Research Article

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2,4-D resistance due to a deletion in *IAA2* in *Sisymbrium orientale* L. carries no apparent fitness penalty

Jenna M Malone,[a*] Hue Thi Dang,[a] Marcelo RA de Figueiredo,[b] Gurjeet Gill,[a] Todd A Gaines[b] and Christopher Preston[a]

Abstract

BACKGROUND: A deletion mutation in the degron tail of auxin coreceptor *IAA2* was found to confer resistance to the herbicide 2,4-D in *Sisymbrium orientale*. Given the importance of auxin signalling in plant development, this study was conducted to investigate whether this deletion mutation may affect plant fitness.

RESULTS: The F_2 progeny of crosses with two resistant populations P2 (P2♂ × S♀) and P13 (P13♂ × S♀) were used in this study. The F_2 plants were grown under competition with wheat in pot-trials and evaluated for biomass and total seed production. Progeny of the F_2 plants were phenotyped by application of 250 g a.e. ha^{-1} 2,4-D and genotyped for the 27 bp deletion in *IAA2*. In a separate experiment, F_4 and F_5 recombinant inbred lines (RILs) with resistant and susceptible phenotypes were grown in monoculture and phenotyped over time for biomass and seed production. Wheat competition reduced biomass and seed production for all genotypes in each population and in each year. A density of 400 plants m^{-2} of wheat reduced *Sisymbrium orientale* biomass by 76–78% in 2016 and by 59–63% in 2017 and total seed production by 80–83% and 60–64% by respective year. For the experiment involving resistant and susceptible RILs, biomass accumulation and seed production were the same between resistant and susceptible for both populations.

CONCLUSIONS: The 27 bp deletion in *IAA2* in *Sisymbrium orientale* does not carry a measurable fitness penalty, as determined by biomass reduction or seed production, either in monoculture or in competition with wheat. As a result, this mutation is unlikely to decrease over time in weed populations if 2,4-D selection pressure were removed.

Keywords: fitness cost; 2,4-D resistance; IAA2 deletion; herbicide resistance

1 INTRODUCTION

Individual plant fitness will dictate the proportion of the future population that will be the progeny of different genotypes.[1] Individual plant fitness is determined by the influence of the collection of traits present in that plant relative to the traits in competing individuals in the population.[2] Traits that provide higher fitness in the environment where the plant population occurs will increase in frequency in the population through increased relative reproduction rate, whereas traits that reduce fitness should decrease in frequency in the population. A change in the environment can favour individuals in the population with different traits leading to a change in the direction of evolution.[3]

Herbicide use can result in a catastrophic change to the environment for target weed species, leading to high mortality and greatly favouring those individuals able to survive the herbicide and reproduce.[4,5] This leads to rapid evolution of herbicide resistant weeds.[6] A general assumption is that herbicide resistance traits are initially very rare because they carry a fitness penalty, otherwise resistant individuals would be common in populations when the herbicide was first used.[7] Understanding the fitness penalty of specific mutations providing resistance is valuable as it is linked to the initial frequency of resistance alleles in populations prior to the herbicide being used, which influences how rapidly resistance can be selected.[6] Also, fitness penalties associated with herbicide resistance traits can, if large enough, be exploited with management practices, such as crop competition, to aid management of resistant populations.[8]

Target site resistance to the photosystem II (PS II) inhibiting triazine herbicides results in about 20% reduction in growth and seed production of plants carrying this trait.[9] Fitness loss was due to reduced electron transfer as a result of the mutation.[10] Fitness costs

* *Correspondence to: J Malone, School of Agriculture, Food and Wine, University of Adelaide, PMB1, Glen Osmond, South Australia 5064, Australia. E-mail: jenna.malone@adelaide.edu.au*

a *School of Agriculture, Food and Wine, University of Adelaide, PMB1, Glen Osmond, Australia*

b *Department of Agricultural Biology, Colorado State University, Fort Collins, CO, USA*

of mutations in other target sites vary from not measurable to a 67% reduction in biomass.[11] In addition to target site mutations, resistance can be due to non-target site mechanisms. In many cases, these non-target site mechanisms have been recorded as having unmeasurable to modest fitness penalties.[12–16]

