Resistance to glyphosate, paraquat and other herbicides in a population of *Lolium rigidum* from South Australia

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ABSTRACT
Lolium rigidum (annual ryegrass) is one of the major weed species in Australian cropping systems. The main method of control for annual ryegrass is treatment with herbicides. Recently a multiple resistant population of *L. rigidum* was identified from a long-term irrigated white clover seed field. Here we report this population has high levels of resistance to the group A (inhibitors of acetyl coenzyme A carboxylase) herbicides clethodim and pinoxaden and the group B (inhibitors of acetolactate synthase) herbicides iodosulfuron-methyl, imazamox/imazapyr and chlorsulfuron. This population was also resistant to paraquat and showed low level resistance to glyphosate. Inheritance studies indicated resistance to paraquat and glyphosate may be due to a single, nuclear gene, whereas clethodim resistance is due to more than one gene.

INTRODUCTION
L. rigidum (annual ryegrass) is one of the major weed species in Australian cropping systems (Jones et al. 2005). Its high genetic diversity (Malone et al. 2014) and successful adaptation to a range of environments (Gill 1996) has made it a widespread problem. The main method of control for annual ryegrass is treatment with herbicides. They provide efficient and cost-effective options in multiple cropping situations. The long-term use of herbicides for weed control has caused the evolution of broad resistance. Currently *L. rigidum* populations with at least nine dissimilar herbicide chemistries have been identified in Australia (Malone et al. 2014).

Resistance to the acetyl-coenzyme A carboxylase (ACCase)-inhibitors, the acetolactate synthase (ALS) inhibitors, 5-enolpyruvulshikimate-3-phosphate synthase (EPSPS) inhibitors, microtubule assembly inhibitors, carotenoid biosynthesis (unknown target) inhibitors, mitosis inhibitors, long chain fatty acid inhibitors and lipid inhibitors have been identified in annual ryegrass. Although these instances of resistance pose a serious problem for weed management, a larger emerging problem is the evolution of populations with resistance to multiple mode of action (MOA) herbicide chemistries. Multiple resistance can be as a result of selection by each of the classes of herbicides or by cross-resistance, where resistance to one class is conferred after selection by an alternate distinct class (Burnet et al. 1991).

Recently a resistant population of *L. rigidum* was identified from a long-term irrigated white clover seed field in Naracoorte, South Australia. This field has a history of two to three applications of 300 g ha^{-1} paraquat from 1999 to 2010 and a 3 year period where ALS and ACCase herbicides may have been used. Here we investigated if this population had resistance to a range of herbicide chemistries and if so, how resistance to the various herbicides was inherited.

MATERIALS AND METHODS

Plant material. A population of *L. rigidum* (annual ryegrass) was identified in Naracoorte, South Australia. This population originated from a field that had been used for irrigated white clover seed production long for over 15 years.

Seed germination and plant growth. Seed was germinated on 0.6% agar in a germination cabinet maintained at 12/12 h light/dark cycle (30 µmol $m^{-2}s^{-1}$) at 20°C/15°C (Lorraine-Colwill et al. 2001). Germinated seedlings were transferred to 9.5 cm x 8.5 cm x 9.5 cm punnet pots (Masrac, South Australia) containing formulated potting mix as described by (Boutsalis et al. 2012). Nine seedlings were transplanted per pot with three replicates per herbicide dose for each population and grown outside during July and August 2013.

Dose response to herbicides. Herbicide was applied using a track sprayer delivering 110.1 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). Plants were treated at the 2-3 leaf stage, growth stage Z12-Z13 (Zadoks et al. 1974) and mortality was assessed 28 d after herbicide application. Clethodim was applied at 18, 30, 60, 120, 240 and 480 g a.i. ha^{-1}. Pinoxaden was applied at 7.5, 15, 30, 60, 120 and 240 g a.i. ha^{-1}. Iodosulfuron-methyl was applied at 5, 10, 20, 40, 80 and 160 g a.i. ha^{-1}. Imazamox/imazapyr was applied at 3.6, 7.2, 14.4, 28.8, 57.6 and 115.2 g a.i. ha^{-1}. Atrazine was applied at 150, 300, 600, 1200, 2400, 4800 g a.i. ha^{-1}. Paraquat was applied at 25, 50, 100, 200, 400 and 800 g a.i. ha^{-1}. Amitrole was applied at 250, 500, 1000, 2000, 4000 and 8000 g a.i. ha^{-1}. Trifluralin was applied at 250 g a.i. ha^{-1}. Glufosinate was applied at 200, 400, 800, 1600 and 3200 g a.i. ha^{-1}. Glyphosate was applied at 142.5, 285, 42.5, 570, 855 and 1140 g a.i. ha^{-1}. Chlorsulfuron was applied at 11.25, 18.75, 37.5 and 56.25 g a.i. ha^{-1}. Malathion where used was applied 30 min prior to herbicide application at 1000 ml ha^{-1}.

