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Multiple herbicide-resistant wild radish (*Raphanus raphanistrum*) populations dominate Western Australian cropping fields

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Abstract. *Raphanus raphanistrum* is a problematic weed, which has become increasingly difficult to control in Australian cropping regions. In 2010, a random survey was conducted across 14 million ha of the Western Australian grain belt to establish the frequency of herbicide resistance in *R. raphanistrum* and to monitor the change in resistance levels by comparing results with a previous survey in 2003. Screening *R. raphanistrum* populations with herbicides commonly used to control this weed revealed that most populations (84%) contained individual plants resistant to the acetolactate synthase-inhibiting herbicide chlorsulfuron, whereas 49% of populations also had plants resistant to the imidazolinone herbicides. Resistance to other mode of action herbicides (2,4-D (76%) and diflufenican (49%)) was also common. Glyphosate, atrazine and pyrasulfotole + bromoxynil remained effective on most *R. raphanistrum* populations. These results demonstrate that resistance to some herbicides has increased significantly since 2003 when the values were 54% for chlorsulfuron and 60% for 2,4-D; therefore, a wide range of weed management options that target all phases of the cropping program are needed to sustain these cropping systems in the future.

Additional keywords: chlorsulfuron, diflufenican, 2,4-D, resistance evolution, resistance survey, weeds.

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Introduction

Crop-infesting weed species are a major threat to productivity of the major grain crops. Herbicides are highly effective for weed control in many field crops and are currently the preferred technology. However, as a result of over-reliance on herbicides and the exposure of large weed populations to these herbicides, there is continual and widespread evolution of herbicide-resistant weed populations (Powles and Yu 2010; Heap 2015). Resistant biotypes from 238 weed species have been reported across 66 different countries for 155 different herbicides (Heap 2015), which poses a significant threat to global food production.

Raphanus raphanistrum L. (wild radish) is a damaging weed that has a major economic impact on crop production in Australia (Alemseged *et al*. 2001; Walsh *et al*. 2007). It is also an alternative host or reservoir for several pathogen and insect pests of grain crops (Cheam and Code 1995), and can cause substantial crop yield reduction (Panetta *et al*. 1988; Blackshaw *et al*. 2002) by competing for nutrients, light and water. *Raphanus raphanistrum* is difficult to control in dicot crops, and even at low densities can markedly reduce crop yields (Code and Reeves 1981). This species persists as a weed mainly due to its prolific seed production, long seed persistence and ability to germinate and survive in a wide range of conditions (Reeves *et al*. 1981; Cheam 1986; Cheam and Code 1995).

Herbicides are the most common choice for control of *R. raphanistrum* in Australian crops and herbicide-resistant populations are present in several states (Heap 2015). Resistance to acetolactate synthase (ALS)-inhibiting herbicides (Hashem *et al*. 2001*a*; Walsh *et al*. 2001), photosystem II-inhibiting herbicides (Hashem *et al*. 2001*b*), synthetic auxins (Walsh *et al*. 2004), phytoene desaturase-inhibiting herbicides (Cheam *et al*. 2000) and multiple herbicide chemistries (Walsh *et al*. 2004, 2007) have also been confirmed in *R. raphanistrum* populations from the Western Australian (WA) grain belt. Resistance to ALS-inhibiting herbicides has also been reported in Brazil and South Africa (Heap 2015).

Our previous large-scale herbicide resistance surveys in WA found that *R. raphanistrum* populations were most frequent in the northern agricultural region (Walsh *et al*. 2001, 2007). A more recent weed flora survey (Borger *et al*. 2012) found that *R. raphanistrum* had moved farther south over a 10-year period and is now a problem weed in the more southern cropping regions. Building on our previous resistance surveys in *R. raphanistrum* populations across the WA grain belt (Walsh *et al*. 2001, 2007), here we report on a third (2010) large-scale survey to update and quantify the geographic extent and spectrum of herbicide resistance in *R. raphanistrum*.

