Seed soaking method to test resistance in *Raphanus raphanistrum* to ALS-inhibiting herbicides

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Abstract

Testing resistance in *Raphanus raphanistrum* to ALS-inhibiting herbicides takes 10 weeks using conventional method where seedlings are raised from collected seeds and sprayed with herbicides. The aim of this study was to develop a reliable method to test for resistance in *R. raphanistrum* populations to ALS-inhibiting herbicides without requiring a foliar spray. Germinable seeds of *R. raphanistrum* were soaked in solutions of ALS-inhibiting herbicides for 24 hours and then sown in pot soil. A 40 mg L^{-1} triasulfuron (714 g a.i. kg^{-1}), 10 mg L^{-1} metosulam (714 g a.i. kg^{-1}), and 20 mg L^{-1} chlorsulfuron (750 g a.i. kg^{-1}) were found to be the best concentrations for screening of the resistant biotypes within the *R. raphanistrum* populations tested in this study. At these concentrations, plants of the susceptible biotypes and susceptible plants within resistant biotypes died or remained in a severely stunted condition often without producing a true leaf, while most plants of the resistant biotypes survived unaffected. The high correlation between the plants that survived herbicide soaking treatments and survival from a subsequent foliar herbicide spray confirmed that the discrimination of the resistant and the susceptible biotypes by seed soaking method was reliable. This method is 4–5 weeks quicker than the conventional foliar spray method and minimizes sources of errors that are likely to occur with that method.

Introduction

Raphanus raphanistrum L. (wild radish) is a serious weed in the cereal and grain legume crops over a range of soil types throughout southern Australia (Cheam and Code 1995). In response to strong selection pressure from the sulfonylurea herbicides, *R. raphanistrum* has evolved widespread resistance to ALS-inhibiting herbicides within the wheatbelt of Western Australia (Hashem *et al.* 2001, Walsh *et al.* 2001). Screening for herbicide resistance in weed species such as *R. raphanistrum* is an important component of herbicide resistance management. In the conventional method to detect resistance in *R. raphanistrum* to ALS-inhibiting herbicide, plants are raised in pots usually under glasshouse conditions, herbicides are sprayed on plants at the 2–3 leaf stage, and plant survival is assessed 4–6 weeks after spraying. This method, once seeds are non-dormant, requires about 10 weeks to complete one cycle.

ALS-resistant weed populations that have evolved target site resistance (Christopher *et al.* 1992, Saari *et al.* 1992, Burnet *et al.* 1994) are likely to be insensitive to small variations in spraying conditions. However, the efficacy of ALS-inhibiting herbicides on susceptible populations may be influenced by a wide range of environmental and plant factors. Growth stage of *R. raphanistrum* at the time of treatment is important to achieve effective results for some ALS-inhibiting herbicides. Precision in output delivery, uniformity in spraying and drift risk may confound herbicide efficacy and, thus, the discrimination between resistant and susceptible populations of weeds. Temperature, relative humidity and soil water content affect weed emergence, development and susceptibility to herbicides (Jensen and Kudsk 1988, Klingaman *et al.* 1992). Thus, any variation in application, absorption and translocation of herbicide may lead to a variation in the discrimination between susceptible and resistant biotypes. A quicker method that will minimize some of these limitations is necessary to increase the efficiency of resistance testing procedure in *R. raphanistrum* for ALS-inhibiting herbicides.

Seed soaking methods have been used to characterize resistance of rapeseed (*Brassica napus)* mutants to sulfonylureas and imidazolinone herbicides (Magha *et al.* 1993). Moss (1999) developed a seed soaking method to detect herbicide resistance in *Aleopecurus myosuroides* Huds, *Lolium multiflorum* L. and *Avena* spp. to fenoxyprop-P-ethyl, diclofop methyl, sethoxydim and pendimethalin. Letouzé and Gasquez (1999) described a rapid test for screening aryloxyphenoxy-propionic acid herbicide resistance in *A. myosuroides* and *Lolium* spp. populations based upon coleoptile length. No information is available in the literature on using a seed soaking method to detect herbicide resistance in dicot weeds such as *R. raphanistrum*. This study was undertaken to investigate the possibility of testing for resistance to ALS-inhibiting herbicides in *R. raphanistrum* using a seed soaking method and to ascertain if this test is quicker than the conventional foliar spray method.

