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ORIGINAL PAPER

Long distance pollen-mediated flow of herbicide resistance genes in *Lolium rigidum*

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Abstract Gene flow promotes genetic exchange among plant populations mediating evolutionary dynamics; yet, the importance of gene flow at distance via pollen movement is poorly understood. A field experiment at the landscape level was conducted with *Lolium rigidum* herbicide-susceptible individuals (population VLR1) placed into an otherwise *Lolium*-free bushland environment at increasing distances from adjacent large commercial crop fields infested with herbicide-resistant *L. rigidum*. Herbicide resistance was used as a marker to quantify the distance and the rate of pollen-mediated gene flow. About 21,245 seeds were produced on the isolated, susceptible mother plants of which 3,303 seedlings were tested for herbicide resistance and 664 seedlings were found to be resistant. Pollen-mediated gene flow occurred at 3,000 m (maximum tested distance). Both Mendelian and molecular analyses (sequencing and CAPS markers) confirmed the introgression of herbicide resistance genes. This is the first documented case of long-distance gene flow in *L. rigidum*. The results are important for future modeling simulations of herbicide resistance evolution and subsequent mobility. The adoption of integrated agronomic strategies, the control of potential receptor plants on fields' margins and conservative use of herbicides can be realistic options to minimize herbicide resistance spread.

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Introduction

Evolution is often perceived as solely a long-term adaptive process. However, gene flow promotes genetic variability, prevents genetic isolation and can enable faster evolutionary dynamics (Ehrlich and Raven 1969). For plants, potential gene flow is the estimation of pollen or seed deposition at distance, whereas actual gene flow refers to the rate of cross-pollination or establishment of individual plants as a function of distance from a source (Levin and Kerster 1974). In plants, gene flow is influenced by the reproductive system, with seed dispersal generally predominant in self-pollinating species while pollen-mediated gene flow can be more important in cross-pollinated species (Darmency 1996; Lu et al. 2007; Watrud et al. 2004). A major consequence of gene flow in agricultural weeds is the potential movement of herbicide resistance genes between and within weed populations and subsequent herbicide resistance evolution if resistance alleles are not present in the population.

Long distance gene flow due to seed dispersal is well documented for tumbleweeds in the Chenopodiaceae family (Borger et al. 2007) or weed species belonging to the Asteraceae family through production of airborne seeds (Dauer et al. 2007; Lu et al. 2007). However, in many weed species, especially in wind-pollinated grasses, the potential of effective pollen-mediated gene flow is much greater than gene flow due to seed dispersal (Balfourier et al. 2000;

Pfender et al. 2007). Yet, the contribution of pollen-mediated gene flow to herbicide resistance dispersal across weed populations is poorly understood for both self- and cross-pollinating species and therefore its importance could be underestimated (Jaseniuk et al. 1996). When reviewed by Levin and Kerster (1974), long-distance gene flow by pollen drift was assumed to be a rare phenomenon occurring at a rate of 0.01% for plants separated by 1.5 km or more. Certainly pollen can move large distances through wind dispersal but, in most Poaceae species pollen remains viable for less than 3 h (Fei and Nelson 2003; Luna et al. 2001; Wang et al. 2004a). For instance, in *Agrostis stolonifera* L. only 1% of pollen is viable after 2 h from emission (Fei and Nelson 2003; Pfender et al. 2007). Therefore, successful cross-pollination at long distances has been considered unlikely due to the loss of pollen viability with effective pollen flow in grass weeds only occurring within a few hundreds meters (Nurminiemi et al. 1998; Rognli et al. 2000; Wang et al. 2004b). However, recent modeling simulations have suggested that pollen drift in *Lolium. perenne* L. can occur over distances up to 1 km (Giddings 2000) or to 3 km in *A. stolonifera* (Pfender et al. 2007). Experimental observations have generally been restricted to small experimental plots or small plant source areas mainly because until recently there were few reliable, cheap, and easy-to-detect markers for estimating gene flow in large-scale studies. The introduction of genetically modified (GM) crops has increasingly generated interest or concerns related to pollen-mediated gene flow with evolutionary, ecological, and agronomic implications discussed in several studies analyzing the potential mobility of transgenic herbicide resistance markers (Gaines et al. 2007; Messeguer et al. 2004; Pfender et al. 2007; Rong et al. 2007). In studies conducted on appropriate experimental scales, or in large commercial fields using herbicide resistance as a marker, pollen-mediated long-distance gene flow has been documented at the landscape level (Rieger et al. 2002; Watrud et al. 2004).