Materials and methods

Seed collection

Seed material was collected as part of a broad-scale survey evaluating herbicide resistance in crop fields in key weed

species (Owen *et al*. 2014). Briefly, before seed collection, farmers were contacted at random for permission (through field days, phone, mail outs and local grower networks) and to provide farm maps. In total, 466 crop fields were visited just before grain harvest (therefore, weed control practices would have been used earlier in the season; however, some fields may not have been treated due to dry conditions at the start of the 2010 season). Crop fields were chosen randomly within the farm and weed seed was collected by two people walking in an inverted 'W' pattern across each field. During sampling, weed density was measured by visually estimating the number of plants m^{-2} over the sample area and recording into category-based classifications (see *Results* section). *Raphanus raphanistrum* seed pods were collected from a large number of plants (15–50 per field) and bulked (within each population) at the time of collection. In fields where less than 10 plants were sighted, no sample was collected. After collection, seed pods were milled to release the seeds and chaff material was separated by aspiration. Seed samples were stored in a warm, dry glasshouse from December to April to relieve any seed dormancy. A total of 96 *R. raphanistrum* populations were collected for subsequent herbicide testing (Fig. 1).

Seed germination

During the growing-season months (May–October 2011 and 2012), seeds from each of the 96 *R. raphanistrum* populations were placed into plastic seedling trays (300 mm by 400 mm by 100 mm) containing potting mix (50% composted pine bark, 25% peat and 25% river sand). Fifty to sixty seeds from each population were sown into each tray for each herbicide, to ensure a high number of plants (~45–50) for herbicide treatment. All plants were watered and fertilised as required.

Herbicide resistance screening

Herbicide resistance status was determined by treating seedlings at the 2- to 3-leaf stage with a range of herbicides using the upper recommended field rates for *R. raphanistrum* control (Table 1), together with appropriate adjuvants, using a