Auxin herbicides such as 2,4-D are selective in grasses mostly due to differential metabolic detoxification[17] and examples of enhanced metabolic detoxification for resistance to auxin herbicides have been identified in weeds.[18,19] Due to the crucial nature of auxins in plant growth and development, it has been regularly suggested that target site mutations providing resistance to the auxin herbicides would carry a large fitness penalty.[20–22] Indeed, a *Kochia scoparia* population with a mutation in the degron domain of *IAA16*, had reduced growth and 80–90% less seed production compared to plants without this mutation.[23] Likewise, a point mutation in *IAA7* in an Arabidopsis mutant carries a 90% fitness penalty.[24] Additional mutations in the degron domain of auxin/indole-3-acetic acid (Aux/IAA) genes have been recently reported in weeds, including the *IAA16* gene in *Chenopodium album*[25] and a splicing shift causing a degron mutation in the *IAA16* gene in *K. scoparia*,[26] as well as a double deletion flanking the degron domain of the *IAA20* gene in *Sonchus oleraceus*.[27] Recently, another type of target site mutation in *IAA2* of *Sisymbrium orientale* from South Australia has been identified. Resistant plants in several populations carry a 27 bp deletion in auxin coreceptor Aux/IAA2, which removes nine amino acids in the degron tail, that is not present in susceptible plants.[28] The degron region is involved in auxin-responsive degradation of multiple Aux/IAA proteins which is essential for plant growth and development.[29]

In this case, the resistant plants show no obvious reduction in growth.[30] The aim of this study was to determine potential fitness costs of the 27 bp deletion in *IAA2* of *Sisymbrium orientale*.

One of the challenges in accurately determining fitness costs of herbicide resistance genes is the differences between resistant and susceptible individuals due to geographic or other management selection. It is essential to reduce or remove these effects by being able to select resistance and susceptible plants with similar genetic backgrounds.[31] Here we have used the F_2 progeny of crosses between resistant and susceptible *Sisymbrium orientale* plants in a competitive neighbourhood experiment with wheat, as well as F_4 and F_5 recombinant inbred lines (RILs) in a monoculture experiment, to examine the potential fitness cost of the *IAA2* 27 bp deletion.

2 MATERIALS AND METHODS

2.1 Plant materials

The two populations of 2,4-D-resistant *Sisymbrium orientale* used in this study, P2 and P13 were collected from near Port Broughton, South Australia, Australia. The susceptible population originated from Roseworthy, South Australia, Australia, about 120 km away from the resistant populations.[30] P2 and P13 plants had previously been found to be more than 20-fold resistant to 2,4-D compared to susceptible plants[30] and contain a deletion in the degron tail of *IAA2*.[28] Seeds of two F_2 families, P2.1 (P2 × S♀) and P13.1 (P13♂ × S♀), generated from previous studies[30] were used in the competition experiment. Briefly, two biparental crosses were made using P2 and P13 as the male parent and the susceptible as the female parent for both crosses. The F_1 progeny were self-pollinated to produce F_2 progeny used in the study.

Seeds of F_4 RILs (derived from the P13♂ × S♀ cross) and F_5 RILs (derived from the P2♂ × S♀ cross) were used in the monoculture experiment. The F_2 individuals from earlier were self-pollinated to produce F_3 progeny, and homozygous resistant and homozygous susceptible F_3 lines were inbred via single-seed descent to create RILs.[28] Resistant and susceptible RILs were phenotyped using 2,4-D and genotyped to ensure uniformity for presence (resistant) and absence (susceptible) of the 27 bp mutation in *IAA2*.

2.2 Fitness cost assessment in F_2 segregating populations

2.2.1 *Competition experiment*

A neighbourhood model was used where all *Sisymbrium orientale* F_2 progenies were evaluated as the target plants while wheat at different densities was the neighbour providing interspecific competition as described in Dang *et al.*[32] Briefly, seeds of *Sisymbrium orientale* were sown into trays containing standard potting mix.[33] At the 3–4 leaf stage, seedlings were transplanted in the middle of an 8.5 L round pot (30 cm diameter × 30 cm high) with one plant (target) per pot, which equates to a constant weed density of 20 plants m^{-2}. Seven densities (0, 20, 40, 60, 120, 200 and 400 plants m^{-2}) of wheat seeds (neighbour) were sown and spatially arranged with each neighbour equidistant from the target plant.