Inheritance of resistance. The susceptible SLR4 and 556 lines were crossed by growing pairs in close proximity to each other, with each pair in an isolated glasshouse room. The plants were grown to maturity and the seed collected from the plants. F_1 plants were taken and used for backcrossing with parental lines. Two F_1 plants from lines determined to be homozygous were potted with each parent population and seed was collected from these plants. Parental, F_1 and backcross populations were treated with 12.5, 25, 50, 100, 150, 200, 300, 400, 800 and 1600 g a.i. ha^{-1} paraquat, 3.8, 7.5, 15, 23, 30, 45, 60, 120, 240 and 480 g a.i. ha^{-1} clethodim and 28.5, 42.75, 57, 99.75, 142.5, 213.8, 285, 427.5, 570 and 855 g a.i. ha^{-1} glyphosate. Dose response experiments were analysed by probit analysis (Pri Probit; Sakuma 1998) as populations 556, SLR4, F_1 pooled, backcross to SLR4 pooled (BC-S) and backcross to 556 (BC-R). The expected response of the backcross populations to herbicides were calculated using the F_1 dose response data based on an expected 1:1 rr and Rr response (backcross to susceptible) and 1:1 Rr to RR response (backcross to resistant).

DNA extraction and sequencing. DNA was extracted from leaf material using a DNeasy Plant Mini Kit (Qiagen, Australia) according to manufacturer's instructions). PCR reactions contained 5x MyFi reaction buffer, 0.4µM of each primer and 2 Units of MyFi DNA Plymerase (Bioline, USA). DNA amplification was carried out in an automated DNA thermal cycler (Eppendorf Mastercycler® Gradient, Germany). DNA was visualised on 1.5% (w/v) agarose gels stained with 1x SYBR®safe DNA gel stain (Invitrogen, Australia). Samples were prepared with 1 x Crystal DNA loading buffer red (Bioline, USA) and electrophoresed in 1x UltraPure™ TAE buffer [2M tris-acetate, 50mM EDTA](Invitrogen, Australia) at 100 volts and photographed under UV light (λ302nm). Gene specific primers were used to

amplify regions containing known mutation sites for the 5-enolpyruvulshikimate-3-phosphate synthase (*EPSPS*), acetolactate synthase (*ALS*) and acetyl-coenzyme A carboxylase (*ACCase*) genes.

RESULTS AND DISCUSSION

This population showed high levels of resistance to the group A (inhibitors of acetyl coenzyme A carboxylase) herbicides clethodim and pinoxaden and the group B (inhibitors of acetolactate synthase) herbicides iodosulfuron-methyl, imazamox/imazapyr and chlorsulfuron (Figure 1). Population 556 was more than 100 times as resistant to clethodim, pinoxaden and iodosulfuron compared with the susceptible population SLR4 (Table 1). Population 556 was 2 to 10-fold resistant to imazamox, glyphosate, atrazine and amitrole.

Previous work has shown that resistance to herbicides from group A and group B chemistries can be via two main mechanisms. Resistance may be due to increased herbicide detoxification, which is thought to be mediated through cytochrome P450 enzymes (Yu et al. 2009) or via reduced herbicide sensitivity caused by a mutation within the target enzyme (Preston et al. 2013). We investigated metabolic resistance indirectly by observing the effect of malathion on resistance. The 556 population showed no change in resistance whereas susceptible plants had less resistance in a dose response with chlorsulfuron (Figure 2). This indicates that metabolism is unlikely play a role in resistance to group B herbicides. The application of malathion did however reduce the resistance of 556 to pinoxaden (Figure 2). Resistance was still higher than the susceptible population and metabolism may complement other resistance mechanisms.

Sequencing of the *ALS* and *ACCase* genes showed the population did not contain any of the target site mutations known to confer resistance in weed species (Table 2). Four non-synonymous single nucleotide polymorphisms (SNPs) were found within the coding region of the *ALS* gene that are not present in susceptible populations. When compared to susceptible sequences 556 contained 12 non-synonymous changes in the *ACCase* gene that differed between the two. These changes may be conferring resistance in the 556 population. Mutations that have not been identified in weed species to date have been shown to confer resistance in other plant species. Site-directed mutagenesis has produced resistance to ALS inhibiting herbicides in yeast, tobacco and *A. thaliana* (Preston 2009). None of the site-directed mutations were identified in the 556 population and the mutations shown here may be a yet unknown site that confers resistance.

Sequencing of the EPSPs gene in these populations showed no Pro to Ser or Thr mutation at amino acid position 106 showing resistance to glyphosate is not due to a target site mutation.

Determining the genetic inheritance of herbicide resistance can give important information toward understanding how resistance can evolve in the field. The pattern of inheritance in population 556 indicates that resistance to paraquat and glyphosate is due to a single, nuclear gene (Figure 3). Segregation ratios in the F_1 and backcross populations are consistent with this inheritance mode. The backcross populations had slightly lower survival than that predicted from parental and F_1 dose results. This may be due to a range of resistance levels within the population. These populations were also screened to test for the mode of inheritance of clethodim resistance. Similar to the results seen in paraquat and glyphosate treatments, the measured backcross curves were slightly lower than the predicted backcross curves for a single gene inheritance model.