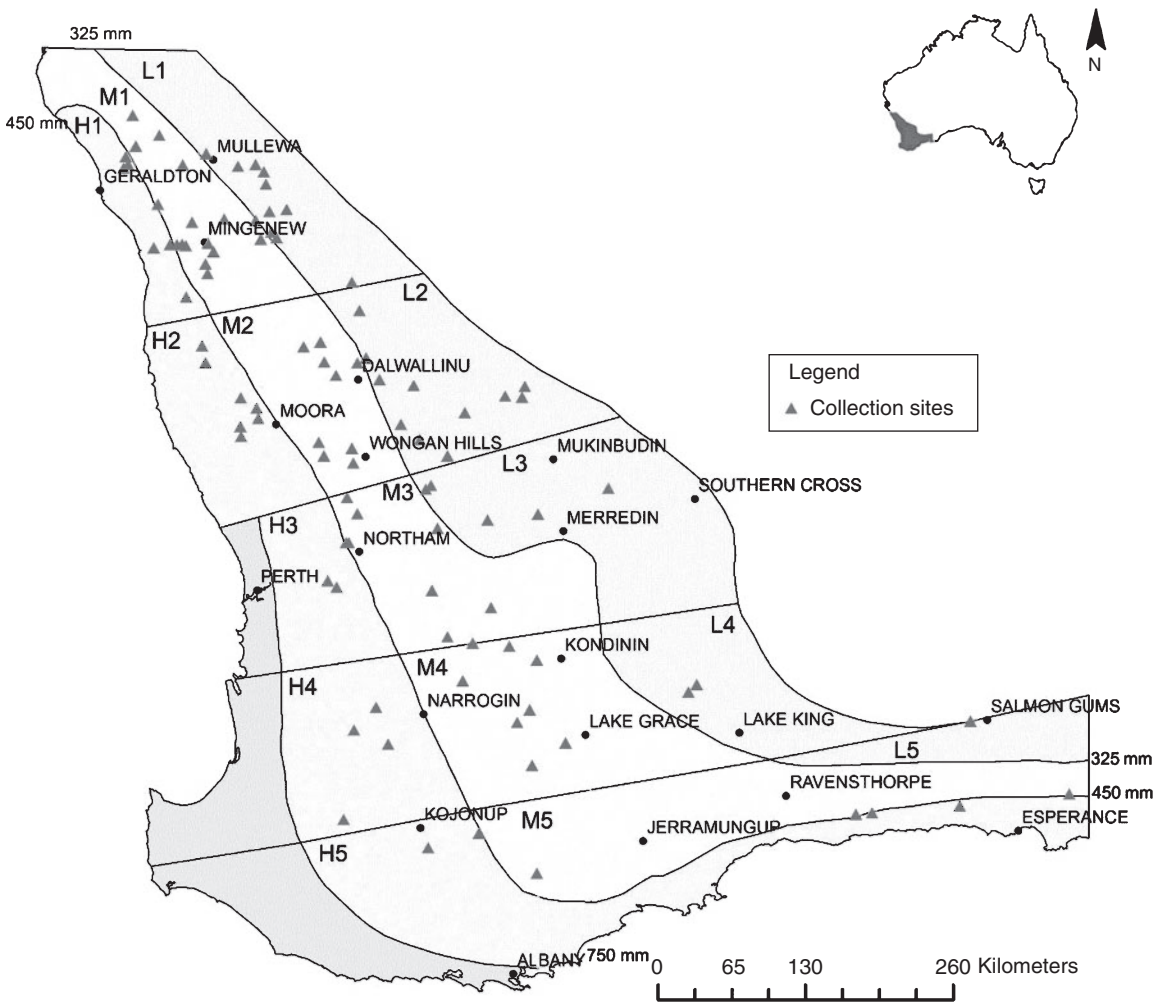

Fig. 1. Map of south-western Western Australia showing the agronomic zones of the grain belt where *Raphanus raphanistrum* samples were collected for herbicide resistance testing. Average annual rainfall isohyets are shown. Rainfall regions are represented by H (High 450–750 mm), M (Medium 325–450 mm), and L (Low <325 mm). Zones are signified by 1 (North), 2 (North central), 3 (Central), 4 (South central), 5 (South).

Table 1. Herbicides and rates used for resistance screening of *Raphanus raphanistrum* populations collected in 2010 from the Western Australian grain belt

Chemical class	Mode of action	Active ingredient	Field rate (g ha^{-1})
Sulfonylureas	Inhibitors of acetolactate synthase	Chlorsulfuron	10
Imidazolinones	Inhibitors of acetolactate synthase	Imazamox + imazapyr	13 + 6
Nicotinanilides	Inhibitors of phytoene desaturase	Diflufenican	100
Phenoxycarboxylic acids (Phenoxys)	Synthetic auxins – disruptors of plant cell growth	2,4-D amine	625
Triazines	Inhibitors of photosynthesis at photosystem II (PS II inhibitors)	Atrazine	900
Pyrazoles + nitriles	Inhibitors of 4-hydroxyphenyl-pyruvate dioxygenase + inhibitors of photosynthesis at photosystem II (PS II inhibitors)	Pyrasulfotole + bromoxynil	25 + 141
Glycines	Inhibitors of 5-enolpyruvylshikimate-3-phosphate synthase	Glyphosate	705

custom-built dual nozzle (TeeJet) cabinet sprayer (see Owen *et al*. 2014; for spraying details). Herbicide resistance status was tested for the ALS-inhibiting herbicides chlorsulfuron (Lusta, 750 g a.i. kg^{-1}, Nufarm, Melbourne, Australia) and imazamox + imazapyr (Intervix, 33 + 15 g a.i. L^{-1}, Nufarm, Australia), and for diflufenican (Brodal Options 500 g a.i. L^{-1}, Nufarm, Australia), 2,4-D (Amicide 625 g a.i. L^{-1}, Nufarm, Australia), pyrasulfotole + bromoxynil (Velocity 37.5 + 210 g a.i. L^{-1}, Nufarm, Australia), atrazine (Nutrazine 900 g a.i. kg^{-1} DF, Nufarm, Australia), and glyphosate (Roundup Powermax 540 g a.i. L^{-1}, Nufarm, Australia) (Table 1).

Plant mortality was assessed 21 days after treatment, by determining if the growing point was chlorotic or new growth was visible. For populations where only a small number (five or less) of individual plants survived treatment, these plants were cut back, re-sprayed and allowed to regrow to confirm that the individual plants were resistant. For diflufenican and 2,4-D, plants were also assessed on whether they progressed to the reproductive stage to show unequivocally that they were still alive and capable of reproducing. Known susceptible (WARR7) and resistant *R. raphanistrum* populations (WARR5, WARR6) (Walsh *et al*. 2004, 2007) were used as controls in all experiments, with 100% control of the known susceptible population and high survival (>80%) of the known resistant populations for each herbicide (data not shown). Selected populations were also used as untreated controls, with no mortality observed in these populations over the duration of the experiments. Herbicide treatments were repeated during the same growing season ~8 weeks after the first treatment, except in cases where seed quantities were too low or for the imidazolinone (IMI) herbicide, and results averaged for each population (small variations between experiments were recorded for some populations; however, there was no change in resistance category). For chlorsulfuron and atrazine only those populations with a small number of surviving plants were re-tested.