The experiments were conducted outdoors in the normal winter growing season for this species (May–November) in 2016 and 2017 at the Waite Campus of the University of Adelaide (34° 58′13.5″S 138°38′22.7″ E). Monthly rainfall and average monthly minimum and maximum temperatures from the nearest weather station for the experimental period for each year are listed in Table 1. There were 16 replicate *Sisymbrium orientale* plants for each wheat density. Plants were randomly selected from the germinated F_2 population to ensure representatives of the different genotypes, resistant, heterozygote and susceptible, were present for each wheat density. Pots were arranged in a completely randomised design in an outdoor bird-proof net house and watered

Table 1. Rainfall and temperature during May–November in 2016 and 2017 when the experiments were being conducted

	Monthly rainfall (mm)		Monthly average maximum temperature (°C)		Monthly average minimum temperature (°C)	
Month	2016	2017	2016	2017	2016	2017
May	88	51.2	19.8	19.2	12.4	9.4
June	95.2	8.6	16.0	17.0	8.9	5.7
July	112	91.2	15.3	16.4	8.1	8.1
August	58.8	87.8	17.7	15.9	7.7	7.9
September	131.2	56.0	17.4	19.4	9.1	10.7
October	81	36.8	21.0	24.3	10.6	12.3
November	33.2	23.2	24.7	28.0	12.1	17

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as required to avoid water-stress for the plants, especially at the later stages when plants reached full-growth.

2.2.2 Competition data collection and analysis

The aboveground biomass of target plants (*Sisymbrium orientale*) was harvested and oven-dried at 65 °C until reaching a constant weight at maturity. Total biomass, total seed weight, and 1000-seed weight by each target plant were recorded. The number of seeds was calculated from total seed weight and 1000-seed weight. A hyperbolic non-linear model was used to analyse the data to describe the response of the *Sisymbrium orientale* plants to increasing density of the neighbour plants for biomass production and the number of seeds of target plants using the following equation based on Park *et al*.[34]

$$Y = C\,(1+ax)^{-b}$$

where Y represents the biomass or seed number of the target plant at neighbour density x, C is the biomass or seed number of the target plant in the absence of competitors (neighbours) ($x = 0$) and a and b are parameters. Data from each year and each population was analysed separately. The curves fitted to the three genotypes for each population in each year were compared with a sums of squares F test and where the result was not significant a single curve representing all three biotypes was plotted.

2.2.3 Target plant genotyping – herbicide susceptibility assessment

Seeds produced by the target plants were germinated by sowing directly onto the soil surface of 9.5 cm × 8.5 cm × 9.5 cm punnet pots (Masrac Plastics, Dry Creek, Australia) and grown outdoors at the Waite Campus, University of Adelaide, Australia. At the four-leaf stage, plants were treated with a single rate of 250 g a.e. ha^{-1} 2,4-D (2,4-D amine 650, FMC). Herbicide was applied using a laboratory moving boom pesticide applicator and applied at an equivalent of 109 L ha^{-1} of water at a pressure of 250 kPa and a speed of 1 m s^{-1} using Tee-Jet 001 nozzles (Tee-Jet 8001E; Spraying Systems Co., Wheaton, IL, USA). Leaf petiole curl was used to assess the resistance status of the seedlings 3 days after treatment.[35] The target parent plant was determined to be homozygous resistant if all progeny showed no petiole curling and were therefore all resistant; heterozygous if there were a mixture of both curled (susceptible) and unaffected (resistant) progeny; and susceptible if all progeny showed curling.[36]