Lolium rigidum Gaud. is a very economically important weed (especially in Australian cropping system) and currently represents the most serious case of herbicide resistance worldwide. *Lolium rigidum* is an anemophilous, self-sterile, cross-pollinated species with massive pollen production (Gill 1996; McCraw and Spoor 1983). Herbicide resistant *L. rigidum* infests millions of hectares of the southern Australian cropping region (Owen et al. 2007), and thus, there is huge potential for gene flow due to wind-mediated pollen dispersal, especially when plants are present at high densities. Gene flow by cross-pollination at distance could allow the spread of rare herbicide resistance traits (e.g. glyphosate or paraquat resistance genes) and favor the development of multiple-resistant populations through the accumulation of different resistance genes in individual plants or populations. Moreover, if there is substantial long-distance gene flow, this could dissuade farmers from adopting measures to minimize herbicide resistance evolution (Llewellyn and Allen 2006) and cause more rapid loss of effective herbicide molecules, leading to weed management practices at higher costs (Weersink et al. 2005).

In Western Australia, crop land infested with *L. rigidum* immediately neighbors an extensive area of undisturbed natural bushland that is free of *L. rigidum*. This landscape pattern provided a unique opportunity to study gene flow due to pollen emanating from fields infested with herbicide-resistant *L. rigidum*. This study determined pollen-mediated gene flow in *L. rigidum* by evaluating the introgression of herbicide resistance genes from crop fields into herbicide-susceptible plants placed in the adjacent bushland.

Materials and methods

Field experiment

A site was chosen as the experimental area near Salmon Gums (32°7′S–121°6′E) at the edge of the Western Australian 'Wheatbelt'. This region was chosen because it contains cleared areas adjacent to undisturbed *L. rigidum*-free natural bushland separated by a long straight boundary between crops and bushland (Fig. 1a). About 50 fields totaling 8,000 ha of cropland were located within approximately 16,500 ha of experimental area (Fig. 1b). This site is representative of Australian commercial agriculture (large farm sizes, about 3,000 ha). A *L. rigidum* population (VLR1), known to be susceptible to all the herbicides recommended for grass control in Australia, was used in this study as the susceptible (mother) population. Seed stocks of this susceptible reference population have been maintained and multiplied since 1985 in absence of herbicide selection and preventing external gene flow.

Before anthesis in September of 2005, 80 large VLR1 plants (hereinafter referred to as susceptible mother plants) were placed into the undisturbed bushland established to be free of *L. rigidum*. Individual susceptible plants were placed at varying distances (0–3,000 m) from the wheat and pasture fields known to be infested with herbicide-resistant *L. rigidum* (pollen source). Homogeneous *Lolium* infestation was present across the experimental area with average densities (visual assessment) ranging from 1 to 10 plants m^{-2}. Most of the susceptible plants placed at the greatest distances from the pollen source were positioned along one main South-North transect (Fig. 1b). In addition, eight groups of paired susceptible plants (1 m apart) were also arranged at 0, 100 and 200 m from the field's edge to

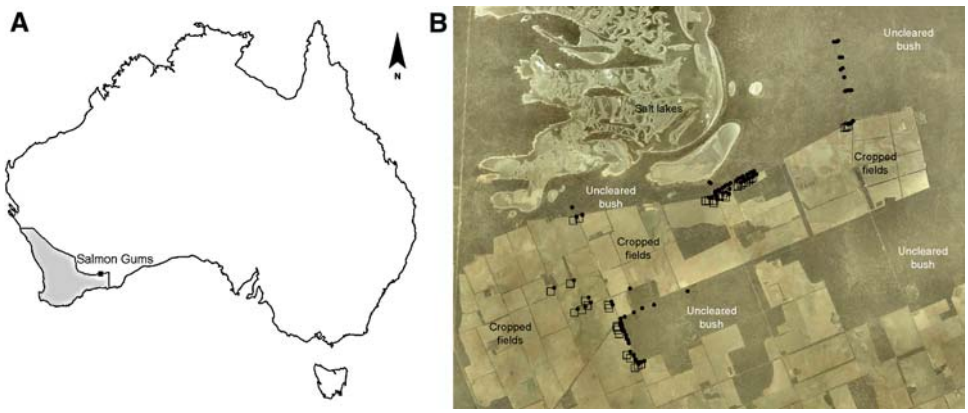

Fig. 1 **a** Experimental location map. The experiment was carried out in Salmon Gums at the edge of the Western Australian 'Wheatbelt' (*grey area*). **b** Experimental site aerial photo (width × height 15 × 11 km^2). *Solid circles* and *open squares* represent the position of individual susceptible mother plants and resident population (RP) seed samples, respectively