Materials and methods

Plant materials

Siliqua from suspected resistant and known susceptible *R. raphanistrum* populations were collected in late spring from field crops in the Western Australia wheatbelt for use in this study. They were dried at room temperature for one week. To release the dormancy of seeds, siliqua walls were removed (Cheam 1986) and clean seeds were stored at 23±3°C before use.

Common screening procedure

Three experiments were conducted during 1998 and 1999 at the Dryland Research Institute, Department of Agriculture, Merredin, Australia. Required quantities of chlorsulfuron (Glean®, 750 g a.i. kg^{-1}, DuPont Australia Ltd.), triasulfuron (Logran®, 714 g a.i. kg^{-1}, Ciba-Geigy Australia Ltd.) and metosulam (Eclipse®, 714 g a.i. kg^{-1}, DowElanco Australia Ltd.) were dissolved in deionized water. About 75 seeds of resistant or susceptible biotype were soaked in 20 mL of the herbicide solution for 24 h in a 30 mL plastic vial. The seeds were shaken 8–10 times during 24 h. After soaking for 24 h, the herbicide solution was drained. Seeds (20–24 pot^{-1}) were sown at about 1.5 cm depth in 1 L pots under glasshouse conditions (23±3°C). The pots were filled with potting mix (Zin Zin quartz sand 20%, jarrah saw dust 35%, crusted pine bark 30%, peat deposit 10% and manure compost 5%) with a 2 cm layer of sandy loam soil at the top. Five days after emergence, the plants were thinned to 12 per pot. Plants were fertilized weekly with a complete nutrient solution.

Where indicated, plants of the R biotypes were sprayed with an overhead compressed-air belt-driven glasshouse sprayer calibrated to deliver 96 L ha^{-1} output at 200 kPa pressure after plant survival assessment five weeks after emergence (WAE). All herbicides were commercial formulations with wetting agents added as recommended by the manufacturer. Plant survival and shoot dry weight of the treated plants were expressed as a percentage of the untreated control plants.

A population was declared completely susceptible if the percentage of surviving individuals was 0 or completely resistant if 100% of the individuals survived. Plant survival percentage in the *R. raphanistrum* populations tested in this study varied from 18 to 94% establishing resistance but with some susceptible individuals remaining in the population.

Experiment 1 (seed soaking resistance test 1)

Using original collections, seeds of the R biotype 1 (RB1) and the S biotype 1 (SB1) were soaked in six concentrations of triasulfuron (0, 5, 10, 20, 40 and 80 mg L^{-1})

and metosulam (0, 1, 3, 5, 10 and 20 mg L^{-1}) for 24 h. The soaked seeds were then sown in 1 L pots. Plant survival of the emerged plants was recorded 5 WAE because most of the RB1 plants that survived seed soaking treatments were growing almost unaffected while most of the SB1 plants died at this stage. A small proportion of plants in the R or S biotype treated with low to medium concentrations of herbicide remained stunted without producing a true leaf at 5 WAE. These stunted plants were considered as survivors.

To confirm the resistance, the RB1 plants that survived soaking treatments were sprayed with label rate of triasulfuron (25 g a.i. ha^{-1}) and metosulam (5 g a.i. ha^{-1}) after plant survival assessment 5 WAE (4–5 leaf stages). Most of the stunted plants that were considered alive at 5 WAE died after the foliar spray at label rates. Plant survival and dry weight of shoots of both the R and S biotypes were recorded 4 weeks after spraying (WAS).

