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Inheritance of 2,4-D resistance traits in multiple herbicide- resistant *Raphanus raphanistrum* populations

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ABSTRACT

A relatively low number of weed species have evolved resistance to auxinic herbicides despite their use for almost 70 years. This inheritance study with two *Raphanus raphanistrum* populations multiple-resistant 2,4-D and the ALS-inhibiting herbicide chlorsulfuron determined the number of genes and genetic dominance of 2,4-D resistance and investigated the association between traits conferring resistance to the two herbicide modes of action. Levels of 2,4-D phenotypic resistance and resistance segregation patterns were assessed in parental populations, F_1 and F_2 families.

2,4-D resistance in *R. raphanistrum* populations is mostly conferred by nuclear-inherited highly dominant trait(s) segregating at one single locus not closely linked to the *ALS* gene, and with minor interference from other loci. The inheritance patterns observed are highly consistent with the unravelled physiological basis of 2,4-D resistance shown in *R. raphanistrum*. Monogenic and highly dominant resistance mechanisms with simple nuclear inheritance can evolve in weeds despite the complexities associated with the selecting herbicide modes of action.

As there is an increased pressure on auxinic herbicides due to the rapid adoption of 2,4-D-resistant transgenic crops future research aimed at reducing the evolution of auxinic herbicide resistance in weeds should unravel the genetic basis of resistance, exploit possible pleiotropic effects of those resistance traits and minimize the spread of multiple-resistant populations by deploying effective herbicidal and non-herbicidal solutions that maximize selection heterogeneity.

1. Introduction

In modern agriculture, the efficacy, reliability and relative low cost of herbicides mean that herbicide use is almost universal to achieve effective weed control and protect crop yield from potentially severe losses [1]. However, over-reliance on herbicides to control large weed populations often results in strong selective pressure which progressively leads to herbicide resistance evolution [2,3]. For example, single nucleotide mutations leading to single-gene modification of an herbicide target-site enzyme can endow herbicide resistance. Such mutations can be rapidly enriched in weed populations under continuous herbicide selection pressure and have been frequently reported as the mechanistic basis of herbicide resistance in weed species [reviewed by [4]]. Target-site resistance (TSR) is well understood at the molecular level and has rapidly evolved in weeds in response to strong selective pressure applied with ACCase-, ALS- and PSII-inhibiting herbicides and others [reviewed by [5,6–9]].

Conversely, non-target-site resistance (NTSR) mechanisms are much less understood. In general, NTSR is endowed by mechanisms that minimize herbicide injury by decreasing the herbicide concentration at its site(s) of action to non-toxic levels. For example, NTSR can be a morphological plant trait (e.g. cuticle thickness) which causes differential herbicide retention or foliar uptake between susceptible and resistant plants [10,11], or more often a physiological change due to up- or down-regulation of constitutive enzymatic families involved in herbicide detoxification and metabolism (e.g. P450, GST, GT enzymes) and/or transporters that can reduce herbicide translocation and/or mediate sequestration (e.g. ABC transporters) [12,13]. In cross-pollinated species such as *R. raphanistrum* multiple traits conferring TSR and NTSR can often be selected and co-exist in weed populations displaying complex patterns of cross- and multiple-resistance [14].

The synthetic auxin herbicide 2,4-D was developed in the 1940's and was the first synthetic herbicide to be used to control dicot weeds in cereal crops [15]. Auxinic herbicides are categorized in different chemical classes including phenoxycarboxylic acids, benzoic acids, pyridinecarboxylic acids, aromatic carboxymethyl deriva-

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tives and quinolinecarboxylic acids [reviewed by [16]]. After several decades of use, resistance has evolved in at least 27 broadleaf and five grass weed species, which is a relatively low number compared to the hundreds of weed populations that have evolved resistance to ACCase- and ALS-inhibiting herbicides [17]. This can be attributed to several factors but it is possible that the initial frequency of resistance to auxinic herbicides is low, the resistance mechanism(s) do not confer high levels of resistance to plants [18], and/or the pleiotropic effects of auxinic herbicide resistance traits may impose a reduction in plant fitness [19].

Synthetic auxinic herbicides interfere with numerous plant pathways and cause complex biochemical and physiological responses in susceptible plants as they mimic the natural plant hormone auxin (indole-3-acetic acid) [16]. Thus far, several putative TSR and NTSR mechanism(s) have been reported to endow resistance to these herbicides, although the exact definition of the complex mechanistic basis of resistance to auxinic herbicide remains to be fully elucidated [20,21].