investigate the effects of pollen competition on cross-pollination at distance. The resident plants, infesting crop and pasture fields, were the herbicide-resistant pollen donors and the 80 bushland herbicide-susceptible mother plants were the receptors. Longitude and latitude coordinates for each plant introduced into the bushland were GPS recorded to enable accurate calculation of the distance from the nearest donor field. Weather data were obtained from the Bureau of Meteorology with automated instrumentation placed in the nearby town of Salmon Gums. Following seed maturation (December) the bushland-placed susceptible mother plants were harvested and their seed collected. Plants were threshed and the number of full seeds (i.e. heaviest seeds), separated by gravity from the chaff fraction, was estimated with the following formula:

$$S_n = \frac{TS_W \times 50}{S_W} \quad (1)$$

where S_n is the total number of seeds, TS_w denotes the total seed weight produced by each plant and S_w represents the mean weight of 50 seeds per plant (Table 1).

Thirty-seven seed samples (100 plants each) were collected within cropped land to establish the level of resistance to acetolactate synthase (ALS)-inhibiting herbicides in the donor resident population (hereinafter referred to as RP).

Herbicide screening

Introgression of herbicide resistance trait into individual susceptible plants was used as a marker for gene flow to quantify the effective pollination distance. In this study, seedlings grown from the seed progeny produced on susceptible mother plants (Progeny 1, referred to as P1) and seedlings of RP plants were tested for sulfometuron resistance. This ALS-inhibiting herbicide is extremely active on *L. rigidum* and is not metabolized by plants (Christopher et al. 1992); therefore, surviving plants are likely to be target-site resistant (i.e. a point mutation in the ALS gene and the subsequent amino acid substitution in the ALS enzyme endows target-site resistance to ALS-inhibiting herbicides). The *L. rigidum* population VLR1 is susceptible to sulfometuron with 100% mortality at 10 g ha^{-1} of sulfometuron (Christopher et al. 1992). However, for confirmation, herbicide screening was conducted with the susceptible population, a known target-site ALS-resistant population (WLR1) and four F1 populations obtained by

Table 1 Total number of plants recovered/introduced, seeds recovered, seeds tested for herbicide resistance (R) to sulfometuron, sulfometuron R seedlings and R frequency to sulfometuron (R seedlings/seeds tested) at each distance established in the field experiment

Distance from pollen source (m)	Plants recovered / plants introduced	Seeds recovered	Seeds tested	Sulfometuron R seedlings	R frequency to sulfometuron (%)
0	18/21	13279	1545	504	33
100	10/21	2693	581	81	14
200	13/20	2528	647	60	9
400	3/3	2538	323	3	1
500	1/2	55	55	2	4
1000	2/5	17	17	5	29
1500	1/1	21	21	2	10
2500	2/3	89	89	6	7
3000	1/4	25	25	1	4
Total	51/80	21245	3303	664	20
Resident Population	37 samples	94400	2327	1091	47

crossing these two populations (VLR1 as receptor mother plant ♀ × WLR1 as pollen donor ♂). From this study 15 g ha^{-1} of sulfometuron clearly discriminated between resistant (WLR1 or F1) and susceptible plants (VLR1). Plants of the susceptible mother population VLR1 were always killed at 10 g ha^{-1} of sulfometuron or at higher rates.

Seeds collected from the bushland-placed susceptible mother plants (P1) and the samples of RP plants were germinated in 0.6% (w/v) agar and one leaf stage seedlings transplanted into plastic pots (30 cm × 20 cm) containing potting mixture (50% sand and 50% peat). Plants were grown outdoors during the normal growing season and were sprayed with 10 g ha^{-1} of sulfometuron (Oust SP 75% a.i. plus 0.25% wetting agent BS1000) at the two-leaf stage. After 20 days, survivors were trimmed and allowed to re-shoot before being re-sprayed with 15 g ha^{-1} of sulfometuron. Plants surviving this double sulfometuron treatment were assessed as resistant.

Inheritance study

Plants assessed as being herbicide resistant (i.e. those that survived the double sulfometuron treatment) were sampled according to representative experimental locations (i.e. different gene flow distances) and arranged in 18 crosses (Table 3). Mature seeds (Progeny 2, referred to as P2) were collected and stored in dry conditions. The number of seeds per plant was estimated with the same formula described above (Eq 1).

