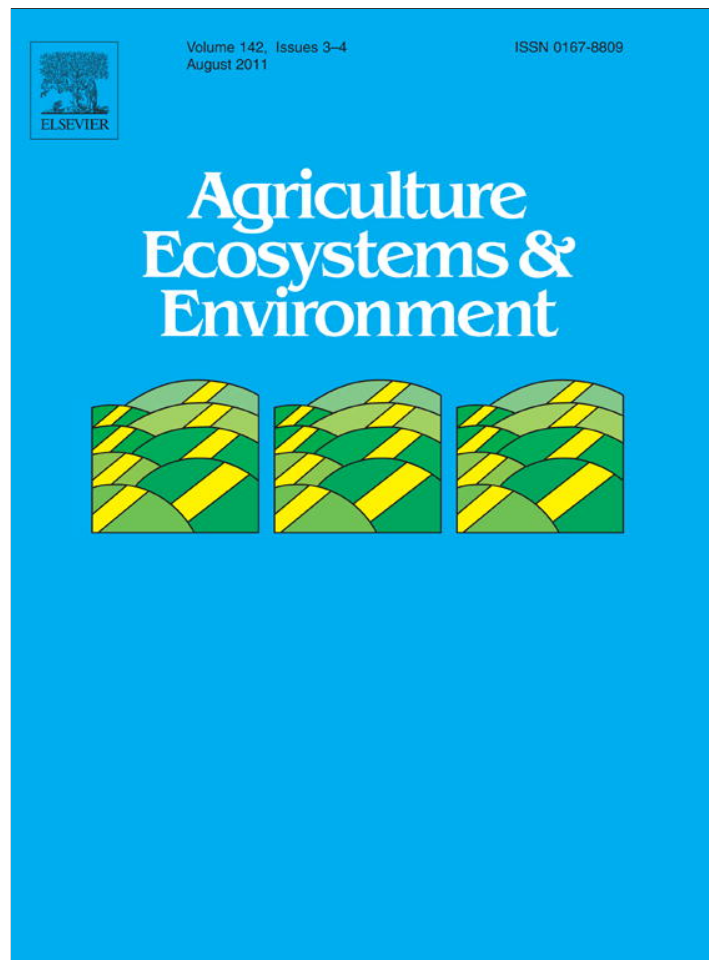


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Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/ageeGene flow increases the initial frequency of herbicide resistance alleles in unselected *Lolium rigidum* populationsRoberto Busi^{a,*}, Severine Michel^b, Stephen B. Powles^a, Christophe Délye^b

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ARTICLE INFO

Article history:

Received 25 January 2011
 Received in revised form 2 June 2011
 Accepted 10 June 2011
 Available online 2 July 2011

Keywords:

ACCase
 ALS
 Gene introgression
 Gene transfer
 Pollen migration
 Seed movement

ABSTRACT

In two different locations of the Western Australian “wheatbelt”, *Lolium rigidum* (rigid ryegrass) seeds were collected from organic fields (no herbicide use) and neighbouring conventional fields (persistent herbicide use), the latter infested with herbicide-resistant plants, to investigate the occurrence of gene flow among field populations as revealed by herbicide resistance gene transfer. Herbicides targeting acetyl-CoA carboxylase (ACCase) or acetolactate-synthase (ALS) were used to detect herbicide-resistant plants. Overall, the frequency of plants resistant to ACCase- or ALS-inhibiting herbicides was, respectively, 21% and 74% in the conventional fields and 2% and 37% in neighbouring organic fields. Mutant, herbicide-resistant ACCase and ALS alleles were detected in 16% and 38% of plants from conventional fields and in 0.53% and 3.7% of plants from organic fields. Identical mutant, herbicide-resistant ALS haplotypes were detected both in conventional and organic fields, supporting the occurrence of gene flow between *L. rigidum* populations in different fields. Gene flow can thus substantially increase the frequency of herbicide-resistant plants in unselected *L. rigidum* populations. Although gene flow cannot be prevented, it can be limited or managed. Hygiene tactics such as clean crop seed, weed seed removal at harvest and seed destruction post-harvest should be considered in order to minimize gene transfer among farms.

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1. Introduction

Weeds can adapt to different environments and evolve strategies to escape control in cultivated crops (Baker, 1974). The evolution of herbicide resistance in modern agro-ecosystems reliant on herbicide use is not only a prominent example of weed adaptation, but is also an increasing problem (Powles and Yu, 2010).

Genetic and bio-ecological factors interplay to determine the dynamics of resistance evolution in weed populations (Warwick, 1991). Herbicide resistance evolution is mainly driven by population genetic principles, and can be considered as a trade-off between the intensity of the herbicide selection, the mutation rate towards resistance alleles and the possible pleiotropic effects decreasing plant fitness (fitness cost) that may be associated with resistance-endowing alleles (Diggle and Neve, 2001; Vila-Aiub et al., 2009). Furthermore, migration of alleles (gene flow) between populations can affect the dynamics of herbicide resistance evolution by connecting plant populations and increasing the initial frequency of resistance alleles in unselected populations (Jasieniuk et al., 1996). In cross-pollinated species with high pollen longevity, pollen is expected to have a greater contribution to resistance dispersal than seeds (Darmency, 1996). However, the impact of gene

flow on the initial frequency of resistance in unselected populations is still not well assessed or understood (Jasieniuk et al., 1996). It was suggested that in predominantly self-pollinated plant species, gene flow frequencies should be extremely low (i.e., ranging between 10^{-9} and 10^{-6}), whereas in cross-pollinated species, gene flow frequencies could be as high as 10^{-3} (Levin and Kerster, 1974). Studies have shown pollen-mediated gene flow at low frequency with limited gene migration in self-pollinated crops such as rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) or weeds such as wild oats (*Avena fatua* L.) (Murray et al., 2002; Gaines et al., 2007b; Rong et al., 2007). Conversely, in cross-pollinated weed species, pollen-mediated flow of herbicide resistance genes can occur over considerable distances at relatively high frequency (Watrud et al., 2004; Zapiola et al., 2007; Beckie and Hall, 2008; Busi et al., 2008).