Experiment 2 (seed soaking resistance test 2)

Using original collections, seeds of resistant biotype 2 (RB2) and resistant biotype 3 (RB3) were soaked in five concentrations of triasulfuron (0, 20, 40, 80 and 160 mg L^{-1}), metosulam (0, 5, 10, 20 and 40 mg L^{-1}) and chlorsulfuron (0, 5, 10, 20, 40 and 80 mg L^{-1}). The soaked seeds were then sown in 1 L pots. In experiment 2, higher concentrations of triasulfuron and metosulam were used than in experiment 1. Plant survival was assessed using the assessment procedure as described above.

To confirm the resistance, the RB2 and RB3 plants that survived soaking treatments were sprayed with label rate of triasulfuron (25 g a.i. ha^{-1}), metosulam (5 g a.i. ha^{-1}) and chlorsulfuron (15 g a.i. ha^{-1}) after plant survival assessment at 5 WAE (4–5 leaf stage). Plant survival and shoot dry weight of all biotypes were recorded 4 WAS.

Experiment 3 (conventional single-dose resistance test)

In experiment 3, plants were grown in 1 L pots, and sprayed at the 2–3-leaf stage with label rate (25 g a.i. ha^{-1} triasulfuron, 5 g a.i. ha^{-1} metosulam, and 15 g a.i. ha^{-1} chlorsulfuron). Plant survival was recorded 4 WAS.

Statistical analysis

The experiments were conducted in a completely randomized design with three replications. Data were subjected to ANOVA and means were compared by LSD. In experiment 1 and 2, correlation analyses were performed between the number of R biotype plants that survived seed soaking treatments of ≥40 mg L^{-1} triasulfuron, ≥10 mg L^{-1} metosulam, or ≥20 mg L^{-1} chlorsulfuron and the number of R biotype plants that survived after foliar spray 5 WAE with label rate of each herbicide.

Results

Experiment 1 (seed soaking resistance test 1)

A clear difference in the response of the RB1 and SB1 plants was found when seed was soaked in different concentrations of ALS-inhibiting herbicides. About 60% of the RB1 plants survived 40 mg L^{-1} triasulfuron (Figure 1A) and 90% survived 20 mg L^{-1} metosulam (Figure 1B). All the SB1 plants died at 40 mg L^{-1} chlorsulfuron or 1 mg L^{-1} metosulam (Figures 1A,B). Most of the plants in SB1 soaked at low to medium concentrations of herbicide died 2–4 WAE and few remained stunted often without producing a true leaf. Most of the surviving plants of the RB1 soaked in different herbicide concentrations were growing unaffected at 5 WAE. A very small proportion of the plants in RB1 soaked at low–medium concentrations of herbicide remained stunted often without producing a true leaf. Most of these stunted plants within the RB1 and the SB1 died after the foliar spray. Shoot dry weight of these biotypes showed similar trends to plant survival (Figures 1C,D).

There was a significant correlation between the number of the RB1 plants that survived seed soaking treatments of ≥40 mg L^{-1} triasulfuron or ≥10 mg L^{-1} metosulam and the number of plants that survived after foliar spray with 25 g a.i. ha^{-1} triasulfuron or 5 g a.i. ha^{-1} metosulam. The r^2 values varied from 0.97 to 0.98 (Table 1).

