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Identification of Triazine-Resistant *Vulpia bromoides*

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In Australia, triazine herbicides have routinely controlled the *Vulpia* species (*Vulpia bromoides*, *Vulpia myuros*, and *Vulpia fasciculata;* collectively referred to as silvergrass). However, a simazine-resistant silvergrass biotype, collected from Pingelly in the Western Australian grain belt in 2014, has been confirmed. Compared to the pooled mortality of three simazine-susceptible silvergrass populations (S1, S2, and S3), the simazine-resistant Pingelly population was > 594-fold resistant at the LD_{50} level. Dose-response screening of the simazine-selected progeny (> 800 g ai simazine ha^{-1}) demonstrated that the simazine resistance mechanism was heritable. Sequencing of the chloroplast *psb*A gene revealed the resistant population is homozygous for a serine 264 to glycine mutation, which confers a high-level triazine resistance. As expected this Ser-264-Gly mutation conferred resistance to atrazine and metribuzin, but not the phenyl-urea diuron. This is the first published report confirming field-evolved triazine resistance in a *Vulpia* population.
Nomenclature: Atrazine; metribuzin; phenyl-urea diuron; simazine; silvergrass, *Vulpia bromoides* (L.) S.F. Gray. VLPBR.
Key words: Evolution, herbicide resistance, silvergrass, simazine, *Vulpia*.

En Australia, los herbicidas triazine han controlado rutinariamente especies del género *Vulpia* (*Vulpia bromoides*, *Vulpia myuros*, y *Vulpia fasciculata*; colectivamente referidas como silvergrass). Sin embargo se ha confirmado un biotipo de silvergrass resistente a simazine, el cual fue colectado en Pingelly, en la faja de granos del Oeste de Australia, en 2014. Al compararse con la mortalidad promediada de tres poblaciones de silvergrass susceptibles a simazine (S1, S2, y S3), la población Pingelly resistente a simazine fue > 594 veces más resistente según el nivel de LD_{50}. Una evaluación de respuesta a dosis de la progenie seleccionada con simazine (> 800 g ai simazine ha^{-1}) demostró que el mecanismo de resistencia a simazine fue heredable. La secuenciación del gen *psb*A del cloroplasto reveló que la población resistente es homocigota para la mutación serine 263 a glycine, la cual confiere un alto nivel de resistencia a triazine. Como se esperaba, esta mutación Ser-264-Gly confirió resistencia a atrazine y metribuzin, pero no a diuron, un herbicida phenyl-urea. Este es el primer reporte publicado confirmando la resistencia a triazine evolucionada en campo en una población de *Vulpia*.

Simazine is a triazine herbicide that competes with plastoquinone (PQ) at the PQ binding site located within the D1 protein of the photosystem II (PSII) complex enzyme (Arntzen et al. 1982; Gronwald, 1994). PSII inhibition stops photosynthesis, leading to plant death. Several herbicide chemistries (e.g., triazine, triazinone, urea, uracil, biscarbamate) also act to inhibit PSII. Triazine herbicides, such as simazine, are important tools, providing effective residual weed control in a variety of crops, pasture, and other uses. Due to their effectiveness, triazine herbicides are routinely used in Australian agricultural systems, especially for PRE and POST weed control in triazine-resistant canola (*Brassica napus* L.). In addition, triazine herbicides are used in a range of recreational and industrial areas. Since the first reported triazine resistance in common groundsel (*Senecio vulgaris* L.) in 1970 (Ryan 1970), triazine resistance has been identified in 72 species (49 dicots, 23 monocots) from over 28 countries (Heap 2015). In Australia, triazine resistance has been confirmed in annual ryegrass (*Lolium rigidum* Gaudin) (Burnet et al. 1991), wild radish (*Raphanus raphanistrum* L.) (Friesen and Powles 2007; Hashem et al. 2001; Walsh et al. 2004), oriental mustard (*Sysimbrium orientale* Torn.), junglerice [*Echinochloa colona* (L.)

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Link], and burning nettle (*Urtica urens* L.) (Heap 2015). Prior to this study, there was a single unconfirmed report of simazine resistance in *Vulpia bromoides* (silvergrass) from the Australian state of Victoria (P Boutsalis, personal communication).