Data analyses

Populations were classified as susceptible if there was zero plant survival. Populations with resistant survivors were classified into two groups: those with ≥20% plant survival and those having <20% survival (this includes all plant survival between 1% and 19%). This classification enables comparisons with previous surveys (Walsh *et al*. 2007) and is used because farmers often visually recognise resistance at a level of ~20% survival in the field. Resistance of ≥20% would result in commercial failure of the herbicide, at which point farmers may stop using the herbicide or consider alternative management options; whereas <20% survival indicates that a sufficiently high number of resistant individuals are present in the population to result in commercial failure the next time this herbicide is applied (Walsh *et al*. 2007). Standard errors were calculated for the two replicates of each herbicide treatment. The standard error for all susceptible controls for each herbicide treatment was 0, whereas the standard errors for each herbicide-resistant control were: 5.2 (2,4-D), 9.1 (diflufenican), 4.2 (chlorsulfuron) and 5.5 (atrazine).

Results

Raphanus raphanistrum was collected from 21% of the 466 crop fields sampled in this large-scale survey (Fig. 1), although it was present in 34% of fields. Wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and oil seed rape (*Brassica napus* L.) were the most common crops, comprising 65%, 12% and 10% of cropped fields, respectively (Owen *et al*. 2014). *Raphanus raphanistrum* was present in 76% of lupin crops and 34% of cereal crops. In the northern agricultural regions (zones H1, M1, L1, H2, M2, L2), *R. raphanistrum* was far more common, with 52% of fields containing this species, compared with 22% in the southern cropping regions. Generally, *R. raphanistrum* was present at low numbers, with 18% of fields containing plants which were difficult to find and 14% of fields having densities of <1 plant m^{-2}. Only 2% of fields had densities of 1–10 plants m^{-2}. In 66% of fields, no *R. raphanistrum* plants were found within the sampling area.

ALS-inhibiting herbicides

The selective ALS-inhibiting herbicides have been widely used to control *R. raphanistrum* in Australia. The survey revealed widespread, high level resistance to the commonly used ALS herbicides. The majority (70 out of 96), of populations screened with the ALS-inhibiting sulfonylurea (SU) herbicide chlorsulfuron exhibited ≥20% (±0.1 to 3.5%) plant survival to this herbicide, with most of these populations having >80% plant survival (Table 2; Fig. 2). A further 11 populations had <20% (±3.1 to 5.0%) plant survival (Table 2). Resistance was widespread across the survey area with most zones having a high percentage of resistant plants (Table 3, Fig. 1). Only 15 populations were completely susceptible to chlorsulfuron, and

these were all found in the higher rainfall southern cropping regions.

Of the 86 populations treated with the IMI class of ALS-inhibiting herbicides (imazamox + imazapyr), 16 populations had ≥20% survival whereas a further 26 populations had <20% survival (Table 2). Resistance was more common in the northern cropping regions (zones M1, L2, L3 and M3) whereas southern coastal areas had less resistance (Table 3). Around half (44) of the populations remained susceptible to this herbicide and were found in most of the cropping zones (Fig. 1).

Phytoene desaturase-inhibiting herbicides

Forty-five of the ninety-one populations treated with diflufenican contained plants with resistance to this herbicide; however, only five of these, originating from the northern grain belt in the M1 zone, had ≥20% (±2.4 to 14.1%) survival. Resistance to this herbicide was nevertheless widespread (i.e. appeared in most zones) across the survey area (Table 3).