2.2.4 Target plant genotyping – genetic assessment

The genotype of the target plants was also confirmed via a genetic assay using the presence or absence of the IAA2 deletion mutation. Genomic DNA was extracted from a pooled sample of tissue from around 30 progeny seedlings from each individual target plant using the DNeasy 96 Plant kit (Qiagen, Clayton, Victoria, Australia) according to the manufacturer's instructions. Phire Green Hot Start II PCR Master Mix (Thermo Scientific, Waltham, MA, USA) was used to amplify a fragment of the *IAA2* gene, producing a 239 bp fragment in wild-type samples and a 212 bp fragment in resistant samples with the deletion mutation[28] to be used as a genotype maker. Polymerase chain reaction (PCR) of 20 μL contained 1× Phire Green Hot Start II Master Mix (containing 1.5 mM magnesium $chloride_2$, 200 μM of each dNTP and Phire Hot Start II DNA polymerase), 0.5 μM of each gene-specific primer (forward primer 5′-AACTCAAATCGTTGGTTGGC-3′ and reverse primer 5′-CTTTATCCTCGTACGTTGGTACG-3′) and 0.5 μL of genomic DNA. Amplification was carried out in an automated DNA thermal cycler (GeneTouch; Bioer Technology, Binjiang, Hangxhou, China) with PCR conditions of: initial denaturing at 98 °C for 30 s; 35 cycles of denaturation at 98 °C for 5 s, annealing at 58 °C for 5 s and elongation at 72 °C for 10 s, followed by a final extension for 1 min at 72 °C. PCR products were loaded into 3% agarose gels and electrophoresed in 1× TAE buffer (40 mM of Trizma base, 1 mM of Na_2EDTA, pH to 8 with glacial acetic acid) at 100 V and photographed under ultraviolet (UV) light ($\lambda = 302$ nm). A low-molecular weight marker (HyperLadder 25 bp; Bioline) was included, as well as DNA fragment of IAA2 known to be either wild-type (239 bp) or contain the deletion (212 bp).

2.3 Fitness cost assessment in resistant and susceptible recombinant inbred lines

To evaluate potential fitness penalty in *Sisymbrium orientale* related to 2,4-D resistance, studies on growth and seed production were performed on RILs derived from crosses between P2 and the susceptible population and P13 and the same susceptible population.[28] There were six replicates of susceptible and resistant individuals from the F_5 RILs of P2, consisting of two independent susceptible RILs and two independent resistant RILs with three biological replicates each. The same replication was used for F_4 RILs of P13 (two RILs of resistant and susceptible with three biological replicates each). The RILs were checked to ensure they were homozygous for the wild-type (susceptible) or homozygous for the 27 bp deletion (resistant) in *IAA2* using PCR as described earlier. Destructive evaluations were done at 21, 28, 35, 42, 49, 56, 63, 70, 84 and 98 days after plant emergence, with three individual biological replicates for each RIL evaluated at each time point. Plants were initially planted in 10 cm × 10 cm square pots until the phenological stage of 4 developed leaves. Plants were then transplanted into 4 L pots (15 cm diameter × 21 cm high) containing potting soil with vermiculite (volume 1:1). The plants were cultivated under glasshouse conditions at the Colorado State University Weed Research Laboratory at temperature of 25 °C, relative humidity of 75%, fertilised every 15 days with 10 g of NPK (nitrogen–phosphorus–potassium) 15:15:15, and watered twice per day. Plants of each line and phenotype were randomly selected for dry mass measurement, roots were washed to eliminate substrate and the entire plant was dried at 60 °C for 72 h, and the dry plant material was weighed. To estimate seed production from each biotype, plants were harvested when fully mature at 126 days after germination. Siliques were collected, opened, and seeds were cleaned using sieves. The calculation of seed production was based on 100-seed weight of each plant. Dry mass accumulation data were analysed using a log-logistic model[37]

$$Y = (M_0 K)/(M_0 + (K - M_0)e^{-rt}))$$

where M_0 is the initial biomass, r is the increase in biomass per unit time (t), and K is a maximum mass obtained. The curves fitted to the two genotypes for each population were compared with a sums of squares F test and where the result was not significant a single curve representing both genotypes was plotted. A t-test was performed on seed production data to compare differences between *IAA2* genotypes.