During May–June 2007, P2 seedlings were tested for segregation of the sulfometuron resistance gene trait. Two-leaf stage seedlings were sprayed with 15 g ha^{-1} of sulfometuron and assessed for mortality 20 days later. Since the introgression of single resistance alleles was assumed, the phenotypic Mendelian segregation of each P2 was tested expecting a 3:1 ratio, corresponding to three resistant (survivors) versus one susceptible (dead) plant. The Mendelian segregation in P2 was used to confirm the introgression of an ALS resistance gene in the susceptible progenitor plants (VLR1) and the subsequent sulfometuron resistance observed in the seed progeny P1.

ALS mutation identification

In order to confirm introgression of an ALS resistance gene from resident plants to susceptible plants, highly conserved regions of the ALS gene containing the 197 and 574 mutation sites, known to endow sulfometuron resistance (reviewed by Tranel and Wright 2002), were amplified, sequenced, and compared between individual P1 and RP plants surviving 15 g ha^{-1} of sulfometuron. Five resistant individual P1 plants, produced from susceptible mother plants placed at the longest distances (500, 1,000, 1,500, 2,500, and 3,000 m from the pollen source), and 20 resistant RP plants were analyzed (Table 4). Bulked samples (50 individuals) from the susceptible mother population (VLR1) without sulfometuron treatment were used as a control. Two pairs of primers were designed based on ALS sequences from *L. rigidum* (accession number EF411170), *Hordeum vulgare* L. (AF059600), *Triticum aestivum* L. (AY210406), *Oryza sativa* L. (AB049822), *L. multiflorum* L. (AF310684), *Alopecurus myosuroides* Huds. (AJ437300), and *Zea mays* L. (X63554). The primer pair ALS197F (5′-ACTCCATCCCCATGGTGGC-3′) and ALS197R (5′-ATCTGCTGCTGGATGTCCTT-3′) was used to amplify a 232-bp fragment flanking the 197 mutation site. The primer pair ALS574F (5′-TGGGCG GCTCAGTATTACAC-3′) and ALS574R (5′-ATAGGC AGCACATGCTCCTG-3′) was used to amplify a 479-bp fragment encompassing the 574 mutation site. Heterozygous individuals were recognized by double peaks at the same position in nucleotide chromatograms of both forward and reverse sequencing. Heterozygosity at the 197 or 574 mutation site was also confirmed using cleaved amplified polymorphic sequence (CAPS) markers.

Cleaved amplified polymorphic sequence (CAPS) markers

A CAPS marker was developed for identification of the Pro to Ser mutation at 197. Within the 232-bp fragment amplified by the primer pair ALS197F/ALS197R, the resistant ALS allele has an *Eco*31I restriction site (GGTCTC) around the Pro197 codon due to the nucleotide C to T mutation, while the susceptible allele does not. This results in one undigested band of 232 bp in homozygous susceptible plants and one digested band of 200 bp in homozygous resistant plants (Fig. 3a lane 1, 4, 8). Heterozygous plants exhibit a combination of these two bands (Fig. 3a lane 2, 3, 5, 6). Another CAPS marker was developed for the Trp to Leu mutation at 574. Within the 479-bp PCR fragment of the primer pair ALS574F/ALS574R, the susceptible allele has two *Bts* I restriction sites (GCAGTG) while the resistant allele only has one restriction site due to the G to T mutation around the Trp574 codon. This results in two closely related bands of 210 and 228 bp in homozygous susceptible plants and one 438-bp band in homozygous resistant plants (Fig. 3b lane 1, 2, 5, 6). Heterozygous plants exhibit a combination of these two bands (Fig. 3b lane 3, 4, 7). Restriction digestions were carried according to the manufacturer's recommendations (*Bts*I from Biolabs and *Eco*31I from Fermentas). Reactions were incubated for 3 h at recommend temperatures and digestion results were resolved on 2–3% agarose gels.

Statistical analysis

In order to determine the relative contribution of two factors such as the density of the acceptor plants (a single plant versus two paired plants) and the distance from the pollen source on the observed rates of gene flow a χ^2 test of independence was performed. After establishing the independence of each of the two entities (density and distance), the actual frequency values of sulfometuron resistance (gene flow) were analyzed and compared by a goodness-of-fit χ^2 test (Table 2).