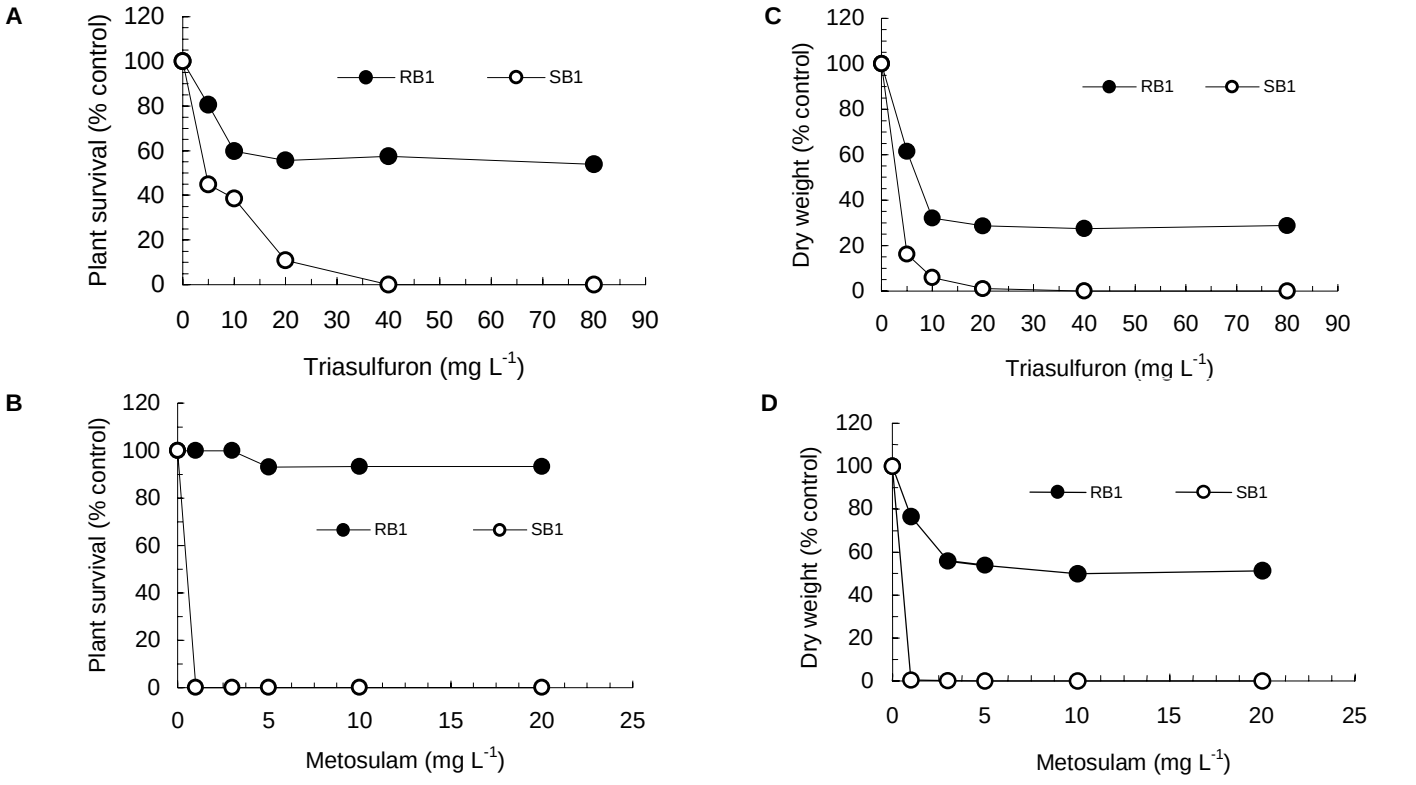

Figure 1. Survival of the treated plants of resistant biotype (RB) and susceptible biotype (SB) of *R. raphanistrum* immediately before foliar spray five weeks after emergence and their shoot dry weight four weeks after foliar spray with label rate of triasulfuron and metosulam. $LSD_{.01}$ for plant survival is 16.58% and that for shoot dry weight is 15.43%.

Experiment 2 (seed soaking resistance test 2)

About 81% of the RB2 plants survived 40 mg L^{-1} triasulfuron (Figure 2A), 82% 10 mg L^{-1} metosulam (Figure 2B), and 85% 20 mg L^{-1} chlorsulfuron (Figure 2C). However, only 20% of the RB3 plants survived 40 mg L^{-1} triasulfuron (Figure 2A), 15% 10 mg L^{-1} metosulam (Figure 2B), and 60% 20 mg L^{-1} chlorsulfuron (Figure 2C). All the SB2 plants died at these concentrations (Figures 2A,B,C). A very small proportion of the plants of the RB2 and RB3 soaked at low concentration of herbicides remained stunted often without producing a true leaf. Most of these stunted plants within RB2 and RB3 died after the foliar spray. Most of the SB2 plants at low herbicide

Table 1. Correlation between number of the resistant *R. raphanistrum* plants that survived seed soaking treatments of ≥40 mg L^{-1} triasulfuron, ≥10 mg L^{-1} metosulam or ≥20 mg L^{-1} chlorsulfuron, and the number of plants that also survived label rate of each herbicide sprayed five weeks after emergence in Experiment 1 and 2.