Table 2. The number of *Raphanus raphanistrum* populations collected in 2010 in each resistance category (fully susceptible, <20% plant survival or ≥20% plant survival) for each herbicide

Herbicide	Susceptible	<20% survival	≥20% survival
Chlorsulfuron	15	11	70
Imazamox + imazapyr	44	26	16
Diflufenican	46	40	5
2,4-D amine	23	37	36
Atrazine	95	0	1
Pyrasulfotole + bromoxynil	94	0	0
Glyphosate	92	0	0

Synthetic auxin herbicides

Of the 96 populations treated with 2,4-D amine, 73 populations contained plants resistant to this herbicide. Of these, 36 populations had ≥20% (±0.1 to 5.7%) survival whereas a further 37 populations had <20% (±0.2 to 9.3%) survival (Table 2). Resistance was generally higher in the higher rainfall zones of the northern agricultural region (Table 3). Only 23 populations were completely susceptible and were generally from the higher rainfall southern cropping zones (Fig. 1).

Photosystem II-inhibiting herbicides

Only one population of the 96 populations tested had ≥20% (±5.8%) plant survival to atrazine applied at and above the recommended field rate, whereas all other populations were susceptible (Table 2).

Other herbicides

All populations tested were completely susceptible (0 ± 0% survival) to the mixture of pyrasulfotole (an inhibitor of 4-hydroxyphenyl-pyruvate dioxygenase, HPPD) and bromoxynil (an inhibitor of photosynthesis at photosystem II). All populations were also susceptible to glyphosate, an inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (Table 2).

Multiple herbicide resistance

A high number of populations exhibited multiple herbicide resistance, with 77 of the 96 populations having plants resistant across at least two herbicide modes-of-action (Table 3). Nearly half (43) of the populations had resistance across two herbicides modes-of-action (generally chlorsulfuron

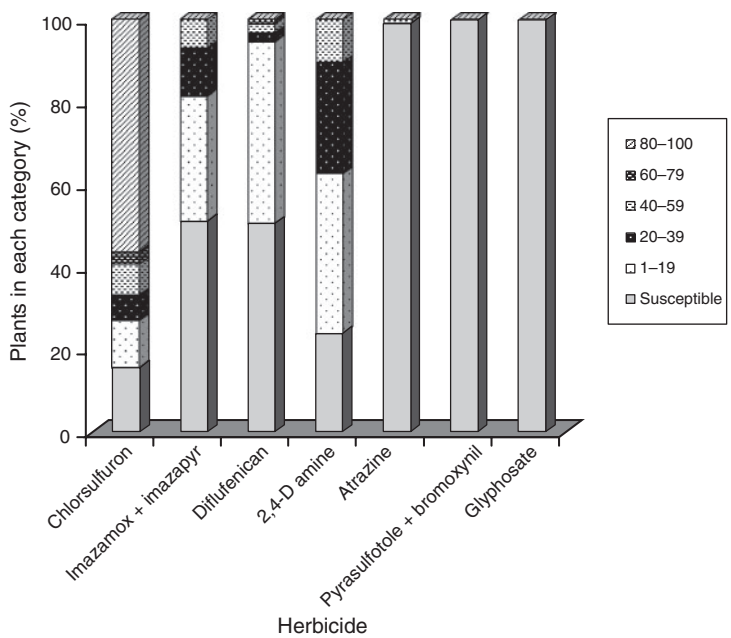

Fig. 2. Severity of resistance to each herbicide tested. Resistance intervals are 0% (all plants are susceptible), 1–19%, 20–39%, 40–59%, 60–79% and 80–100% of plants surviving each herbicide treatment.

Table 3. The percentage of *Raphanus raphanistrum* populations in the high-resistance (≥20% surviving plants (H)), low-resistance (<20% surviving plants (L)) or fully susceptible (0% surviving plants (S)) categories by agronomic zone (refer to Fig. 1) to herbicides tested in this survey