The intensity of gene exchange between plant populations mainly depends on the plant breeding system (Darmency, 1996), the respective demographic and pollen migration dynamics of donor and recipient populations (Damgaard and Kjellsson, 2005; Délye et al., 2010a), and the distances among populations (Busi et al., 2008). Multiple, independent selection of resistance alleles under persistent herbicide application in geographically heterogeneous environments has been reported in the cross-pollinated weed species *Alopecurus myosuroides* Huds. (Délye et al., 2010b). Thus, at a broad geographical scale, the selection of resistance alleles from within the local existing genetic variation seems to play the most important part in the evolution of herbicide resistance.

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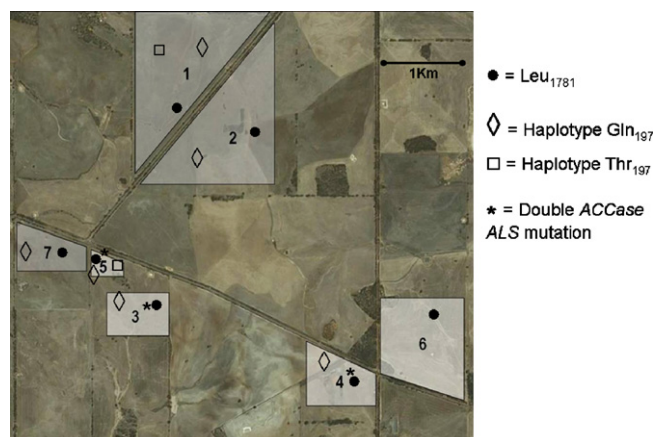


Fig. 1. Aerial Google Earth® photo of the Dumbleyung site showing the sampled conventional (3, 4, 5 and 6 in light grey) and organic fields (1, 2 and 7 in dark grey) and the spatial distribution of resistant ACCase and ALS alleles. Only resistant alleles and haplotypes found in both conventional and organic fields are shown. Mean field size was 60 ha.

Yet, at a local farm scale, the movement of resistance alleles by pollen flow has been reported to substantially affect the evolution of resistance (Délye et al., 2010a).

Highly effective herbicides inhibiting key enzymes of fatty acid (acetyl-coenzyme A carboxylase – ACCase) or branched-chain amino acid (acetolactate synthase – ALS) biosynthesis were introduced to world agriculture in 1980 and have been adopted for weed control in major crops worldwide, which has led to the selection of resistance mechanisms in at least 149 species (Heap, 2011). Resistance to ACCase- or ALS-inhibiting herbicides is often conferred by mutant alleles of the ACCase or ALS gene, respectively. Resistance to each class of herbicides has been characterized in several plant species at the phenotypic, enzymatic and molecular level (Tranel and Wright, 2002; Délye, 2005; Powles and Yu, 2010). In particular, molecular markers to identify resistance-endowing mutant ACCase or ALS alleles have been developed for several weed species and can provide an excellent tool to investigate the migration of resistance alleles in weed populations (Délye et al., 2010a).

In this study, the frequency of plants resistant to ACCase and ALS inhibitors was assessed in populations of the cross-pollinated grass weed *L. rigidum* Gaud. collected from conventional fields where these herbicides have regularly been used, and from nearby organic fields where herbicides have not been used in the last 20 years, or at all. The aim was to investigate the occurrence of gene flow from *L. rigidum* populations in which resistance genes are present in substantial frequencies, to those growing in neighbouring fields without herbicide selection, and to understand whether gene flow could affect the frequency of herbicide resistance genes in these latter populations.

2. Materials and methods

2.1. Sample collection from conventional and organic fields

Mature seed samples were collected in early summer 2007 (December) as described by Owen et al. (2007) from two locations approximately 430 km apart in the Western Australian wheat belt: Dumbleyung (33°18'38.11"S, 117°49'11.79"E) and Moora (30°33'55.47"S, 116°3'52.41"E). The mean field size was 60 ha in Dumbleyung (Fig. 1) and 25 ha in Moora (Fig. 2). *L. rigidum* seed samples were collected from 11 fields under conventional farming with intensive use of herbicides (source populations

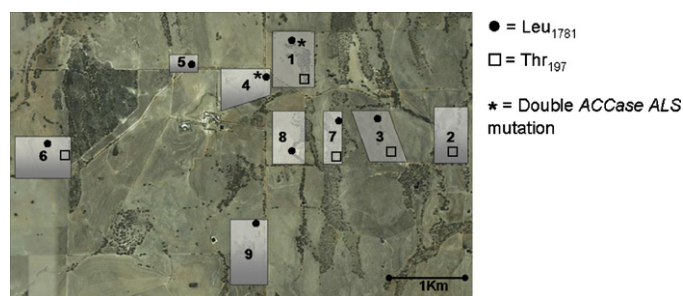


Fig. 2. Aerial Google Earth® photo of the Moora site showing the sampled conventional (2, 4, 5, 6, 7, 8 and 9 in light grey color) and organic fields (1 and 3 in dark grey color) and the spatial distribution of resistant ACCase and ALS alleles. Only resistant alleles found in both conventional and organic fields are shown. Mean field size was 25 ha.

for resistance alleles) and from five fields where organic crops have been grown without herbicide use for at least 20 years (recipient populations for resistance alleles) (see Table 1). In Dumbleyung seeds from organic recipient fields were collected both from field margins and the centre. In each location seed samples were collected from organic receptor fields of one organic farm (i.e. managed by one grower), whereas conventional source fields were part of different conventional farms (Figs. 1 and 2). A total of 20 seed samples (12 from conventional and 8 from organic fields, Table 1) were collected. Each seed sample consisted of 100 *L. rigidum* spikes (one spike/plant). Each seed sample was assigned with a specific code referring to the site, the field number on our sampling list, the position of collection point within the field, and the management strategy used (Table 1). Geographical coordinates for each seed sample were recorded by a GPS.