Herbicides	Correlation coefficients (r^2)		
	Experiment 1	Experiment 2	
	R biotype 1	R biotype 2	R biotype 3
Triasulfuron	0.98**	0.99**	0.98**
Metosulam	0.97**	0.91**	0.80**
Chlorsulfuron	–	0.96**	0.80**

** Statistically significant at P>0.01. A dash indicates the experiment was not undertaken.

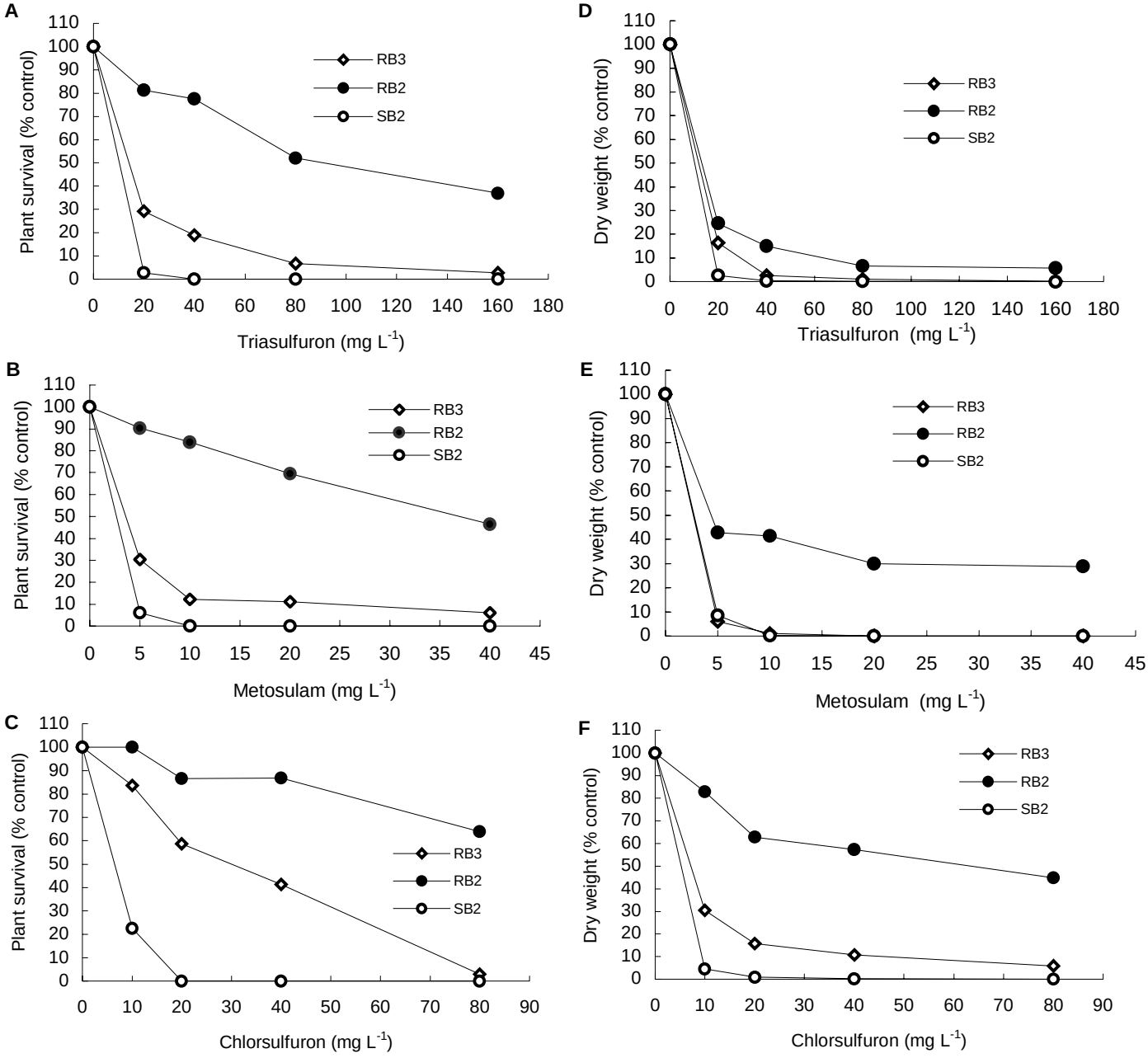

Figure 2. Survival of the treated plants of resistant biotype (RB) and susceptible biotype (SB) of *R. raphanistrum* immediately before foliar spray five weeks after emergence and their shoot dry weight four weeks after foliar spray with label rate of triasulfuron, metosulam and chlorsulfuron. $LSD_{.01}$ for plant survival is 15.11% and that for shoot dry weight is 15.36%.

concentrations died 2–4 WAE and few remained severely stunted often without producing a true leaf. Shoot dry weight of these biotypes showed similar trends to plant survival (Figures 2D,E,F).

There was significant correlation between the number of the RB2 and RB3 plants that survived seed soaking treatments of ≥40 mg L^{-1} triasulfuron, ≥10 mg L^{-1} metosulam or ≥20 mg L^{-1} chlorsulfuron and the number of plants that survived after foliar spray with label rates (25 g a.i. ha^{-1} triasulfuron, 5 g a.i. ha^{-1} metosulam, or 15 g a.i. ha^{-1} chlorsulfuron). The r^2 values varied from 0.80 to 0.98 (Table 1).

Experiment 3 (conventional single-dose resistance test)

The conventional single-dose resistance test established that populations SB1 and SB2 were completely susceptible while populations RB1, RB2, and RB3 were resistant to triasulfuron, metosulam and chlorsulfuron (Table 2).

Discussion

A clear difference in response of the R and S biotypes was found when seeds were soaked in different concentrations of ALS-inhibiting herbicides and then allowed to emerge and grow in pot soil. A 40 mg L^{-1} triasulfuron (714 g a.i. kg^{-1}), 10 mg L^{-1} metosulam (714 g a.i. kg^{-1}), and 20 mg L^{-1} chlorsulfuron (750 g a.i. kg^{-1}) were found to be the best concentrations for screening of resistant biotypes within the *R. raphanistrum* populations by the seed soaking method.