Data are not shown for herbicides where no resistance was detected

Zone	No. populations tested	Chlorsulfuron			Imazamox + imazapyr			Diflufenican			2,4-D amine			Atrazine		
		H	L	S	H	L	S	H	L	S	H	L	S	H	L	S
H1	7	86	14	0	0	43	57	0	71	29	14	72	14	0	0	100
M1	15	87	13	0	31	38	31	38	31	31	60	40	0	7	0	93
L1	10	100	0	0	12	50	38	0	44	56	60	40	0	0	0	100
H2	7	86	14	0	0	43	57	0	71	29	43	43	14	0	0	100
M2	9	78	11	11	11	22	67	0	22	78	56	22	22	0	0	100
L2	11	100	0	0	56	22	22	0	50	50	45	55	0	0	0	100
H3	3	33	0	67	0	33	67	0	67	33	33	0	67	0	0	100
M3	8	88	0	12	29	29	42	0	62	38	38	38	24	0	0	100
L3	5	100	0	0	67	33	0	0	0	100	60	40	0	0	0	100
H4	4	25	25	50	0	25	75	0	50	50	0	0	100	0	0	100
M4	7	29	29	42	14	0	86	0	43	57	0	29	71	0	0	100
L4	2	50	50	0	0	50	50	0	50	50	0	50	50	0	0	100
H5	4	0	25	75	0	0	100	0	25	75	0	25	75	0	0	100
M5	3	0	33	67	0	50	50	0	0	100	0	33	67	0	0	100
L5	1	0	0	100	0	0	100	0	100	0	0	100	0	0	0	100

and 2,4-D), whereas a further 33 populations had resistance across three modes-of-action. Only seven populations were susceptible to all herbicides tested (these were generally from the southern agricultural region) (Table 3).

Change in resistance levels from 1998 to 2010

Our previous resistance surveys were conducted in the same region in 1998 for chlorsulfuron and in 2003 for chlorsulfuron, diflufenican, 2,4-D and atrazine. During the 7 years between the 2003 and 2010 surveys, the number of populations with resistance to the ALS-inhibiting and auxin herbicides have increased dramatically (by 30 and 16 percentage points, respectively), whereas resistance to diflufenican has remained relatively steady, and the number of populations with resistance to atrazine has remained low (Fig. 3). Over the 12-year period since the first survey in 1998 on resistance to the ALS-inhibiting SU herbicide chlorsulfuron, the incidence of resistance has increased dramatically from 21% of populations in 1998 to

Fig. 3. Changes in herbicide resistance status between 2003 and 2010 (chlorsulfuron, 2,4-D amine, diflufenican, atrazine) for *Raphanus raphanistrum* populations collected from the same regions of the Western Australian grain belt. Resistance values for 2003 and 2010 include both (*a*) <20% surviving plants and (*b*) ≥20% surviving plants, reflecting the categories used by Walsh *et al*. (2007).

54% in 2003 and 84% in 2010 (Fig. 3). Alarmingly, 49% of populations tested in 2010 also contained individuals resistant to the ALS-inhibiting IMI herbicide mixture of imazamox + imazapyr (Table 2).

Discussion

In this survey, over a large geographic area, *R. raphanistrum* was found in ~34% of WA cropping fields and was more prevalent in the northern cropping regions. A recent survey of the WA cropping area found that over the period between 1997 and 2008, the incidence of *R. raphanistrum* in cropping regions had increased significantly (Borger *et al*. 2012), to levels similar to those found in the present study. *Raphanus raphanistrum* had an 11% increase in incidence throughout the northern and central grain belt and had spread south from the northern agricultural region (Borger *et al*. 2012). The spread of this weed may be associated with its herbicide resistance, and the increased prevalence of intensive cropping in the southern regions is likely to be exerting greater herbicide selection pressures on these weed populations. *Raphanus raphanistrum* is less prevalent in cropping regions of eastern Australia, being found in only 4–21% of fields in New South Wales (Lemerle *et al*. 1996; Osten *et al*. 2007; Broster *et al*. 2012) and in 14% of fields in southern Queensland (Osten *et al*. 2007).