The silvergrasses (*Vulpia bromoides*, *Vulpia myuros*, *Vulpia fasciculata*), are economically important weeds of crops and pastures throughout southern Australia, especially in light- to medium-textured acidic soils (Borger et al. 2012; Espeland et al. 2010; Lemerle et al. 1996) in the higher-rainfall zones (Lemerle et al. 1996). Moreover, silvergrass species are not nutritious or palatable to livestock (Allden 1959) making silvergrasses persistent in pastures (Bolland 1985; Chapman et al. 2003; Waller et al. 2001).

While a persistent component in pastures, silvergrass has not generally been considered a problem in cereal crops (Velthuis and Amor 1983). However, with widespread adoption of no-tillage cropping systems in Australia, silvergrass is becoming increasingly prevalent, causing estimated wheat (*Triticum aestivum* L.) yield reductions of 10 to 30% (Dowling and Wong 1993; Wallace 1988). Unlike nearly all grass weed species, the silvergrasses are naturally tolerant to acetyl-CoA carboxylase (ACCase)-inhibiting aryloxyphenoxypropionate and cyclohexanedione-inhibiting herbicides (Bowran and Wallace 1996; Dowling and Wong 1993; Leys et al. 1988; Yu et al. 2004). Consequently, in dicot crops and pastures, triazine herbicides are relied upon to control silvergrass (Bowran and Wallace 1996). This study reports the first confirmed case of simazine resistance in a population of silvergrass collected from the grain belt of Western Australia.

Materials and Methods

Collection of Susceptible and Triazine-Resistant Silvergrass Populations. In 2014, a field at Pingelly, Western Australia (32.53°S, 117.08°E) was identified to contain distinct patches of silvergrass uncontrolled by simazine at rates > 450 g ai ha^{-1}. Mature seed was collected from a minimum of 50 surviving plants and stored in a controlled-temperature room (> 35 C) for 6 mo to break dormancy. In 2015, three putative herbicide-susceptible silvergrass populations were collected from nonagricultural areas of the Western Australian agricultural grain belt near Northam (31.39°S, 116.39°E; referred to hereinafter as S1), Miling (30.29°S, 116.225°E; referred to hereinafter as S2), and Serpentine (32.36°S, 115.98°E; referred to hereinafter as S3), where there had been no known herbicide application history.

Whole Plant Resistance. In January 2015, seed from the putative simazine-resistant (Pingelly) and herbicide-susceptible (S1) populations were sown into three replicate 300- by 350-mm plastic trays containing washed river sand and maintained in cooled conditions (10 to 15 C) for 3 wk. Following emergence, 20 seedlings per pot were transplanted into 180-cm pots containing washed river sand and maintained in a phytotron at 15 C. At the three-true-leaf stage, seedlings were treated with simazine (Gesatop Herbicide™, 900 g kg^{-1}, Syngenta, Macquarie Park, New South Wales, Australia). The susceptible population (S1) was treated with simazine at 0, 50, 100, 200, 400, 1,600, and 3,200 g ha^{-1}, while the Pingelly population was treated at 0, 200, 400, 800, 1,600, 3,200, 6,400, and 12,800 g simazine ha^{-1}. Plant survival was assessed 21 d after treatment (DAT) by inspecting treated plants for active growth.

This dose-response assay was repeated in March 2015. Three susceptible populations (S1, S2, S3) and the field-collected Pingelly population were established as previously described into five replicate 180-cm pots containing washed river sand. Each pot contained 20 seedlings and pots were maintained in a phytotron at 15 C. At the three-leaf stage, the S1 to S3 populations were treated at 0, 25, 50, 100, 200, 400 g simazine ha^{-1}, while the field-collected triazine-resistant Pingelly population was treated with simazine at 0, 400, 800, 1,600, 3,200, 6,400, 12,800, 25,600, and 51,600 g ha^{-1}. Plant survival was assessed 21 DAT.