2.2. Identification of herbicide-resistant plants

In 2008 during the winter growing period (May–August), seedlings from all the samples collected from the Moora and Dumbleyung fields were simultaneously tested for resistance to ACCase- or ALS-inhibiting herbicides. As resistance to herbicides can be due to mutation(s) at the herbicide target site and/or to other genes causing a reduction in the amount of herbicide reaching its target, in particular by causing enhanced herbicide metabolism by the plant (Délye, 2005; Powles and Yu, 2010), screening for resistant plants was conducted with two types of herbicides. Diclofop-methyl (an ACCase inhibitor) and chlorsulfuron (an ALS inhibitor) can be metabolized by *L. rigidum* plants (Christopher et al., 1992) and were used to assess the total frequency of resistant plants. Sethoxydim and sulfometuron are respectively ACCase and ALS inhibitors for which metabolism-based resistance has never been reported, and survival of treatment with these herbicides is thus likely due to the occurrence of mutant alleles.

Plants were grown in pots containing a standard potting mixture (50% sand, 30% peat moss and 20% pine bark). Plants were kept in an outdoor environment simulating field conditions well watered and fertilized. A total of 7609 plants were treated with herbicides to identify resistant individuals. Plants from each collection point were treated at the two-leaf stage with the label rate of one of the following herbicides: diclofop-methyl at 375 g ha⁻¹ (Hoegrass 50% EC, Bayer CropScience), sethoxydim at 93 g ha⁻¹ (Sertin 18.6% EC, Bayer CropScience), chlorsulfuron at 20 g ha⁻¹ (75% WDG, 4Farmers Ltd.) or sulfometuron at 20 g ha⁻¹ (Oust 75% WDG, Dupont). Plants from the well-characterized *L. rigidum* population VLR1 are susceptible to all herbicides. This population was included in all

Table 1

Lolium rigidum seed samples collected at Dumbleyung (Du) and Moora (Mo), centre (c) and margins (m), in conventional (Conv) and organic (Org) fields. The frequency of herbicide-resistant plants was investigated using two ACCase-inhibiting herbicides (diclofop-methyl and sethoxydim) and two ALS-inhibiting herbicides (chlorsulfuron and sulfometuron). Mutant, herbicide-resistant ACCase or ALS alleles were identified using genotyping and/or sequencing.

Population	Resistance to ACCase inhibitors		Mutant ACCase allele(s) detected	Resistance to ALS inhibitors		Mutant ALS allele(s) detected
	Diclofop-methyl (%)	Sethoxydim (%)		Chlorsulfuron (%)	Sulfometuron (%)	
Du1c-Org ^c	1.3	0.0	None	53.1	7.8	Gln ₁₉₇
Du1m-Org ^c	1.7	0.4	Leu ₁₇₈₁	31.4	2.2	Thr ₁₉₇
Du2c-Org ^c	0.0	0.3	Leu ₁₇₈₁	28.9	3.0	Gln ₁₉₇
Du2m-Org ^c	1.8	0.0	None	39.5	0.9	None
Du7c-Org	– ^b	4.6	Leu ₁₇₈₁ , Asn ₂₀₄₁	– ^b	5.6	Gln ₁₉₇
Du7m-Org	– ^b	0.0	None	– ^b	6.9	Codon ₁₉₇ , not identified ^a
Du3m-Conv	37.8	51.9	Leu ₁₇₈₁ ^c	85.7	65.0	Gln ₁₉₇
Du4c-Conv	31.8	25.1	Leu ₁₇₈₁ ^c	100.0	80.2	Gln ₁₉₇ ; Thr ₁₉₇ ; Leu ₁₉₇
Du5c-Conv	20.0	7.6	Leu ₁₇₈₁ ; Arg ₂₀₈₈ ^c	94.4	72.9	Gln ₁₉₇ ; Thr ₁₉₇
Du5m-Conv	12.5	2.2	None	84.4	49.1	Ser ₁₉₇ ; Thr ₁₉₇
Du6c-Conv	16.0	3.2	Leu ₁₇₈₁ ^c	73.9	41.5	None
Mo1c-Org	0.0	0.7	Leu ₁₇₈₁ ; Arg ₂₀₈₈	50.0	2.9	Thr ₁₉₇
Mo3c-Org	9.1	1.1	Leu ₁₇₈₁	21.7	2.6	Thr ₁₉₇
Mo2c-Conv	0.0	1.3	None	20.0	1.3	Thr ₁₉₇
Mo4m-Conv	– ^b	27.0	Leu ₁₇₈₁ ^c	– ^b	44.4	Ser ₁₉₇
M5c-Conv	17.6	20.2	Leu ₁₇₈₁	75.0	40.8	Codon ₁₉₇ , not identified ^a
Mo6c-Conv	50.0	30.0	Leu ₁₇₈₁ ; Gly ₂₀₇₈	100.0	38.5	Thr ₁₉₇
Mo7c-Conv	14.8	4.0	Leu ₁₇₈₁	31.6	1.6	Thr ₁₉₇
Mo8c-Conv	16.7	2.8	Leu ₁₇₈₁	61.5	3.8	Codon ₁₉₇ , not identified ^a
Mo9c-Conv	26.5	10.6	Leu ₁₇₈₁	80.0	6.1	Codon ₁₉₇ , not identified ^a
Control VLR1	0.0	0.0	None	25.9	0.0	None

^a Mutant alleles detected using dCAPS only no sequencing performed.

^b Limited seed stocks available prevented assessment of the frequency of herbicide-resistant plants.