In the inheritance study the observed segregation frequencies for each progeny P2 were compared to the 3:1 Mendelian segregation ratio by a goodness of fit χ^2 test. Probability values (P) were obtained indicating the probability of type II error in rejecting the null hypothesis (H_o = the introgression of one single gene endows resistance to sulfometuron in progeny P1 and the progeny P2 segregates according to Mendelian laws). In order to increase the power of the statistical test the significance level was increased to $\alpha = 0.1$ and only P values $\geq \alpha$ were accepted to statistically validate the one gene segregation model for each progeny P2 tested. A pooled χ^2 value was calculated considering all the progeny samples tested as a single progeny P2 and a heterogeneity χ^2 test was performed to compare the segregation frequencies obtained (Sokal and Rohlf 1969).

Results

Field experiment

Isolated single susceptible mother plants placed into the native bushland produced viable seed. It is important to emphasize that *L. rigidum* is an obligate cross-pollinated species and there were no *L. rigidum* plants growing naturally in the bushland. Therefore, as *L. rigidum* is a self-incompatible cross-pollinated species, the seed produced on these isolated bushland plants suggests pollen successfully moved by wind up to 3,000 m (maximum tested distance) emanating from the donor source (crop and pasture fields) and caused cross-pollination in the *L. rigidum* plants placed in the bushland. Southerly prevailing winds (speed 13 m s^{-1} at the maximum wind gust) were recorded during the experimentation period. As some of the donor plants growing in fields were herbicide-resistant, the seed produced on the susceptible bushland plants were screened for herbicide resistance to provide concrete evidence of effective gene flow. Approximately 21,245 seeds were recovered from the bushland plants. A different proportion of seed was tested at each distance depending on seed germinability and total number of seeds recovered. In total, 3,303 seedlings were grown and treated with sulfometuron to detect the presence of herbicide resistance and provide evidence of gene flow.

Table 2 Comparison of sulfometuron resistance frequency (equivalent to gene flow rate %) found in single susceptible mother plants versus two paired plants at different distances: 0, 100, 200 m

	Resistance frequency to sulfometuron (%)		
Distance (m)	Single plant	Paired plants	*P*
0	37.8 (463 in 1226)	12.9 (41 in 319)	<0.0001
100	21.2 (79 in 373)	0.96 (2 in 208)	<0.0001
200	19.4 (59 in 304)	0.29 (1 in 343)	<0.0001
P	<0.0001	<0.0001	

The actual number of sulfometuron-resistant and tested seedlings is given in brackets. Probability values (P) for χ^2 goodness-of-fit analyses are reported for each density (*column*) and distance (*row*)

Sulfometuron resistance

ALS herbicide resistance was common in the resident plants (RP) infesting the donor crop/pasture fields with the resistance frequency being 47% (i.e. proportion of plant genotypes surviving sulfometuron treatment). Little variation was observed in the resistance level among RP samples collected (±5% as standard error). According to Hardy–Weinberg equilibrium, an estimated 27% of the pollen [calculated as $(1-p)^2 = 0.53$], emitted by the RP donor plants, carried a resistant form of the ALS gene. We established that a point mutation of the ALS gene was the basis of ALS herbicide resistance in these donor plants. We found known resistance-endowing mutations in RP individuals at the 197 and 574 loci (see below) displaying 33 and 66% heterozygosity, respectively (Table 4). ALS herbicide resistance was found in 78% of the susceptible mother plants placed within the bushland that produced seed (i.e. 40 seed samples of P1 harvested). In total, 664 seedlings were scored as resistant to sulfometuron. The resistance frequency found in these P1 samples was on average 20% and this indicates the overall rate of pollen-mediated gene flow in this study (Table 1). The mean number of herbicide-resistant seeds produced on bushland-susceptible plants placed at distance from the resistant donors indicates a rapid decline of successful cross-pollination with distance from the source (Fig. 2).

As expected, where there was pollen competition by having paired susceptible plants considerably less sulfometuron resistance was observed compared to a single isolated plant at the same distance (Table 2). The χ^2 test of independence revealed that sulfometuron resistance

frequency was strongly dependent on two factors: density of acceptor plants and distance from pollen source ($P < 0.0001$). The difference of actual values of sulfometuron resistance frequency (gene flow) found in a single plant versus paired plants at each distance was highly significant ($P < 0.0001$). Also, within single or paired plants, a highly significant difference was observed when comparing the frequencies at different distances (Table 2). No statistical difference in sulfometuron gene flow frequency was obtained comparing single plants or paired plants at 100 versus 200 m, respectively (data not shown).