Table 2. Number of parent plants of *Sisymbrium orientale* grown in 2016 and 2017 assigned to each genotype for each population along with the *G* statistic and probability (*P*) for fit to a 1:2:1 ratio for a single gene effect

Year	Population	Susceptible	Heterozygote	Resistant	*G* Statistic	*P*
2016	P2	26	58	28	0.219	0.90
	P13	37	45	24	5.22	0.07
2017	P2	28	51	18	2.45	0.29
	P13	27	63	14	8.88	0.01

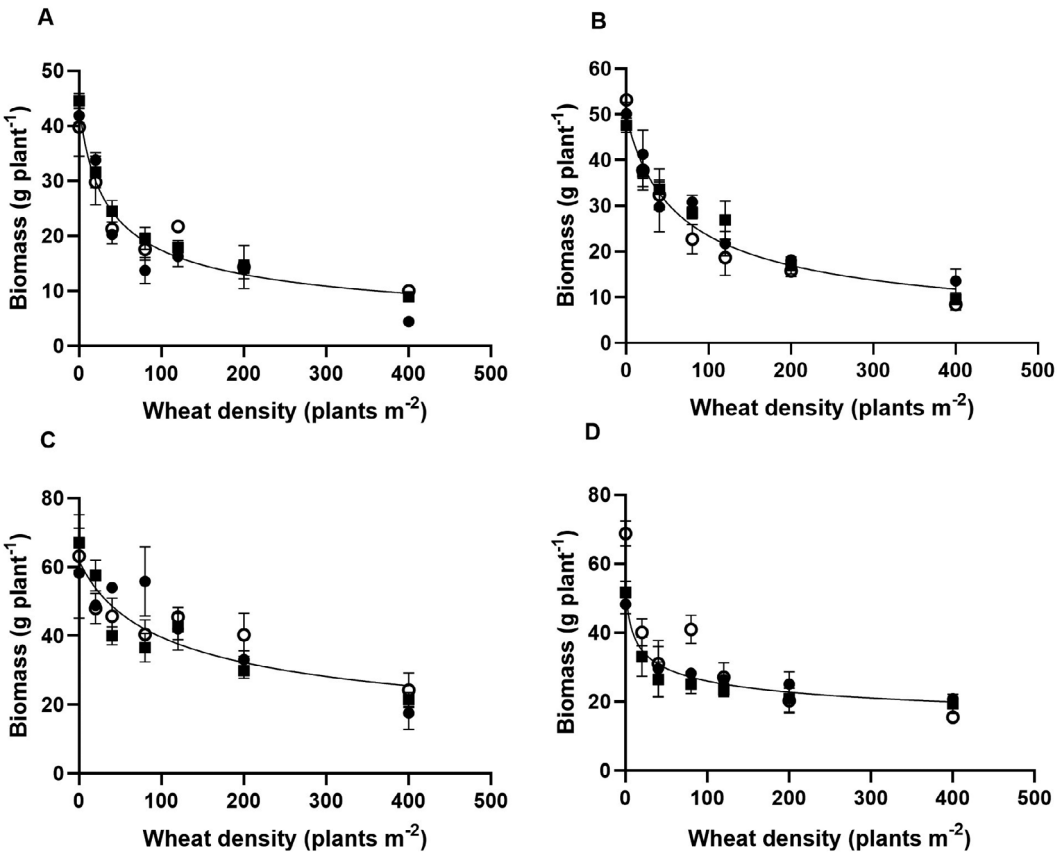

Figure 1. Biomass production for susceptible (○), heterozygotes (■), and resistant (●) genotypes of *Sisymbrium orientale* as influenced by wheat density for population P2 (A and C) and P13 (B and D) in 2016 (A and B) and 2017 (C and D). The curves plotted are the joint response of the three genotypes (Table 3).