^c Fields that have never received herbicide selection.

assays as the susceptible control. Surviving plants were recorded 21 days after herbicide treatment. Some individuals which survived treatments with sethoxydim or sulfometuron and grew vigorously, indicating the likelihood of target-site resistance, were randomly chosen and used for ACCase and ALS genotyping and sequencing. A Z-test was used to compare the frequencies of herbicide resistant plants from two independent groups to determine if they were significantly different for $\alpha < 0.01$.

2.3. Detection and characterization of mutant ACCase and ALS alleles

DNA was extracted as described in Yu et al. (2008) from individuals having survived applications of either sethoxydim or sulfometuron.

Using *A. myosuroides* amino-acid numbering (Délye, 2005), mutant ACCase alleles conferring herbicide resistance bear a non-synonymous mutation at codons 1781 (most frequent), 1999, 2027, 2041, 2078, 2088 or 2096 (Délye, 2005; Powles and Yu, 2010). Thirty individuals having survived sethoxydim application were genotyped using a previously described allele-specific PCR assay detecting an isoleucine-to-leucine substitution at ACCase codon 1781 (Délye et al., 2002). When no mutant ACCase allele was detected at this position, a 406-bp ACCase fragment containing codons 1999–2096 was amplified by PCR and sequenced using primers ACcp4 and ACcp2R as described by Délye and Michel (2005).

Using *Arabidopsis thaliana* amino-acid numbering (Tranel and Wright, 2002), the most common mutant ALS alleles identified so far that confer herbicide resistance in grass weeds bear a non-synonymous mutation at codon 197 or 574 (Powles and Yu, 2010). Forty-six individuals having survived sulfometuron application were genotyped using previously described dCAPS assays detecting the occurrence of amino-acid substitutions at each codon (Délye et al., 2009). In plants identified as containing mutant ALS allele(s), a 218-bp fragment of the ALS gene encompassing codon 197 was amplified and sequenced using primers RG205F and RG205R as described by Délye et al. (2009). Some plants contained

two different ALS haplotypes (heterozygous plants), as deduced from the occurrence of double-peaks on sequence chromatograms. After sequencing a 218-bp fragment encompassing codon 197, primer sequences and flanking low-quality nucleotides were discarded, resulting in a 135-bp fragment spanning nucleotides 388–522 in the *Lolium* ALS cDNA sequence (GenBank/EMBL accession AF310684) that encode residues Arg₁₆₀ to Asp₂₀₄ (according to *A. thaliana* amino-acid numbering). The sequences of individual haplotypes were statistically inferred with the program PHASE, which implements a Bayesian method for estimating haplotypes from population sequence data (Stephens et al., 2001). This was performed on the basis of the sequences obtained from the homozygous plants in the current study, and from plants previously sequenced (Délye et al., 2009). Editing and alignment were performed using BioEdit (Hall, 1999), and sequence polymorphism was analysed using DNASP v5 (Librado and Rozas, 2009).

3. Results

3.1. Herbicide resistance in plants from conventional and organic fields

L. rigidum plants from the 20 samples collected in the two sites of Dumbleyung and Moora were treated with four different herbicides (two ACCase and two ALS inhibitors). Diclofop-methyl (596 plants treated) and chlorsulfuron (557 plants treated) were used to determine the total frequencies of ACCase and ALS herbicide resistance, respectively. Sethoxydim (3223 plants treated) and sulfometuron (3233 plants treated) were used to specifically indicate the frequencies of plants likely to have mutation(s) of ACCase or ALS genes, respectively. Some of the plants having survived these two herbicides were further analysed to identify the mutations endowing herbicide resistance (Table 1).

3.2. Frequency of plants resistant to ACCase-inhibiting herbicides

As expected, for the known herbicide-susceptible population (VLR1), the ACCase-inhibiting diclofop-methyl caused 100% mor-

tality. However, from conventional fields in Dumbleyung (herbicide history), 23% of the plants were diclofop-methyl-resistant, whereas from nearby organic fields (no herbicide history), only 1.2% were resistant ($Z=6.93$; $P<0.01$). Similarly, from Moora, 18% of the plants from conventional fields were diclofop-methyl-resistant compared to 4.5% in nearby organic fields ($Z=1.81$; $P=0.07$). Overall, resistant plant frequency was the same in seed samples collected along field margins compared to the centre of the fields in both conventional ($Z=1.17$; $P=0.12$) and organic ($Z=0.27$; $P=0.39$) fields.

As was the case for diclofop-methyl, sethoxydim gave 100% mortality in the known susceptible population VLR1. However, there was 25% resistance in conventional Dumbleyung fields versus 0.42% in organic fields ($Z=17.6$; $P<0.01$). From the Moora site, the frequency of resistant plants in conventional fields (8.3%) was also significantly higher than the 0.94% in organic fields ($Z=4.48$; $P<0.01$). There was no difference in sethoxydim resistance frequency between organic fields with no herbicide history (Du1 and Du2) versus fields which had last received herbicide more than 20 years ago (Du7) ($Z=1.24$; $P=0.21$). Again, the frequency of sethoxydim-resistant plants was not significantly different in the centre or margins of fields (0.6% vs. 0.2%) ($Z=0.747$; $P=0.33$).

3.3. Frequency of plants resistant to ALS-inhibiting herbicides

For the control herbicide-susceptible VLR1 population there was 26% plant survival at the chlorsulfuron rate employed. It is known that at this chlorsulfuron rate there is some survival of susceptible plants when treated in pots at the two-leaf stage (Busi et al., 2011), whereas pre-emergence chlorsulfuron at the same dose is generally more effective for *L. rigidum* control. A marginally higher 37% survival rate was found in plants from the organic fields in both Dumbleyung and Moora ($Z=1.12$; $P=0.13$). However, in conventional fields, there was a massive 87% resistance ($Z=9.21$; $P<0.01$) in Dumbleyung and 53% ($Z=1.79$; $P=0.046$) in Moora. In both conventional and organic fields there was no difference between resistance frequency in seed samples collected along the field margin or centre ($Z=0.268$; $P=0.39$).