Multiple herbicide-resistant *R. raphanistrum* is now widespread and prevalent across most of the WA cropping region. Nearly all populations showed a high level of survival to the ALS-inhibiting SU herbicide chlorsulfuron, consistent with the previously reported distribution of resistance (Hashem *et al*. 2001*a*; Walsh *et al*. 2007). However, resistance to another ALS-inhibiting herbicide class (IMI) has previously not been evaluated. The lower frequency of resistance to the IMI herbicides identified in the present survey could be due to the type of ALS mutation(s) and/or metabolic resistance present in the populations (reviewed in Yu and Powles 2014). Many known ALS mutations confer resistance to the SU herbicides (Tan and Medd 2002; Yu *et al*. 2003), and/or the IMI herbicides. Therefore, the observed pattern of *R. raphanistrum* resistance to SU plus IMI herbicides versus SU only could be due to the presence of different specific ALS mutations (together with potential metabolic resistance mechanisms) in different populations (Powles and Yu 2010; Han *et al*. 2012; Yu *et al*. 2012). For example, in *R. raphanistrum*, the ALS Trp-574-Leu and Ala-122-Tyr mutations both confer resistance to all three classes of ALS inhibitors, whereas the Asp-376-Glu mutation confers high level resistance to the SU and triazolopyrimidine herbicides with only low to moderate resistance to the IMI herbicides (Han *et al*. 2012; Yu *et al*. 2012). Although field histories were not collected in this survey, Hashem *et al*. (2001*a*) found that resistance was more common in fields where wheat (*Triticum aestivum* L.) and lupin (*Lupinus* spp.) was the main rotation, with resistance to the ALS-inhibiting herbicides evident after only five applications.

Although chlorsulfuron-resistant populations of *R. raphanistrum* have previously been identified in WA, with a targeted survey revealing 43% of *R. raphanistrum* populations having resistance in 1996 (Hashem *et al*. 2001*a*), the three large-scale, entirely random surveys carried out over the past 12 years have identified an increase in chlorsulfuron resistance (undoubtedly due to continued selection pressure) from 21% in 1998 (Walsh *et al*. 2001) to 84% in 2010. Hashem *et al*. (2001*a*) found no resistance to 2,4-D in 1996, whereas the random surveys in 2003 (Walsh *et al*. 2007) and 2010 (present work) found that 60% and 74%, respectively, of populations had plants with resistance to 2,4-D. This could reflect increased reliance on 2,4-D in response to the increased difficulty in controlling *R. raphanistrum* with the ALS-inhibiting herbicides. Similarly, triazine herbicide resistance was detected in one population in the present random survey but was absent in the 1996 survey (Hashem *et al*. 2001*a*); however, 15% of populations had some level of resistance in the 2007 survey (Walsh *et al*. 2007). Several more populations with probable resistance to this mode of action have subsequently been identified in the northern cropping region by farmers having control problems (P. Newman, pers. comm.).

Importantly, all populations collected in 2010 remained susceptible to the HPPD-inhibiting herbicide pyrasulfotole (mixed with bromoxynil), and to glyphosate. *Raphanus raphanistrum* populations collected in the 2003 survey (Walsh *et al*. 2007) were also tested for glyphosate and pyrasulfotole and found to be susceptible (M. J. Owen, unpubl. data). Pyrasulfotole is the first HPPD herbicide used for weed control in Australia and was released to the Australian market in 2008. Resistance to the HPPD-inhibiting herbicides has been reported in *Amaranthus palmeri* and *A. tuberculatus* in the United States (Hausman *et al*. 2011; McMullan and Green 2011; Ma *et al*. 2013; Heap 2015). Glyphosate-resistant *R. raphanistrum* populations have recently been identified in the WA grain belt in a targeted study of two problem fields (Ashworth *et al*. 2014). Further targeted surveys of fields with incomplete weed control would probably reveal a greater number of populations with resistance to herbicides such as atrazine (whereas only one resistant population was found in the 2010 random survey), glyphosate and possibly HPPD-inhibiting herbicides.

The distribution of *R. raphanistrum* has increased throughout the WA grain belt (Borger *et al*. 2012) with this present study showing a high proportion of populations resistant to two or more herbicides, highlighting the need to focus on control techniques which aim to reduce seed production and seed return to the soil seed bank. With the continued increase in resistance to herbicides commonly used to control this species, long-term strategic weed management systems using herbicide mixtures with different modes-of-action, as well as non-chemical control options such as competitive crops and crop rotation, should be employed (Matthews 1994). Newer strategies targeting weed seed removal at crop harvest range in efficacy from 93% to 99%. Use of a combination of methods such as narrow windrow burning, baling chaff, carting/collecting chaff and seed crushing with the Harrington Seed Destructor (Walsh *et al*. 2013), which prevent seed return to the soil seed bank, will become vital for the sustainability of cropping regions in the future.

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