NTSR traits endowing reduced translocation of the auxinic herbicide 2,4-D have been similarly reported in the two weed species *Raphanus raphanistrum* L. and *Lactuca serriola* L. [22,23]. Specifically in *R. raphanistrum* (in the population designated as R_2 in the current study), loss of function of a plasma membrane ABCB-type auxin transporter was hypothesised as the mechanism inhibiting long-distance transport of 2,4-D, and another minor resistance mechanism (currently unknown) was also detected [23]. Despite putatively weak 2,4-D resistance mechanisms, a recent (2010) randomly-conducted geographical survey of circa 500 Western Australian crop fields indicates that the frequency of 2,4-D resistance in *R. raphanistrum* populations is widespread (>70% of 2,4-D tested seed samples) [24].

Inheritance studies allow define the eco-evolutionary context in which herbicide-resistant weeds evolve [25], improve our general understanding of the intrinsic diversity of traits conferring herbicide resistance and support simulation modelling predictions [26,27].

Thus, the aim of this classical Mendelian inheritance study with 2,4-D resistant *R. raphanistrum* was to determine the number of genes, genetic dominance and association and mode of inheritance associated with 2,4-D resistance in two distinct *R. raphanistrum* populations which also display resistance to ALS-inhibiting herbicides endowed by specific *ALS* gene mutations. Plants of the two 2,4-D resistant populations were hybridized by crossing 2,4-D-surviving resistant parental plants with untreated herbicide-susceptible parental plants from a well characterized herbicide-susceptible population. Levels of 2,4-D phenotypic resistance and resistance segregation patterns were assessed in a final experiment with parental populations and F_1, F_2. families.

2. Materials and methods

2.1. Plant material

The *R. raphanistrum* population WARR12 (hereinafter referred to as R_1) originated from Nabawa WA (28.52S, 48.81 E), the population WARR20 (hereinafter referred to as R_2) was collected from a field in Wongan Hills WA (30.88S, 116.51 E), and both populations were established as 2,4-D resistant in previous herbicide screenings [28]. Similarly here, the resistance phenotype was identified following the same detailed methodological protocols which are described elsewhere [24,28]. In these two populations most individuals (>68%) displayed 2,4-D resistance at the Australian recommended label rate for *R. raphanistrum* control in wheat [corresponding to 500 g 2,4-D ha^{-1} delivered through 715 ml of Amicide Advance 700 (Nufarm, Laverton North, Vic 3026)]. The *R. raphanistrum* population (WARR7, hereinafter referred to as S), collected from Yuna WA (28.34S, 115.01 E), is a known and well characterized herbicide-susceptible standard, and S plants were used as the herbicide-susceptible parents.

2.2. Generation of F_1, pseudo-F_2 populations

Plants of 2,4-D resistant and susceptible parental populations were grown in 17-cm diameter pots containing a potting mix (50% peatmoss:50% river sand) and maintained outdoors during the growing season (May–October) for *R. raphanistrum*. R_1 and R_2 parental plants that survived the recommended rate of 2,4-D (500 g ha^{-1}) were kept for pair crossing. Single R_1, R_2 and S plants growing individually in pots were paired according to floral synchronicity and enclosed within a plastic cage (80 cm × 80 cm × 1.5 m height) covered by a Teflon sheet which excluded foreign pollen. Manual pollination with cotton wool was carried out at three day intervals for at least two months during the flowering period and this ensured cross-pollination only between the R and S plants within each pair-cross. At maturity, seeds were collected from each mother plant of the seven reciprocal crosses between R_1 and S and seven reciprocal crosses from R_2 and S parents, thus generating 28 F_1 families comprising seven F_1 maternal R_1 (hereinafter referred to as F_1 R_1S) and seven maternal S (F_1 SR_1), seven F_1 maternal R_2 (F_1 R_2S) and seven maternal S (F_1 SR_2). Following herbicide screening of each individual F_1 family and statistical analysis indicating no significant differences, the seven F_1 families were pooled into each of the four groups F_1 R_1S, F_1 SR_1, F_1 R_2S and F_1 SR_2 for subsequent herbicide dose-response testing.

Pseudo-F_2 families (hereinafter referred to simply as F_2) were generated not by selfing a single F_1 plant but by performing F_1 pair-crosses with 50 2,4-D-resistant individuals each from different F_1 R_1S and F_1 R_2S families. This is because *R. raphanistrum* is reported to be a sporophytically self-incompatible plant [29,30] and we could not obtain a sufficient amount of seed by selfing individual F_1 plants (data not shown). F_1 plants were grown individually in pots and, following treatment with 500 g 2,4-D ha^{-1}, survivors randomly paired and enclosed at flowering time as described above. In total a sufficient amount of seed was recovered from pair-crosses generating 36 distinct F_2 families (23 originating from R_1 and 13 from R_2 parental plants). Seed from the two plants of each pair cross was pooled and stored under laboratory conditions until the subsequent herbicide study to assess 2,4-D resistance and phenotypic segregation.