Table 3. Parameters fitted to the hyperbolic equation $Y = C\,(1 + ax)^{-b}$ for plant biomass for each population of *Sisymbrium orientale* in each year and the probability (*P*) of the *F* test for whether the curves for the three genotypes are the same

Year	Population	*P*	*C*	*a*	*b*
2016	P2	0.36	43.8	0.061	0.469
	P13	0.32	49.5	0.025	0.603
2017	P2	0.16	61.9	0.021	0.400
	P13	0.06	54.6	0.442	0.195

3 RESULTS

3.1 Target plant genotyping

Individual target (parent) plants were genotyped by the response of their progeny to 2,4-D and the presence or absence of the deletion using PCR on a pooled sample of seedlings. The genotype results for both the spray test and the PCR test matched, showing that either could be used to determine the parent phenotype. Some plants could not be genotyped due to poor seed viability and were removed from the analysis.

The numbers of each genotype determined for each population and year are listed in Table 2. For both populations in 2016 and for P2 in 2017 the proportion of homozygous susceptible,

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Table 4. Parameters fitted to the hyperbolic equation $Y = C(1 + ax)^{-b}$ for total seed production for each population of *Sisymbrium orientale* in each year and the probability (*P*) of the *F* test for whether the curves for the three genotypes are the same

Year	Population	*P*	*C*	*a*	*b*
2016	P2	0.30	52 929	0.103	0.443
	P13	0.43	43 200	0.010	1.148
2017	P2	0.21	36 228	0.041	0.359
	P13	0.07	20 340	0.389	0.183

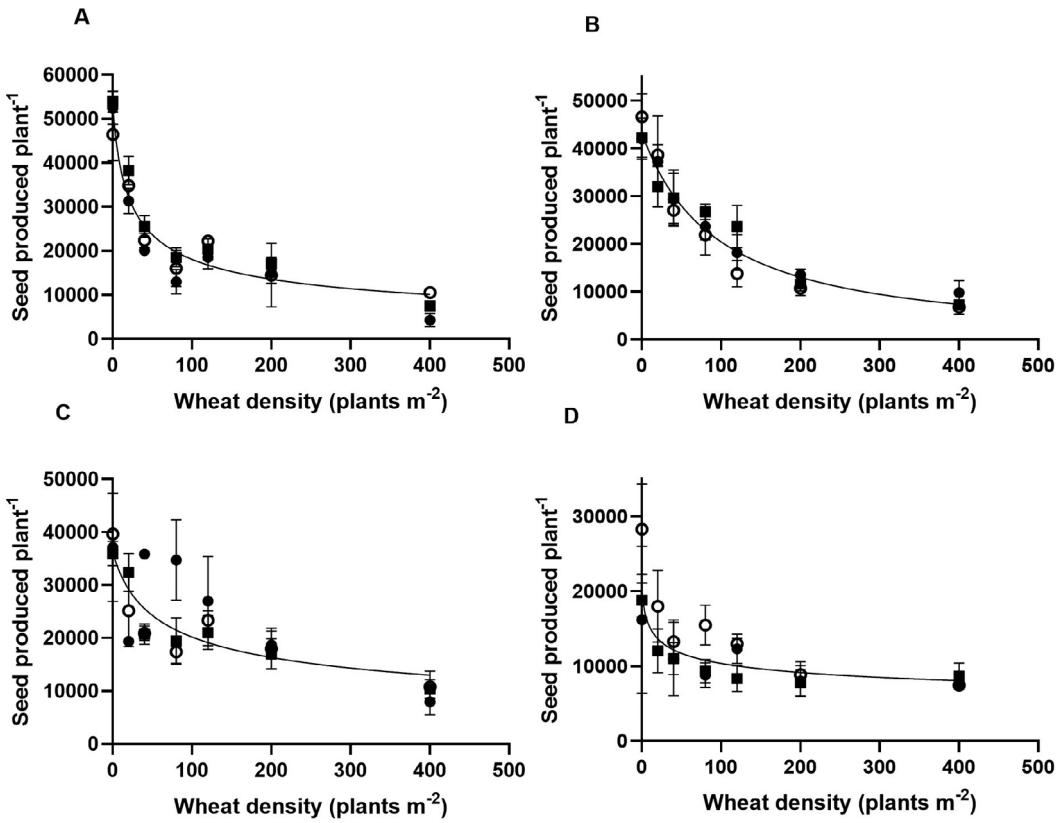

Figure 2. Total seed production for susceptible (○), heterozygotes (■), and resistant (●) genotypes of *Sisymbrium orientale* as influenced by wheat density for population P2 (A and C) and P13 (B and D) in 2016 (A and B) and 2017 (C and D). The curves plotted are the joint response of the three genotypes (Table 4).

heterozygous and homozygous resistant individuals fitted a 1:2:1 ratio (Table 2). However, for population P13 in 2017, there were fewer homozygous resistant individuals than expected.