Inheritance of sulfometuron resistance

Some of the resistant P1 plants were grown to produce seed. On average each P1 individual produced 273 ± 46 seeds. These seeds germinated normally and the P2 seedlings were grown outdoors and sprayed at the two-leaf stage with 15 g ha^{-1} of sulfometuron. As the P1 progeny were heterozygous for the resistant ALS gene the progeny P2 were expected to segregate according to a phenotypic ratio of 3:1 (survival:mortality). The evaluation of *P*-values associated with the χ^2 test performed validated the results obtained in the previous herbicide screening on P1 seed samples at each distance (Table 3). The pooled χ^2 value calculated as a 3:1 ratio for the sum of all surviving and dead plants also resulted in $P > 0.1$. The heterogeneity test was not statistically significant, demonstrating that all the families segregated similarly (Table 3). The results obtained with this Mendelian inheritance test confirmed that pollen-mediated gene flow occurred at 3,000 m from the nearest pollen source.

Sequencing of ALS gene

Sequence results demonstrated that three P1 plants carried the Pro197-Ser mutation and two plants carried the Trp574-Leu mutation (Table 4). Partial sequencing chromatogram of ALS fragment amplified by primer pair ALS197F/ALS197R and ALS574F/ALS574R from resistant plants of progeny P1 revealed heterozygosity at the Pro197 codon and the Trp574 codon by the presence of two peaks at each single 197 and 574 site, respectively (data not shown). Heterozygosity at the 197 or 574 mutation site in P1 individual plants was also further verified by using CAPS markers and the presence of resistant allele 197-Ser or 574-Leu was confirmed (Fig. 3a lane 2, 3, 5, b lane 3, 4). The presence of the same ALS-resistant alleles from the resident (RP) plants (Fig. 3a, lane 6, 8, b, lane 6, 7) was also detected by the CAPS markers and confirmed by sequencing. In total, 20 plants were investigated and three of them were found carrying the Trp574-Leu and six the Pro197-Ser mutation (Table 4).

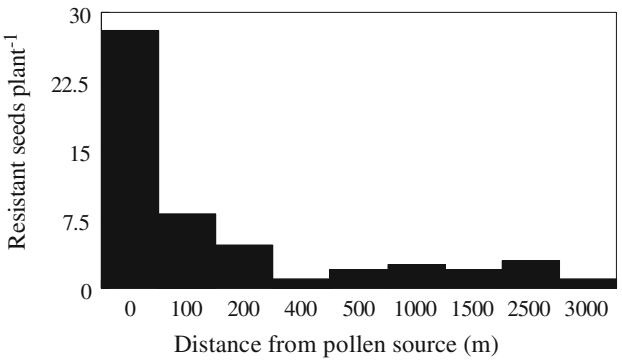

Fig. 2 Mean number of resistant seeds found on susceptible mother plants at different distance from the pollen source

Discussion

Evidence of landscape level pollen-mediated gene flow

In this study individual herbicide susceptible *L. rigidum* mother plants placed in the bushland produced seed progeny that were ALS herbicide resistant. This could only occur by effective cross-pollination from distant resident plants. Overall, in the resident population (RP) 47% of the plants were resistant to the ALS-inhibiting herbicide sulfometuron. Thus, the resident population had a high frequency of ALS resistance and the observed value of 20% sulfometuron resistance frequency in P1 plants gave high confidence on the degree of cross pollination occurring.

Target-site herbicide resistance can be a strong marker that can be detected with a simple herbicide treatment test (Rieger et al. 2002; Watrud et al. 2004). The ALS herbicide screening conducted in this study constituted a clearly discriminating test and the expression of the ALS herbicide resistance trait in P1, and subsequently in P2, was easily quantified. A single herbicide dose identified resistance and thus plant survival was related to pollen-mediated gene flow and herbicide resistance mobility at the landscape level in *L. rigidum* (Table 1). Target-site resistance to ALS-inhibiting herbicides is well known to segregate as a single gene trait (reviewed by Tranel and Wright 2002). This Mendelian analysis in the progeny P2 was definitive in corroborating gene flow occurring at all investigated distances (Table 3). Moreover, the characterization of sulfometuron resistance at the molecular level for both P1 and the RP plants confirmed the unequivocal introgression into susceptible mother plants of two ALS resistant alleles carried by pollen movement from the resistant field source. (Fig. 3, Table 4).