In the seed soaking method, seeds were soaked in herbicide solutions for 24 h and then sown in pots. Plant survival was assessed 5 WAE because most of the R biotype plants that survived seed soaking treatments were growing almost unaffected while most of the S biotype plants died at this stage. A small proportion of plants in the R or S biotypes at low concentrations of herbicide remained stunted without producing a true leaf up to 5 WAE. Although these plants were considered as survivors in this study, most of them died following the foliar spray 5 WAE. Such a foliar spray, which was applied only as a check to confirm resistance status of the surviving RB plants, will not be necessary in the routine resistance test procedure. Therefore, this soaking method required about 5–6 weeks from soaking to plant survival assessment compared to 10 weeks by the conventional foliar spray method.

These results confirmed that the populations identified as the R or S by the soaking method were indeed resistant to each of the three ALS-inhibiting herbicides. The results of conventional single dose screening method are clearly comparable with the results of the seed soaking method. These results also clearly indicated that RB3 was more heterogeneous than RB1 or RB2. The quantity of herbicide required in the soaking method was reduced by 10–100-fold compared to the conventional foliar spray method.

Table 2. Resistance status of the five *R. raphanistrum* populations to three ALS-inhibiting herbicides at label rates (25 g a.i. ha^{-1} triasulfuron, 5 g a.i. ha^{-1} metosulam and 15 g a.i. ha^{-1} chlorsulfuron) sprayed 5 weeks after emergence.