Sulfometuron caused 100% plant mortality in the VLR1 population. In samples from Dumbleyung, there was 66% sulfometuron resistance in conventional fields versus 3.9% resistance in organic fields ($Z=28.76$; $P<0.01$), whilst in Moora the resistance was 9.6% and 2.7%, respectively ($Z=3.85$; $P<0.01$). As seen for the ACCase herbicide sethoxydim, the frequency of resistant plants in *L. rigidum* populations from organic fields without a herbicide history (Du1 and Du2) was not significantly different from populations that had received herbicide more than 20 years ago (Du7) ($Z=1.24$; $P=0.107$).

3.4. Detection of mutant, herbicide-resistant ACCase and ALS alleles

3.4.1. ACCase alleles

Samples from conventional (24 plants) and organic fields (6 plants) surviving the ACCase-inhibiting herbicide sethoxydim were genotyped to detect Leu₁₇₈₁ ACCase mutations known to endow sethoxydim resistance. The majority (22 out of a total of 30) were found to be heterozygous for this mutation, whereas two plants were homozygous. The mutant alleles were detected in ten (conventional) and three (organic) plants from fields in Dumbleyung, and in eight (conventional) and one (organic) plants from fields in Moora (Table 1; Figs. 1 and 2). The remaining eight plants were homozygous wild-type (susceptible) at codon 1781, but sequencing identified other known resistance-conferring ACCase mutations at Asn₂₀₄₁ (one plant), Gly₂₀₇₈ (one plant) and Arg₂₀₈₈

(two plants). The remaining four plants did not carry any known ACCase gene mutations. Six mutants Leu₁₇₈₁ ACCase alleles were also identified in six ALS mutant plants resistant to ALS-inhibiting herbicide sulfometuron, confirming the presence of mutations at both the ACCase and the ALS genes in the same individual plants (Figs. 1 and 2).

3.4.2. ALS alleles

In total, 46 plants surviving sulfometuron were analysed using dCAPS assays targeting codons Pro₁₉₇ and Trp₅₇₄. No mutations at Trp₅₇₄ were found; however, resistance-endowing mutations at Pro₁₉₇ were detected in 38 plants, six of which were homozygous for ALS mutations and the remainder being heterozygous. Eleven different amino acid substitutions at ALS codon Pro₁₉₇ are known to result in herbicide resistance (Powles and Yu, 2010). Partial sequencing of the ALS gene was performed in 14 of the plants carrying mutant ALS alleles, including the six homozygous mutant plants, to identify the specific amino-acid changes at Pro₁₉₇. Sequencing identified four types of mutant ALS alleles (Leu₁₉₇, Ser₁₉₇, Thr₁₉₇ and Gln₁₉₇). Among these, Gln₁₉₇ and Thr₁₉₇ alleles were observed in both organic and conventional fields from Dumbleyung, whilst Thr₁₉₇ alleles were also observed in resistant plants from conventional and organic fields in Moora (Table 1; Figs. 1 and 2). ALS resistance mutations were also found in six ACCase mutants Leu₁₇₈₁ resistant to sethoxydim (Figs. 1 and 2).

3.5. Identification of mutant ALS haplotypes

Among the 22 sequences obtained from the 14 plants analysed for ALS mutations, a total of 19 polymorphic sites were identified. Polymorphisms consisted of 16 nucleotide substitutions, 11 of which had two alleles and 5 had three alleles. In total, five different haplotypes had silent mutations encoding Pro₁₉₇. The five remaining haplotypes all harboured a nucleotide substitution encoding an amino acid replacement at codon Pro₁₉₇, consisting of one Gln₁₉₇, two Thr₁₉₇, one Leu₁₉₇ and one Ser₁₉₇ haplotype (Fig. S1).

Identical mutant Gln₁₉₇ haplotypes were found in one plant in each of the conventional fields Du3m, Du4c and Du5c, and in the organic fields Du1m, Du2c and Du7c (Table 1; Fig. S1). The minimum distances between organic and conventional fields varied from 200 m to 2.5 km (fields Du4c and Du1m; Fig. 1). Similarly, two resistant plants collected from the conventional fields Du5c and Du5 m contained the same Thr₁₉₇ haplotype also found in a resistant plant collected from the organic field Du1m (Fig. S1). The fields were separated by a minimum distance of 1 km (Fig. 1).

4. Discussion

4.1. Herbicide resistance frequency in *L. rigidum* populations within organic fields with no herbicide history

In this study the frequency of herbicide-resistant individuals in *L. rigidum* populations was characterized in fields with a persistent herbicide history versus organic fields without herbicide history. In all fields analysed, the frequency of plants resistant to ALS-inhibiting herbicides was higher than that of plants resistant to ACCase-inhibiting herbicides (Table 1). This is consistent with the herbicide resistance levels reported by Owen et al. (2007) in *L. rigidum* in a major geographical random survey of the Western Australian "wheatbelt". There is positive correlation between strength of the source and gene flow probability (Damgaard and Kjellsson, 2005). Thus, a higher frequency of ALS-resistant plants in conventional fields likely resulted in greater spread of herbicide resistance in neighbouring organic fields.