Table 3 Segregation of ALS-inhibiting herbicide resistance at 15 g ha^{-1} of sulfometuron in 18 P2 progenies selected from progeny P1 survivors at representative distances

	Observed phenotype				Expected phenotype			
Distance (m)	Sprayed	Survived	Dead	Survival (%)	Survived (R/R + R/S)	Dead (S/S)	χ^2	*P*
0[a]	429	314	115	73.2	322	107	0.75	0.39
100	36	25	11	69.4	27	9	0.59	0.44
200	33	25	8	75.8	25	8	0.01	0.92
400	31	23	8	74.2	23	8	0.01	0.92
500	34	22	12	64.7	26	9	1.92	0.17
1000	48	37	11	77.1	36	12	0.11	0.74
1500	47	35	12	74.5	35	12	0.01	0.93
2500	28	20	8	71.4	21	7	0.19	0.66
3000	35	22	13	62.9	26	9	2.75	0.10
Pooled	721	523	198		541	180	2.33	0.13
Heterogeneity							4.01	0.91

Chi-square analysis (χ^2) and associated probability values (*P*) are displayed. *S/S* homozygous susceptible, *R/S* heterozygous resistant, *R/R* homozygous resistant

[a] Pooled data of ten samples

Table 4 ALS mutations and resistant alleles identified in sulfometuron resistant plants in the resident population (RP) and in the first progeny (P1) obtained from susceptible mother plants after the field experiment

Distance from pollen source (m)	Mutation identified	Genotype (no. plants)
Resident population		
--	Pro197-Ser	R/S (2)
--	Pro197-Ser	R/R (4)
--	Trp574-Leu	R/S (2)
--	Trp574-Leu	R/R (1)
Progeny P1		
500	Pro197-Ser	R/S (1)
1000	Pro197-Ser	R/S (1)
1500	Trp574-Leu	R/S (1)
2500	Trp574-Leu	R/S (1)
3000	Pro197-Ser	R/S (1)

R/S heterozygous resistant, *R/R* homozygous resistant

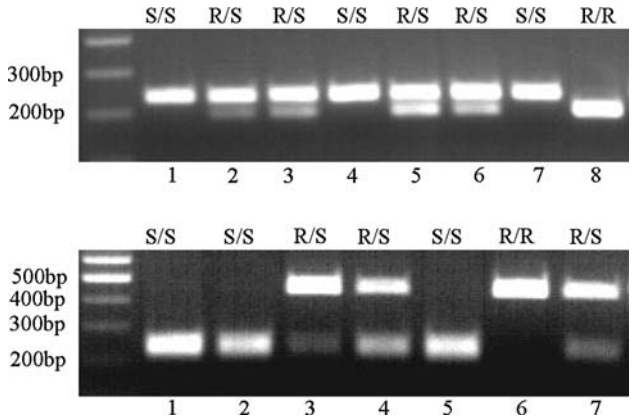

Fig. 3 Cleaved amplified polymorphic sequence (CAPS) analysis. **a** ALS Pro197-Ser mutation in resistant plants of resident population (RP) (*lane 6*, *8*) and in resistant plants of progeny P1 [*lane 2* (3,000 m), *3* (500 m), *5* (1,000 m)]. **b** ALS Trp574-Leu mutation in resistant plants of resident population (RP) (*lane 6*, *7*) and in resistant plants of progeny P1 [*lane 3* (2,500 m), *4* (1,500 m)]. *S/S* homozygous susceptible, *R/S* heterozygous resistant, *R/R* homozygous resistant

Limitation of the study

Lolium rigidum is present across millions of hectares in the Australian wheatbelt and herbicide resistance is frequent (Owen et al. 2007). The presence of resident resistant plants, sometimes at high densities, represented a very large and relatively homogeneous source for production of resistant pollen. Prevailing southerly winds were recorded and this presumably facilitated the drift of viable pollen from the source into the bushland to cross-pollinate other *L. rigidum* plants at distance. Therefore, it is likely that this large source strongly contributed to the observed pollen-mediated gene flow in *L. rigidum* occurring at significant rate over long distances. This is in contrast to small plot or pot studies previously conducted with *Lolium* spp. (Cunliffe et al. 2004; Ghersa et al. 1994; Giddings et al. 1997).

The RP plants were characterized for the presence of the marker (i.e. sulfometuron resistance) at the whole plant and molecular level. Through the Hardy–Weinberg genetic equilibrium the expected proportion of background resistant pollen was estimated. However, the observed sulfometuron resistance frequency in the progeny P1 at each distance was generally lower than 27% and was variable (Table 1). Only the resistance frequencies observed at 0 and 1,000 m were higher or not statistically

different for $\alpha < 0.05$ (Table 1). This could be due to some degree of self-pollination in *L. rigidum*, although it is known to be very low (about 0.1%) (McCraw and Spoor 1983; Vila-Aiub, unpublished), and the rate of self-fertilization proportionally more important as the external pollen availability declines. A distinct possibility is that some cross-pollination among introduced susceptible bushland-spaced plants may also have occurred.