2.3. Herbicide screening of F_1 families to assess genetic dominance

The F_1 hybrids obtained from both reciprocal crosses between R_1 or R_2 and S parental plants were assessed for 2,4-D resistance. Plants from parental lines (R_1, R_2 and S) and four F_1 families (F_1 R_1S, SR_1, R_2S and SR_2) were grown outdoors during the normal growing season for *R. raphanistrum*. At the two-leaf stage, seedlings were treated with 0, 63, 125, 250, or 500 g 2,4-D ha^{-1}. There were three replicates per treatment (dose), the individual pot represented the experimental unit and pots were arranged in a completely randomized experimental design. Plant survival was evaluated 21 days after herbicide treatment. The most discriminative dose was identified as 500 g 2,4-D ha^{-1} which corresponds to the recommended label dose used in the field to control *R. raphanistrum*.

2.4. Segregation of 2,4-D phenotypic resistance in F_2 families

F_2 families were tested for phenotypic segregation at a specific herbicide dose of 500 g 2,4-D g ha^{-1}. R_1, R_2 and S parental and F_1 plants were included as controls. The experiment was repeated in

Table 1
Herbicide treatments to evaluate multiple-resistance to different modes of action in parental R_1, R_2, and S and pooled F_1R_1 and F_1R_2 families.

Active ingredient	Herbicide mode of action	Dose (g ha^{-1})	Recommended dose range (g ha^{-1})
Atrazine	PSII inhibitor	2000	1000–2000
Bromoxynil + Pyrasulfotole	PSII + HPPD inhibitor	105 + 17.5	141 + 23
Chlorsulfuron	ALS inhibitor	15	10–15
Diflufenican	PDS inhibitor	75	50–200
Glyphosate	EPSPS inhibitor	720	540–720
MCPA	Auxinic herbicide	500	Up to 600

time for a total of two experiments and data were pooled prior to analysis.

2.5. Association of 2,4-D and chlorsulfuron phenotypic resistance in single plants in F_2 families obtained under 2,4-D selection

In a separate study we assessed the potential for genetic drift of traits endowing resistance to the ALS inhibitor chlorsulfuron under 2,4-D selection. F_2, and S control plants at the two leaf stage were treated with the recommended label dose of 2,4-D (500 g ha^{-1}) or chlorsulfuron (15 g ha^{-1}). Plants were grown and maintained outdoors as described previously and assessed 21 days after herbicide treatments. After the first survival assessment, plants treated with 2,4-D were re-treated with chlorsulfuron and vice versa. At 28 days after the second herbicide treatment, plant survival was assessed. The null hypothesis was that genetic association between two major genetic traits conferring 2,4-D or ALS resistance in single plants would result in a non-significant decrease of plant survival after two sequential herbicide treatments (2,4-D followed by chlorsulfuron or vice versa).

2.6. Herbicide screening to assess multiple-resistance in parental R_1, R_2, S and F_1 lines

In a separate study, each parental R_1, R_2 and S population and four F_1 families were also treated with the recommended label dose of various herbicides of dissimilar modes of action (Table 1). Plants were grown and maintained outdoors and assessed for survival 21 days after herbicide treatments.

2.7. Genetic models and statistical analysis for F_2 resistance segregation

F_2 families were treated at 500 g ha^{-1} 2,4-D. This dose was chosen as it corresponds to the dose at which *R. raphanistrum* plants are selected in the field and it was also the most discriminative dose. On average 25 plants were treated in each F_2 in two replicates. The experimental null hypothesis was that 2,4-D resistance segregated as controlled by one locus with two alleles, R (resistant) and S (susceptible). Similar to that described by Tabashnik [31], the segregation analysis of F_2 families was based on the observed survival as resistant (alive) or susceptible (dead) plants compared to the expected survival/mortality of plants assuming one resistance-endowing locus (Table S1). The expected number of surviving plants in F_2 families was calculated using the following equations Eq. (1):

$$F_2\ \text{survivors (expected)} = 0.25\,W_{R1,2} + 0.5W_{F1} + 0.25W_S \quad (1)$$

where $W_{R1,2}$, W_{F1} and W_S are the observed survival (number of plants) of the presumed R_1, R_2 parents, F_1 and S parent phenotypes at the 2,4-D dose tested.

