS inhibitors; however, a second mechanism also appears to be present. Most broadleaf weed populations with resistance to AHAS inhibitors have been shown to be resistant as a result of a target-site modification. Non–target-site resistance has been reported for grass weeds, but is rare in broadleaf weeds (Preston 2004); however, in this case it appears that non–target-site resistance could be present in the resistant population. The evolution of non–target-site resistance can lead to unexpected cross-resistance across herbicidal modes of action (Petit et al. 2010), greatly reducing the number of herbicide options available. This may have happened in the present example; however, whether resistance to AHAS inhibitors and hormone herbicides are genetically linked in the oriental mustard population remains to be elucidated.

Acknowledgments

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