3.2 Competitive responses of *Sisymbrium orientale* genotypes to wheat

For both populations and in both years increasing the number of neighbouring wheat plants reduced the biomass of *Sisymbrium orientale* plants (Fig. 1). There was no significant difference in the curves for the three genotypes for either population or in either year (Table 3). The highest wheat density of 400 plants m^{-2} reduced biomass of *Sisymbrium orientale* plants by 76–78% in 2016 and by 59–63% in 2017 (Fig. 1).

Total seed production of individual *Sisymbrium orientale* plants in the absence of competition from wheat ranged from 43 000 to 53 000 in 2016 but was lower in 2017 (Table 4). Wheat competition greatly reduced seed production of *Sisymbrium orientale* in both years (Fig. 2). At 400 plants m^{-2} of wheat, seed production of *Sisymbrium orientale* was reduced by 80–83% in 2016 and by 60–64% in 2017. There was no difference between the three *Sisymbrium orientale* genotypes in the response of seed production to increasing wheat competition for either population or in either year (Table 4) and a single curve described the response for each population in each year (Fig. 2).

3.3 Monoculture recombinant inbred line growth analysis

Biomass of resistant and susceptible F_4 and F_5 RILs were measured over a time course of 21 days to 98 days after emergence. The accumulation of biomass over time for the susceptible and resistant F_5 RILs from the cross between P2 and the susceptible population fitted a single curve ($P = 0.37$) with a maximum of 79 g $plant^{-1}$ (Fig. 3). Likewise, accumulation of biomass for the susceptible and resistant F_4 RILs from the cross between P13 and the

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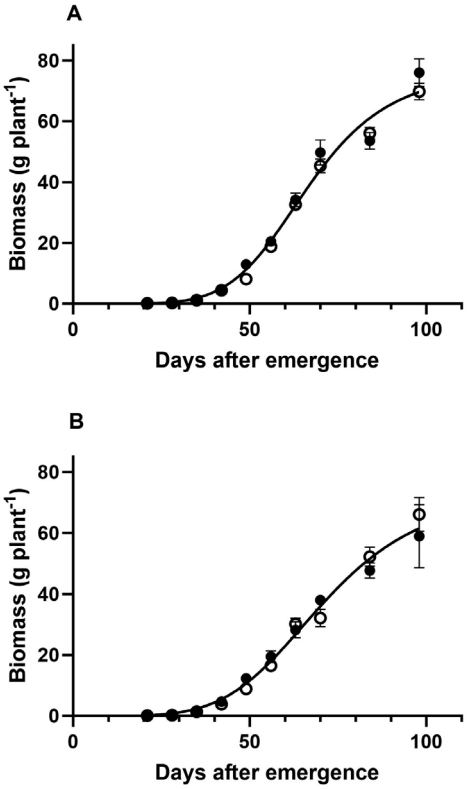

Figure 3. Time course evaluation of plant dry biomass for resistant (●) and susceptible (○) F_5 recombinant inbred lines (RILs) for crosses between the susceptible population and P2 population (A) and for resistant and susceptible F_4 RILs for crosses between the susceptible population and P13 population (B) with six replicates. The resistant RILs are homozygous for a 27 bp mutation in IAA2, while susceptible RILs are homozygous for the wild-type allele.