Following the first dose-response in January 2015, 10 surviving plants from the Pingelly population (surviving simazine rate > 6,400 g ha^{-1}) and two samples comprising five randomly selected plants from the nontreated susceptible population (S1) were transplanted into individual 180-mm pots and numbered. Upon further growth, 2-cm leaf samples were cut from each plant and placed into individual 1.5-ml microcentrifuge tubes containing 700 µl of extraction buffer with a metal bead and stored at −70 C. The S1 and Pingelly

Table 1. Details of herbicides used in cross resistance study.

Herbicide common name	Trade name	Herbicide formulation	Herbicide rate	Manufacturer
			g ai ha^{-1}	
Atrazine	Gesaprim 900DF	900 g kg^{-1}	990	Syngenta, Macquarie Park, New South Wales, Australia
Metribuzin	Lexone Xtruded	750 g L^{-1}	135	Dupont, North Sydney, New South Wales, Australia
Diuron	Diuron 900DF	900 g kg^{-1}	495	Nufarm Australia, Laverton North, Victoria Australia
Paraquat dichloride	Gramoxone	250 g L^{-1}	50	Syngenta, Macquarie Park, New South Wales, Australia
Glyphosate	Roundup attack	570 g L^{-1}	540	Nufarm Australia, Laverton North, Victoria Australia
Glufosinate-ammonium	Basta	200 g L^{-1}	400	Bayer Crop-science, Hawthorn East, Victoria, Australia
Propyzamide	Kerb 500SC	500 g L^{-1}	500	DOW Agro-science, Frenchs Forest, NSW, Australia

populations were then isolated to prevent the ingress of foreign pollen and allowed to produce seed. The progeny from the S1 and Pingelly populations were treated with simazine at 800 g ha^{-1} to confirm the heritability of the resistance gene trait.

Response to Alternative Herbicides. Seedlings were sprayed with herbicides known to control silvergrass (Table 1). In March 2015, 20 seedlings from the original field-collected Pingelly populations and the susceptible population (S1) were established into three replicate 180-mm plastic pots containing washed river sand. Plants were maintained as previously described with herbicide applied at the three-leaf stage. Plant survival was assessed 21 DAT.

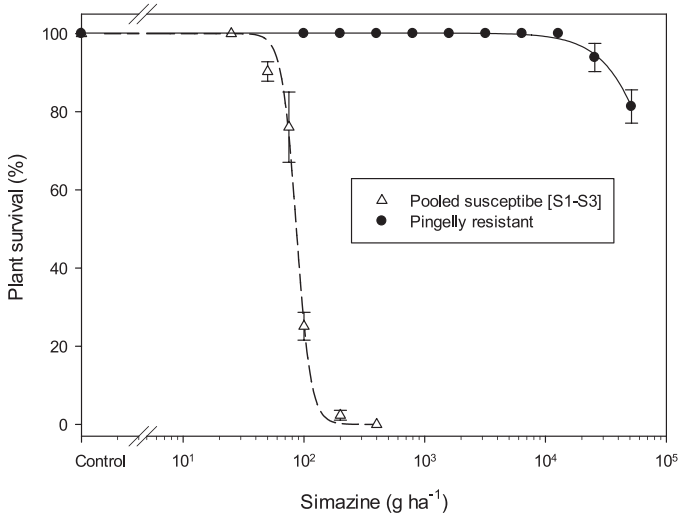

Figure 1. Survival dose-response curves for the pooled susceptible silvergrass populations S1, S2, S3 (...Δ...) and the simazine-resistant Pingelly population (_ _•_ _). Each symbol represents the mean of seven treatments in susceptible and eight treatments in the resistant. The plotted lines are predicted survival curves using a three-parameter log-logistic model (Equation 1). Vertical bars represent mean ± SE ($n = 5$).