In organic fields, the frequency of herbicide-resistant *L. rigidum* individuals was much higher than could be explained by the back-

ground mutation frequency (10^{-9} to 10^{-6}) (Jasieniuk et al., 1996), expected for target-site ACCase gene mutations. In an Australian context, it has been shown that the frequency of plants resistant to the Australian label rate of the ACCase inhibiting herbicide diclofop-methyl in unselected and genetically isolated *L. rigidum* populations can range from 2×10^{-3} to 4.6×10^{-3} (Matthews and Powles, 1992; Neve and Powles, 2005). Thus, the observed resistance frequency to diclofop-methyl in plants from organic fields in the current study is still 3- to 10-fold higher than what can be expected in the absence of gene flow. A similar picture emerges for target-site ALS mutations. In genetically isolated, unselected *L. rigidum* populations from Australia, frequencies of mutants resistant to the label rate of sulfometuron ranged from 2.2×10^{-5} to 1.2×10^{-4} (Preston and Powles, 2002). In our study, the frequency of *L. rigidum* mutant plants resistant to sulfometuron collected in organic field populations was at least 300-fold greater (Table 1). It is thus clear that, especially when considering resistance to the ALS herbicide sulfometuron, the *L. rigidum* populations collected from organic fields with neighbouring conventional fields cannot be considered pristine and genetically isolated.

4.2. Herbicide resistance gene flow

We have previously documented effective pollen-mediated flow of resistance genes occurring at distances of up to three kilometres in isolated *L. rigidum* individuals (Busi et al., 2008). Similarly, effective transfer of resistance genes among fields has been observed in wind-pollinated grasses such as *Agrostis stolonifera* L. and *A. myosuroides* (Watrud et al., 2004; Délye et al., 2010a). Thus, it is suggested that gene flow from *L. rigidum* populations persistently selected with herbicides and containing resistant plants has increased the frequency of herbicide resistance in nearby unselected populations in organic fields. Use of some herbicides in the early 80s in organic fields cannot be discarded. However, noticeable frequencies of herbicide-resistant *L. rigidum* plants in Moora and Dumblebung were not recorded (J. Holmes, personal communication) and thus, most likely, herbicide use did not select for resistance alleles that may have persisted since then. As expected, there was no difference in the frequency of herbicide-resistant plants in *L. rigidum* populations from organic fields where herbicides were never used and in those from the fields where herbicide use was discontinued about 25 years ago. All this suggests that the presence of resistance alleles in unselected populations from organic fields was not due to the persistence of resistance alleles selected 25 years earlier. This, along with the detection of plants carrying identical mutant ALS haplotypes in neighbouring conventional and organic fields, is in agreement with our hypothesis that high frequencies of resistance alleles in organic populations are predominantly caused by introgression of these alleles from nearby conventional fields.

We thus, propose that gene flow from populations containing high frequencies of resistant plants is the main cause for the increased frequency of resistant plants observed in the organic unselected populations. Under this hypothesis, populations of *L. rigidum* growing in neighbouring fields are connected by gene flow that is substantial enough that we could detect its effects on the genetic makeup of populations (i.e., occurrence of resistant plants in organic fields).

4.3. Dynamics of gene flow

Pollen is a major vehicle for gene flow, and single, isolated *L. rigidum* plants can capture pollen from considerable distance (Busi

et al., 2008). Plant density in the recipient population together with the separating distance can substantially affect gene flow (Rognli et al., 2000). If the source population is large enough and has a high frequency of herbicide resistance genes, then gene flow can cause effective migration of these genes to distant populations with a low plant density, as previously demonstrated using isolated, herbicide-susceptible *L. rigidum* acceptor plants (Busi et al., 2008). However, our present study was conducted in large commercial fields of up to 60 ha in size, containing 1–50 *L. rigidum* individuals m^{-2} with a fairly homogeneous spatial distribution. *L. rigidum* can be effectively controlled by herbicide treatments in conventional fields (e.g. glyphosate and paraquat applications in pastures), or in both conventional and organic systems by heavy sheep grazing during a pasture phase in rotation with crops or over summer following crop harvest. Thus, dramatic fluctuations in *L. rigidum* density have very likely occurred from year to year (Waller et al., 1999). It has been hypothesized that drastic population demographic declines may favour resistance gene introgression from adjacent fields (Délye et al., 2010a). Thus, agricultural practices (rotations, grazing, etc.) which caused *L. rigidum* populations in organic fields to collapse likely favoured pollen-mediated gene flow from dense herbicide-resistant populations in neighbouring conventional fields.

Although identical resistant ALS haplotypes were observed in organic and conventional fields in Dumblebung that were separated by distances between 200 m and 2.5 km, it was impossible to estimate effective distances of gene flow between fields. Indeed, ACCase- and ALS-inhibiting herbicides have been in use for 30 years in both of the sites investigated in this study. The repeated use of ACCase- and ALS-inhibiting herbicides has been shown to result in a high frequency of herbicide resistant plants in conventional field populations from the Western Australian wheatbelt (Owen et al., 2007). Over this period of time, pollen-mediated gene migration may have gradually occurred from plant to plant (in a short-distance “stepping-stone” process) if *L. rigidum* plants occurred in between the fields sampled, or directly from field to field (long-distance). Patches of non-arable land such as native pristine bushland, rocky scald and salty lakes are still present today and are scattered among crop fields (Figs. 1 and 2) following the anthropogenic landscape disturbance that constitutes the Western Australian wheatbelt. In those bush patches there are very few *L. rigidum* plants, often replaced by *Vulpia* plants in a short period of time, yet it is impossible to absolutely exclude their presence between the fields investigated. Thus, it is reasonable to assume those areas did not represent a physical barrier to gene flow. The absence of differences in the frequency of herbicide-resistant plants between the margins and centre of the fields sampled (Table 1) suggests that a stepping-stone resistance gene transfer process probably occurs within a field population following gene introgression of resistance alleles in that field.

Seeds can also be a vector for herbicide resistance gene flow at the local landscape level. In *L. rigidum*, seed movement by harvest combines has been shown to be a phenomenon occurring over short distances (from zero to a few tens of meters) (Blanco-Moreno et al., 2004). However, seed contamination can occur under several different circumstances, mainly by accidental transport with grains at harvest (Gaines et al., 2007a), sowing of crop seeds contaminated with *L. rigidum* seeds (Gaines et al., 2007a; Michael et al., 2010), contaminated hay as feed for sheep or by planting *L. rigidum* pasture seed infested with a percentage of herbicide-resistant individuals (Broster and Pratley, 2006). Seeds may have been transported from conventional to organic fields by farm machinery, in cases where conventional and organic fields were part of the same farm (conventional fields 2, 7 and 8 were part of the same farm as organic fields 1 and 3 in Moora) (Fig. 2) or organic fields were adjacent to main roads connecting different farms.