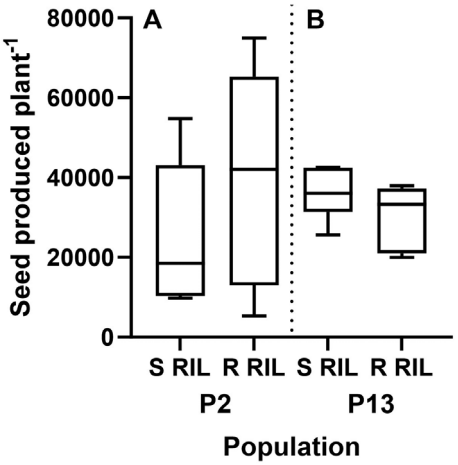

Figure 4. Seed production in resistant (R) and susceptible (S) F_5 recombinant inbred lines (RILs) for crosses between the S population and P2 population (A) and for R and S F_4 RILs for crosses between the S population and P13 population (B) with six replicates. The R RILs are homozygous for a 27 bp mutation in *IAA2*, while S RILs are homozygous for the wild-type allele. For seed production, plants were harvested 126 days after emergence. The line across the box represents the median value with the box lower limit being the 25th percentile, the box upper limit being the 75th percentile and the whiskers are the minimum and maximum values. No difference was found in total seed production between R and S.

susceptible population fitted a single curve ($P = 0.16$) with a maximum of 75 g plant^{-1}.

Seed production at 126 days after emergence was not significantly different ($P = 0.30$) for the susceptible and resistant F_5 RILs from the cross between P2 and the susceptible population with 25 000 to 40 000 seeds produced per plant (Fig. 4). For the susceptible and resistant F_4 RILs from the cross between P13 and the susceptible population seed production was also not different ($P = 0.22$) with between 30 000 and 36 000 seeds produced per plant.

4 DISCUSSION AND CONCLUSIONS

The fitness penalty against herbicide resistance alleles is an important factor in maintaining these alleles at low frequencies in the absence of herbicide use.[6,7] Alleles that carry a high fitness penalty will occur at lower frequencies than those with smaller fitness penalties.[6] There can be considerable difficulties in identifying a fitness penalty for herbicide resistance in weed populations, due to the different selection history of the resistant and susceptible populations chosen.[31] Not managing for genetic differences between populations can result in flawed conclusions. Here we have used segregating F_2 populations and RILs that have allowed us to remove the effect of the background genetic variation.

In the competition experiments, wheat, as expected reduced both biomass and seed production of the *Sisymbrium orientale* populations (Figs 1 and 2). Despite the dramatic reduction in biomass and seed production caused by wheat competition, there was no difference between the three genotypes in their response to competition for either population and in either year (Tables 3 and 4). Both populations of *Sisymbrium orientale* are resistant to 2,4-D due to a 27 bp deletion in *IAA2*.[28] The lack of a fitness penalty was confirmed in growth experiments on F_4 and F_5 RILs (Figs 3 and 4). The lack of a fitness penalty resulting from the 27 bp deletion in *IAA2* contrasts with the large reduction in seed production in *K. scoparia* and *Arabidopsis thaliana* mutants resistant to auxinic herbicides through point mutations in the degron region of IAA genes.[23,24]

Despite causing a reduction in auxin binding affinity,[28] the 27 bp deletion in *IAA2* in *Sisymbrium orientale* did not cause any apparent fitness penalty when plants were grown either alone or in competition with wheat. One potential explanation for the lack of reduced fitness is that the *IAA2* gene in *Sisymbrium orientale* has relatively low expression in an untreated condition and is transiently induced in resistant plants after treatment with 2,4-D.[28] If a different type of stress occurred that activated auxin biosynthesis and induced *IAA2* expression, a fitness penalty due to reduced auxin binding affinity might manifest. The presence of the degron tail mutation in what appears to be an auxin treatment-inducible Aux/IAA gene may provide a selective balance between the fitness advantage of 2,4-D resistance and any potential negative effects due to reduced auxin binding as *IAA2* may not be an important regulator of plant growth and development under typical conditions. The lack of a fitness penalty associated with this resistance mutation suggests that the mutation frequency would be unlikely to decrease in populations with high resistant allele frequency if 2,4-D selection pressure were removed for several generations, similar to the results from a multi-generational fitness cost experiment using several different resistance mechanisms in *Amaranthus tuberculatus*.[16]

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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