Genomic DNA Extraction, PCR Amplification, and Partial Sequencing of *psb*A Gene. Shoot material was obtained from 10 individual survivors of the Pingelly population and two bulked samples of the susceptible population (S1), consisting of five plants for genomic DNA extraction according to the method by Yu et al. (2008). A pair of primers were designed based on homologous regions of *psb*A sequences from *Arabidopsis thaliana* L. (GenBank accession number X79898.1), alfalfa (X04973.1), canola (M36720.1), faba bean (X17694.2), and soybean (X00152.1) containing the known potential *psb*A mutation sites (Pan et al. 2012). A polymerase chain reaction (PCR) fragment of 626 bp with the forward primer 5′-CGTGAGTGG GAACTTAGTTT-3′ and reverse primer 5′-TGAGCATTACGTTCATGCAT-3′ was amplified, sequenced, and compared between the S1 and Pingelly populations. PCR was conducted in a 25-µl volume that consisted of ~ 100 ng of genomic DNA, 0.5 mM of each primer, and 12.5 ml of 2× GoTaq Green Master Mix (Promega, Madison, WI). The PCR was run in a Mastercycler (Eppendorf, Hamburg, Germany) using the following profile: 94 C for 4 min; 40 cycles of 94 C for 30 s, 51 C for 30 s, and 72 C for 1 min; followed by a final extension step of 7 min at 72 C. The PCR product was then purified from Wizard SV agarose gel and cleaned using the PCR Clean-Up System (Promega). The DNA samples were sequenced from both ends using the AB-Big Dye Terminator (Thermo Fisher, Waltham, MA) system using a commercial sequencing service.

Table 2. Parameter estimates for survival for three-parameter log-logistic model (Equation 1) used to calculate estimated rate at which 50% mortality is achieved (LD_{50}) and their resistant susceptible ratio values for herbicide-susceptible silvergrass control populations (S1, S2, S3) and the field-collected simazine resistant Pingelly population, treated with a range of simazine doses.[a]

Population	A	b	e (LD_{50})	R : S[b]
			g ai ha^{-1}	
S1	100	12.50 ± 1.40 SE	93.51 ± 0.91 SE	—
S2	100	11.25 ± 1.25 SE	93.77 ± 0.95 SE	—
S3	100	8.89 ± 0.85 SE	69.06 ± 1.44 SE	—
Pooled control	100	10.04 ± 0.3 SE	86.74 ± 1.66 SE	—
Pingelly	100	1.82 ± 0.15 SE	> 51,600	> 594

[a] Abbreviations: A, upper asymptotic value; e, estimated LD_{50} parameter; b, slope of the curve around the LD_{50} parameter.

[b] R : S, resistant : susceptible ratios calculated between the parameters based upon the pooled susceptible populations and the maximum rate applied to the field-selected population.

Statistics. The observed dose-response plant survival and biomass data were fitted to a three-parameter log-logistic model (Equation 1) where the upper limit is fixed to 1.0 in R 3.0.0 (R Foundation for Statistical Computing, Vienna, Austria) (Price et al. 2012; Streibig et al. 1993):

$$Y = c + \{1 - c / (1 + \exp[b(\log x - \log e)]\} \quad [1]$$

where Y denotes plant survival expressed as a percentage of the untreated control in response to herbicide dose x. The characters c is the lower asymtopic values of Y, e is the estimated lethal dose 50% (LD_{50}) or growth reduction 50% (GR_{50}), and b is the slope of the curve around the LD_{50} or GR_{50} parameter. The resistant and susceptible populations were compared using a resistant : susceptible ratio of the estimated LD_{50} values. The aboveground biomass was expressed as a percentage of the mean nontreated control. Data was checked for homogeneity of variance, normality, and independence of residuals as described by Onofri et al. (2010) using a two-way ANOVA (Genstat version 6.1.0.200, BVSN International Ltd, Hamel, Hempsted, UK). Nonlinear regression analysis was performed using Equation 1 and t tests were used to statistically compare the means of each population curve using R 3.0.0 (R Foundation for Statistical Computing). Data were plotted using SigmaPlot v.12 (Systat Software Inc., San Jose, CA).