5. Conclusions

This study with the obligate cross-pollinated grass weed species *L. rigidum* established that herbicide resistance genes transferred between plants in agricultural fields with herbicide use to organic neighbouring fields and farms that did not use herbicides. This information was reported for *A. myosuroides*, a major grass weed in Europe, where the agricultural context is rather different, with much smaller field size, more complex agricultural landscapes, more intensive agriculture (winter and summer crop rotations) and isolated organic fields scattered among conventional fields (Délye et al., 2010a). Thus, at least in cross-pollinated weed species, herbicide resistance evolution in fields with herbicide history can flow via pollen or seed to nearby fields devoted to organic agriculture (no herbicide usage allowed). A combination of good farm hygiene and management practices can limit seed-mediated flow of herbicide resistance genes and subsequently slow the occurrence and spread of resistance. Conversely, the control of pollen-mediated gene flow is clearly a very difficult task within the growing season. Pollen production can only be minimized by maximising control of *L. rigidum* density in the system. Thus, the best way to prevent the dispersal of resistance is to cooperatively prevent the selection for resistance by implementing diversity in weed control methods such as alternation of herbicide modes of action, diversification of crop rotation and use of non-chemical weed control across adjacent farms (Beckie, 2006). Otherwise, at the end of the growing season management practices limiting seed set in recipient *L. rigidum* plants after cross-pollination (herbicide treatments on late-emerged plants) or removal of mature seed during harvest operations (seed catching) followed by post-harvest seed destruction (Walsh and Powles, 2007), can be a good strategy to minimize herbicide resistance following resistance gene migration from neighbouring farm fields.

Acknowledgements

The authors are grateful to Sueli Amprino for field collection help, Karelle Boucansaud and Fanny Pernin (INRA Dijon) for skilled technical assistance, to Colin Gardiner (Moora) and Dayle Lloyd (Dumbleyung) for field sample collection and farm records, to Dr. Danica Goggin for feedbacks on early version of this manuscript and John Holmes for valued historical agronomy perspectives. The Australian Herbicide Resistance Initiative (AHRI) is funded by the Grains Research and Development Corporation of Australia (GRDC). This study was also funded by the Australian Weed Research Centre (AWRC 08-31).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.agee.2011.06.012.