The large crop land area (source of donor plants) is a landscape in which *L. rigidum* is naturalized in crop/pasture fields. This environment is characterized by low rainfall (350 mm per year) and such meteorological conditions of low rainfall and low relative humidity can be detrimental to pollen viability and longevity (Ahloowalia 1973; Luna et al. 2001). Therefore, perhaps as expected, the production of seeds on isolated plants in bushland was low (some plants producing less than ten seeds). However, notwithstanding these limitations it is clear that effective pollen flow and cross-pollination occurred to considerable distance into the bushland. It is emphasized that anthesis almost perfectly overlapped between the resident resistant field donor plants and the introduced susceptible bushland plants.

Pollen competition and modeling applications

Pollen-mediated gene flow at distance must be lower than near the source because the majority of pollen is deposited within a few meters from the source (van Treuren et al. 2006). Most studies report a negative exponential distribution with varying degree of leptokurtosis (Levin and Kerster 1974; Pfender et al. 2007). However, there are important factors influencing pollen-mediated gene flow. These include the magnitude and abundance of the source, the isolating distance, the size of the sink as number of acceptor plants, the environment and the presence of pollen competition (Rognli et al. 2000). Little information is available on the extent to which gene flow is affected by pollen competition among acceptor plants (Rognli et al. 2000, Baker and Preston 2006). We used single isolated plants to assess the maximum potential of a *L. rigidum* plant to be pollinated at distance and therefore, except for the paired plants, there was not pollen competition among acceptor plants. Much less gene flow was evident when there was paired versus single plants at each distance examined. Also, as expected, the distance from the pollen source was a significant factor overall. However, with presence of pollen competition, gene flow at distances greater than 100 m was extremely low and significant differences were not noticeable between 100 m and 200 m (data not shown). The results are consistent with the existing literature (Baker and Preston 2006) although, we suggest specific studies should be performed to better understand the dynamics of pollen competition and impact on pollen-mediated gene flow. Those studies should be complemented by modeling simulations of resistant pollen migration and subsequent establishment of resistant plants (Devaux et al. 2005).

Simulation models on herbicide resistance dynamics in weed populations give no or little attention to the movement of resistance genes at distance (reviewed by Diggle and Neve 2001). Diggle et al. (2003) and Roux et al. (2008) discuss the importance of genetic isolation in herbicide resistance modeling but, do not consider pollen-mediated gene flow occurring at significant frequencies at long distance. As there is an extensive literature on key factors associated with herbicide resistance evolution in *L. rigidum* we believe this plant could be used as a model cross-pollinated species to investigate the contribution of long-distance gene flow on evolutionary dynamics of herbicide resistance and its subsequent dispersion at the landscape level.

Implications of long distance pollen-mediated gene flow in *L. rigidum*

This study establishes that herbicide resistance mobility over long distances by pollen drift is a reality in cross-pollinated *L. rigidum*. Therefore, herbicide resistance in this species can move considerable distances within and across farm enterprises. Given this mobility, growers may be reluctant to invest in herbicide resistance prevention (Llewellyn and Allen 2006). This would be detrimental to herbicide sustainability in this agro-ecosystem, especially for such herbicides as glyphosate and paraquat which still provide excellent, inexpensive weed control, and to which a relatively small number of *L. rigidum* populations have evolved resistance. In the context of global agriculture this can be relevant, especially, where GM glyphosate-resistant crops are grown.

Theoretically all the different strategies for weed management that keep weed density low in the cropped fields could be effectively used for gene flow management. For example, a more competitive and dense crop can reduce weed seed production and minimize pollen-mediated gene flow (Goggi et al. 2007; Murray et al. 2002). However, the control of single plants or small patches of *L. rigidum* volunteers in marginal areas within the agricultural landscape (fence line or fire breaks) should also be regarded as important to prevent gene introgression from long distances. This study reaffirms the importance of integrated management practices and pre-emptive strategies to delay herbicide resistance evolution, sustain herbicide efficacy, and thus prevent herbicide resistance gene flow. These strategies would be more effective if applied at the landscape level rather than by individual farmers.

This study documented pollen-mediated gene flow to considerable distance at the landscape level in the important weed species *L. rigidum*. The results are relevant to theoretical concepts on herbicide resistance evolution, in validating new approaches in dispersal simulation modeling and promoting strategies for herbicide resistance management.

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