Results and Discussion

This study confirms triazine resistance in the silvergrass population collected from Pingelly within the southern grain belt of Western Australia. Initial simazine treatment of the putative simazine-resistant Pingelly population (400 g ha^{-1}) resulted in 100% survival, while the known susceptible S1

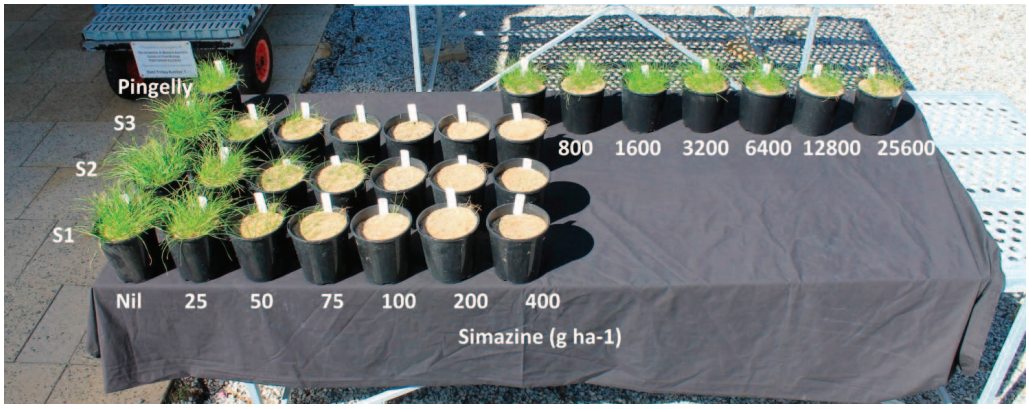

Figure 2. Photographic representation of population dose-response for the susceptible populations S1, S2, and S3 and the simazine-resistant population, Pingelly. (Color for this figure is available in the online version of this article.)

Table 3. Parameter estimates for biomass for three-parameter log-logistic model (Equation 1) used to calculate the estimated rate at which growth is reduced by 50% (GR_{50}) and their resistant susceptible ratio values for herbicide-susceptible silvergrass control populations (S1, S2, S3) and the field-collected simazine-resistant Pingelly population, treated with a range of simazine doses.[a]

Population	A	b	e (GR_{50})	R : S[b]
			g ai ha^{-1}	
S1	100	0.86 ± 0.31 SE	43.91 ± 14.09 SE	—
S2	100	0.91 ± 0.25 SE	32.08 ± 8.38 SE	—
S3	100	1.37 ± 0.17 SE	31.97 ± 2.92 SE	—
Pooled control	100	0.99 ± 0.16 SE	34.45 ± 4.91 SE	—
Pingelly	100	0.72 ± 0.08 SE	10,790 ± 1,487 SE	313

[a] Abbreviations: A, upper asymptotic value; e, estimated GR_{50} parameter; b, slope of the curve around the GR_{50} parameter.

[b] R : S, resistant : susceptible ratios calculated between the parameters based upon the pooled susceptible populations and the field-selected population.

population was fully controlled. Subsequent dose-response studies confirmed the Pingelly population to be simazine-resistant, with 100% survival at the simazine rate of 12,800 g ha^{-1}, whereas the susceptible populations (S1 to S3) were killed at 400 g ha^{-1} (Figure 1). This Pingelly population was found to be > 594-fold (LD_{50}) more resistant to simazine, when compared with the pooled values of the three susceptible populations (S1 to S3) included in this study (Figure 1; Table 2). The biomass of the Pingelly population was not reduced by high rates of simazine with a GR_{50} of 10,790 g ha^{-1}, while the pooled GR_{50} of the S1 to S3 populations was 34 g ha^{-1} (Table 3; Figure 2).

Triazine herbicide resistance is globally widespread (Gronwald 1994). Since triazine resistance was first characterized by Hirschberg and McIntosh (1983), target-site triazine resistance has primarily been endowed by a single amino acid substitution Ser-264- Gly in the PQ binding site, conferring very high-level triazine resistance (Arntzen et al. 1982; Gronwald 1994; Powles and Yu 2010). To check for this mutation, a single PCR fragment of 626 bp from both the triazine-resistant (Pingelly) and triazine-susceptible genotype (S1) was amplified, encompassing the known PSII resistance-conferring mutation sites (codon 129–337) of the chloroplast *psb*A gene. Sequence alignment of the Pingelly and S1 genotypes confirmed that all the plants from Pingelly population contained a homozygous single amino acid base change from serine to glycine in the 264 codon. No other amino acid substitutions were identified between resistant and susceptible populations. The high level of simazine resistance (Figure 1) exhibited by this Pingelly population is typical of this Ser-264-Gly binding-site mutation, which modifies the D1

Figure 3. Photographic representation of the biomass of the non–herbicide-treated susceptible populations S1, S2, and S3 and the non–herbicide-treated simazine-resistant population, Pingelly. (Color for this figure is available in the online version of this article.)