References

- Baker, H.G., 1974. The evolution of weeds. *Ann. Rev. Ecol. Evol. Syst.* 5, 1–24.
- Beckie, H.J., Hall, L.M., 2008. Simple to complex: modelling crop pollen-mediated gene flow. *Plant Sci.* 175, 615–628.
- Beckie, H.J., 2006. Herbicide-resistant weeds: management tactics and practices. *Weed Technol.* 20, 793–814.
- Blanco-Moreno, J.M., Chamorro, L., Marsalles, R.M., Recasens, J., Sans, F.X., 2004. Spatial distribution of *Lolium rigidum* seedlings following seed dispersal by combine harvesters. *Weed Res.* 44, 375–387.
- Broster, J.C., Pratley, J.E., 2006. A decade of monitoring herbicide resistance in *Lolium rigidum* in Australia. *Aust. J. Exp. Agric.* 46, 1151–1160.
- Busi, R., Vila-Aiub, M.M., Powles, S.B., 2011. Genetic control of cytochrome P450-based enhanced herbicide metabolism in a *Lolium rigidum* population. *Heredity* 106, 817–824.
- Busi, R., Yu, Q., Barrett-Lennard, R., Powles, S.B., 2008. Long distance pollen-mediated flow of herbicide resistance genes in *Lolium rigidum*. *Theor. Appl. Genet.* 117, 1281–1290.
- Christopher, J.T., Powles, S.B., Holtum, J.A.M., 1992. Resistance to acetolactate synthase-inhibiting herbicides in annual ryegrass (*Lolium rigidum*) involves at least two mechanisms. *Plant Phys.* 100, 1909–1913.
- Damgaard, C., Kjellsson, G., 2005. Gene flow of oilseed rape (*Brassica napus*) according to isolation distance and buffer zone. *Agric. Ecosyst. Environ.* 108, 291–301.
- Darmency, H., 1996. Movement of resistance genes among plants. In: Brown, T.M. (Ed.), *Molecular Genetics and Evolution of Pesticide Resistance in Plants: Biology and Biochemistry*. Lewis Publishers, Boca Raton, FL, pp. 209–220.
- Délye, C., Calmès, É., Matějček, A., 2002. SNP markers for black-grass (*Alopecurus myosuroides* Huds.) genotypes resistant to acetyl CoA-carboxylase inhibiting herbicides. *Theor. Appl. Genet.* 104, 1114–1120.
- Délye, C., 2005. Weed resistance to acetyl coenzyme A carboxylase inhibitors: an update. *Weed Sci.* 53, 728–746.
- Délye, C., Michel, S., 2005. 'Universal' primers for PCR-sequencing of grass chloroplastic acetyl-CoA carboxylase domains involved in resistance to herbicides. *Weed Res.* 45, 323–330.
- Délye, C., Boucansaud, K., Pernin, F., Le Corre, V., 2009. Variation in the gene encoding acetolactate-synthase in *Lolium* species and proactive detection of mutant, herbicide-resistant alleles. *Weed Res.* 49, 326–336.
- Délye, C., Clément, J.A.J., Pernin, F., Chauvel, B., Le Corre, V., 2010a. Gene flow homogenises the evolution of arable weed populations at the landscape level. *Basic Appl. Ecol.* 11, 504–512.
- Délye, C., Michel, S., Berard, A., Chauvel, B., Brunel, D., Guillemin, J.P., Dessaint, F., Le Corre, V., 2010b. Geographical variation in resistance to acetyl-coenzyme A carboxylase-inhibiting herbicides across the range of the arable weed *Alopecurus myosuroides* (black-grass). *New Phytol.* 186, 1005–1017.
- Diggle, A.J., Neve, P., 2001. The population dynamics and genetics of herbicide resistance. A modeling approach. In: Powles, S.B., Shaner, D.L. (Eds.), *Herbicide Resistance and World Grains*. CRC Press, FL, pp. 61–99.
- Gaines, T., Preston, C., Byrne, P., Henry, W.B., Westra, P., 2007a. Adventitious presence of herbicide resistant wheat in certified and farm-saved seed lots. *Crop Sci.* 47, 751–756.
- Gaines, T.A., Byrne, P.F., Westra, P., Nissen, S.J., Henry, W.B., Shaner, D.L., Chapman, P.L., 2007b. An empirically derived model of field-scale gene flow in winter wheat. *Crop Sci.* 47, 2308–2316.
- Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41, 95–98.
- Heap, I.M., 2011. International Survey of Herbicide Resistant Weeds, Available on: <http://www.weedscience.org> [accessed 05.11].
- Jasieniuk, M., BruleBabel, A.L., Morrison, I.N., 1996. The evolution and genetics of herbicide resistance in weeds. *Weed Sci.* 44, 176–193.
- Levin, D.A., Kerster, H.W., 1974. Gene flow in seed plants. *Evol. Biol.* 7, 139–220.
- Librado, P., Rozas, J., 2009. DNASP v5: a software for comprehensive analysis of DNA polymorphism. *Bioinformatics* 25, 1451–1452.
- Matthews, J.M., Powles, S.B., 1992. Aspects of the population dynamics of selection for herbicide resistance in *Lolium rigidum* (Gaud). *Proc. 1st Int. Weed Cont. Cong.*, p. 318.
- Michael, P.J., Owen, M.J., Powles, S.B., 2010. Herbicide-resistant weed seeds contaminate grain sown in the Western Australian grainbelt. *Weed Sci.* 58, 466–472.
- Murray, B.G., Morrison, I.N., Friesen, L.F., 2002. Pollen-mediated gene flow in wild oat. *Weed Sci.* 50, 321–325.
- Neve, P., Powles, S., 2005. High survival frequencies at low herbicide use rates in populations of *Lolium rigidum* result in rapid evolution of herbicide resistance. *Heredity* 95, 485–492.
- Owen, M.C., Walsh, M.J., Llewellyn, R.S., Powles, S.B., 2007. Widespread evolution of multiple herbicide resistance in annual ryegrass (*Lolium rigidum*) populations within the Western Australian wheat belt. *Aust. J. Agric. Res.* 58, 711–718.
- Powles, S.B., Yu, Q., 2010. Evolution in action: plants resistant to herbicides. *Annu. Rev. Plant Biol.* 61, 317–347.
- Preston, C., Powles, S.B., 2002. Evolution of herbicide resistance in weeds: initial frequency of target site-based resistance to acetolactate synthase-inhibiting herbicides in *Lolium rigidum*. *Heredity* 88, 8–13.
- Rognli, O.A., Nilsson, N.O., Nurminiemi, M., 2000. Effects of distance and pollen competition on gene flow in the wind-pollinated grass *Festuca pratensis* Huds. *Heredity* 85, 550–560.
- Rong, J., Liu, B.-R., Song, Z., Su, J., Snow, A.A., Zhang, X., Sun, S., Chen, R., Wang, F., 2007. Dramatic reduction of crop-to-crop gene flow within a short distance from transgenic rice fields. *New Phytol.* 173, 346–353.
- Stephens, M., Smith, N.J., Donnelly, P., 2001. A new statistical method for haplotype reconstruction from population data. *Am. J. Hum. Genet.* 68, 978–989.
- Tranel, P.J., Wright, T.R., 2002. Resistance of weeds to ALS-inhibiting herbicides: what have we learned? *Weed Sci.* 50, 700–712.
- Vila-Aiub, M.M., Neve, P., Powles, S.B., 2009. Fitness costs associated with evolved herbicide resistance alleles in plants. *New Phytol.* 184, 751–767.
- Waller, R.A., Quigley, P.E., Saul, G.R., Sale, P.W.G., Kearney, G.A., 1999. Tactical versus continuous stocking for persistence of perennial ryegrass (*Lolium perenne* L.) in pastures grazed by sheep in south-western Victoria. *Aust. J. Exp. Agric.* 39, 265–274.
- Walsh, M.J., Powles, S.B., 2007. Management strategies for herbicide-resistant weed populations in Australian dryland crop production systems. *Weed Technol.* 21, 332–338.

- Warwick, S.I., 1991. Herbicide resistance in weedy plants: physiology and population biology. *Ann. Rev. Ecol. Syst.* 22, 95–114.
- Watrud, L.S., Lee, E.H., Fairbrother, A., Burdick, C., Reichman, J.R., Bollman, M., Storm, M., King, G., Van de Water, P.K., 2004. Evidence for landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with CP4 EPSPS as a marker. *Proc. Natl. Acad. Sci. U.S.A.* 101, 14533–14538.
- Yu, Q., Han, H., Powles, S.B., 2008. Mutations of the ALS gene endowing resistance to ALS-inhibiting herbicides in *Lolium rigidum* populations. *Pest Manag. Sci.* 64, 1229–1236.
- Zapiola, M.L., Campbell, C.K., Butler, M.D., Mallory-Smith, C.A., 2007. Escape and establishment of transgenic glyphosate-resistant creeping bentgrass *Agrostis stolonifera* in Oregon USA: a 4-year study. *J. Appl. Ecol.* 45, 486–494.