Populations	Triasulfuron	Metosulam	Chlorsulfuron	Resistance status
	Per cent plant survival			
S biotype 1	0	0	0	Susceptible
S biotyp1 2	0	0	0	Susceptible
R biotype 1	81	81	94	Resistant
R biotype 2	30	18	35	Resistant
R biotype 3	78	81	90	Resistant

The extent of reduction in plant survival of the R and S biotype plants of *R. raphanistrum* at 10 mg L^{-1} metosulam and 40 mg L^{-1} triasulfuron varied between Experiment 1 and 2. Such variation could be attributed to the variability in populations within each experiment. Letouzé and Gasquez (1999) also observed such variation in resistance status among the various populations of *A. myosuroides* at 138 or 690 g a.i. ha^{-1} of fenoxoprop-P-ethyl.

Conclusions

The results show that the seed soaking method developed in this study is a reliable test to identify *R. raphanistrum* populations that are resistant to ALS-inhibiting herbicides. This method has several advantages over the conventional foliar spray method. First, this method is 4–5 weeks quicker than the current conventional method. Second, there is less variation in the output delivery in the new method than conventional foliar spray treatment. Third, herbicide is absorbed by seed and subsequently translocated to other parts of the plant as the plant grows. Thus, the possibility of reduced uptake and translocation by stressed or older plants under the conventional method is minimized. Fourth, this method requires less quantity of herbicides than conventional foliar spray method. Finally, this method is simple and does not require any spraying equipment.

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References

Burnet, M.W.M., Christopher, J.T., Holtum, J.A.M., and Powles, S.B. (1994). Identification of two mechanism of sulfonylurea resistance within one population of rigid ryegrass (*Lolum rigidum*) using a selective germination medium. *Weed Science* 42, 468-73.

Cheam, A.H. (1986). Seed production and seed dormancy in wild radish (*Raphanus raphanistrum* L.) and some possibilities for improvement. *Weed Research* 26, 405-13.

Cheam, A.H. and Code, G.R. (1995). The biology of Australian weeds 24. *Raphanus raphanistrum* L. *Plant Protection Quarterly* 10, 2-13.

Christopher, J.T., Powles, S.B. and Holtum, J.A.M. (1992). Resistance to acetolactae synthase-inhibiting herbicides in annual ryegrass (*Lolium rigidum*) involves at least two mechanisms. *Plant Physiology* 100, 1909-13.

Hashem, A., Bowran, D., Piper, T. and Dhammu, H. (2001). Resistance of wild radish (*Raphanus raphanistrum*) to acetolactate synthase-inhibiting herbicides in the Western Australia Wheat Belt. *Weed Technology* 15, 68-74.

Jensen, P.K. and Kudsk, P. (1988). Prediction of herbicide activity. *Weed Research* 28, 473-78.

Klingaman, T.E., King, C.A. and Oliver, L.R. (1992). Effect of application rate, weed species, and weed stage of growth on imazethapyr activity. *Weed Science* 40, 227-32.

Letouzé, A. and Gasquez, J. (1999). A rapid reliable test for screening aryloxy-phenoxy-propionic acid resistance within *Alopecurus myosuroides* and *Lolium* spp. populations. *Weed Research* 39, 37-48.

Magha, M.I., Guerche, P., Bregeon, M. and Renard, M. (1993). Characterisation of spontaneous rapeseed mutant tolerant to sulfonylureas and imidazolinone herbicides. *Plant Breeding* 111, 132-41.

Moss, S.R. (1999). Rothamsted rapid resistance test: For detecting herbicide resistance in black-grass, wild oats, and Italian ryegrass, pp. 1-14. (IACR-Rothamsted, Harpenden, UK).

Saari, L.L., Cotterman, J.C., Smith, W.F. and Primiani, M.M. (1992). Sulfonylurea herbicide resistance in common chickweed, perennial ryegrass, and Russian thistle. *Pesticide Biochemistry* 42, 110-18.

Walsh, Michael J., Duane, Ryan D. and Powles, Stephen B. (2001). High frequency of chlorsulfuron-resistant wild radish (*Raphanus raphanistrum*) populations across the Western Australian Wheatbelt. *Weed Technology* 15, 199-203.