Table 4. Multiple resistance traits in the field-collected Pingelly silvergrass population. (Zero indicates fully susceptible populations; 1 to 19%, developing resistance; and > 20% survival results in classification as a resistant population.)

Mode of action	Herbicide active ingredient	Herbicide rate	S1[a] mean	Pingelly mean
		g ai ha^{-1}	% survival	% survival
Inhibitor of photosynthesis at photosystem II	Atrazine	990	0	94 ± 3 SE[b]
Inhibitor of photosynthesis at photosystem II	Metribuzin	135	0	100 ± 0 SE[b]
Inhibitor of photosynthesis at photosystem II	Diuron	495	0	0
Inhibitor of photosynthesis at photosystem I	Paraquat	50	0	0
EPSPS inhibitor	Glyphosate	540	0	0
Inhibitors glutamine synthase	Glufosinate	400	0	0
Inhibitors of tubulin formation	Propyzamide	500	0	0

[a] S1 indicates herbicide-susceptible population.

[b] Herbicide-resistant (> 20% survival).

protein in the region of the quinone-binding niche in a way in which specific triazine herbicides cannot bind effectively (Gronwald 1994). This Ser-264-Gly mutation has previously been identified in many grass species including hood canarygrass (*Phalaris paradoxa* L.) (Schönfeld et al. 1987), barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.] (Chodová and Salava, 2004), downy brome (*Bromus tectorum* L.) (McNally et al. 1987), and the dicot species wild radish (Friesen and Powles 2007). However, whilst *psb*A gene mutations endow strong herbicide resistance, this Ser-264-Gly mutation compromises PSII activity, reducing photosynthetic capacity, limiting plant fitness (Gronwald 1994). This reduced fitness was indicated by the nontreated Pingelly population having a lower mean biomass (Pingelly: 0.05 g plant^{-1} 40 d after emergence) compared to the mean biomass of all nontreated herbicide susceptible populations(S1, 0.08 g plant^{-1}; S2, 0.08 g plant^{-1}; and S3, 0.09 g plant^{-1} 40 d after emergence) in this study (Figure 3). This study did not examine for any other possible triazine resistance mechanisms in the Pingelly population. As expected, the Ser-264-Gly mutation conferred resistance to atrazine and metribuzin, but not the phenylurea herbicide diuron (Table 4). As well as exhibiting susceptibility to diuron, the Pingelly biotype was susceptible to all other dissimilar modes of action tested, including glyphosate, glufosinate, paraquat, and propyzamide (Table 4).

Overreliance on herbicides without sufficient diversity in weed management practices is the primary cause of herbicide resistance evolution worldwide (Powles and Yu 2010). At the Pingelly site, triazine herbicides were likely used eight times in the past 12 yr. The cropping history at the Pingelly site was lupin (*Lupinus angustifolius* L.) (2003), wheat (2004), triazine-tolerant (TT) canola (*Brassica napus* L.) (2005), barley (*Hordeum vulgare* L.) (2006), legume pasture (2007, 2008), wheat (2009), TT canola (2010), wheat (2011), legume pasture (2012, 2013), and TT canola (2014). The persistent use of triazine herbicides throughout this rotation leading to triazine-resistant silvergrass evolution highlights the risks of recurrently using a single herbicide mode of action on large weed populations.

Resistance in this population is endowed by the well-known Ser-264-Gly *psb*A gene mutation. In order to limit the evolution of resistance in other weed populations, greater herbicide and nonherbicide diversity is required, including alternating herbicide modes of action and the use of full labeled rate mixtures (Lagator et al. 2013; Norsworthy et al. 2012), deep seed burial using tillage (Chauhan et al. 2006; Dillon and Forcella 1984), harvest weed seed control (Walsh et al. 2013), and improved crop competition (Dillon and Forcella 1984).

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