For example, the expected number of survivors of each F_2 family was calculated with the total number of 2,4-D-treated plants multiplied by the theoretical one locus segregation ratio [(e.g. for the one locus model that ratio is (*1R1:2F1:1S*))] multiplied by the observed survival ratios in R_1, R_2 respectively, F_1 and S populations (no. of plants × segregation ratio × survival ratio; see example in Table S1). Details of two additional two-loci genetic models used to fit 25% of F_2 populations are given in Table S2.

2.8. Statistical analysis

2.8.1. Herbicide dose-responses in F_1 and parental families to establish dominance and mode of inheritance

Plant survival data were transformed and expressed as percentages, and data sets were fitted to a three-parameter non-linear logistic model Eq. (2):

$$Y = \frac{d}{1 + \exp[b(\log x - \log e)]} \quad (2)$$

where Y denotes the plant survival or produced aboveground biomass relative to the untreated control, d is the upper asymptotic value of Y, respectively, b is the slope of the curve, e is LD_{50} or GR_{50}, and x is the herbicide dose [32]. Statistical differences in plant survival response between the parental R_1, R_2 and F_1 lines was analysed to establish the level of dominance of resistant allele(s). Also, differences in plant survival response between F_1 families collected from different mother plants (R_1, R_2 or S) were established to infer the nuclear vs maternal mode of inheritance of 2,4-D resistance genes. Chi-square (χ^2) analysis was used to test hypotheses for dominance of 2,4-D resistance by comparing survival rates of R_1, R_2 parental and F_1 lines at the recommended label rate and also across a gradient of 2,4-D doses by analysis of LD_{50} values. Statistical differences in estimated LD_{50} values of the each selected progeny and the unselected parental population were assessed by using the selectivity index (SI) function in the drc package of the statistical software R (version 3.02; R Core Team). A *t*-test was conducted under the null hypothesis of no difference between the estimated LD_{50} values of the selected progeny versus the unselected parent, or equivalently, that their ratio was equal to one [33].

2.8.2. Goodness of fit χ^2 test

For each segregating F_2 family a goodness of fit χ^2 test was used to compare the observed plant survival to the expected calculated values according to one resistance locus segregation models (see a worked example in Table S1). *P*-values were obtained indicating the probability of type I error in rejecting the null hypothesis (H_o = the F_2 family segregates as 1RR:2F_1:1S, respectively, for one locus with two alleles) (see Table S1). The significance level was $\alpha = 0.05$ (two-sided).

Multiple comparisons among survival proportions were assessed by a chi-square (χ^2) heterogeneity test performed using the statistical software *R* (version 3.02) with the command *prop.test*. Differences in survival data for resistance in parental populations and families in response to different herbicide modes of action were subjected to similar chi-square (χ^2) analysis.

3. Results

3.1. Dominance and nuclear inheritance of 2,4-D resistance in R. raphanistrum

Plantsurvival after 2,4-D treatments was assessed in the 2,4-D resistant parental R_1 and R_2 populations and the herbicide susceptible standard S population and contrasted with pooled F_1 families F_1 R_1S, F_1 SR_1, F_1 R_2S and F_1 SR_2. As expected, the S plants were well controlled by a range of 2,4-D doses and >94% mortality was

Table 2
Herbicide dose-response studies to assess resistance to 2,4-D in parental R_1, R_2, pooled F_1R_1, pooled F_1R_2 and S populations. Estimated LD_{50} values are defined as g 2,4-D ha^{-1} and standard errors reported in parentheses. Resistance index (RI) of each parental R_1, R_2 and F_1 line are compared to the S population. Probability values (*P*) on differences between R_1, R_2 parental, pooled F_1 populations and the S parent or between the two maternal lines within each F_1 family in response to 2,4-D were calculated with the SI function in the *drc* package (R statistical software version 3.02). Parameters *b, d* and *e* of Eq. (2) are given for each population tested.

Population	LD_{50} (g ha^{-1})	*b*	*d*	*e*	RI	*P*
R_1	1001 (679)	1.59 (1.5)	96 (6.9)	1001 (679)	**19**	0.21[a]
R_2	1201 (776)	1.03 (0.7)	93 (7.6)	1201 (776)	**23**	0.19[a]
F_1R_1 pooled	615 (108)	1.98 (0.9)	90 (5.0)	615 (108)	**12**	0.006
F_1R_2 pooled	512 (69)	0.71 (0.2)	101 (5.4)	512 (69)	**10**	0.019
S	52 (16)	1.26 (0.4)	102 (8.4)	52 (16)	–	–
F_1 R_1S	580 (69)	2.1 (1.4)	90 (5.5)	580 (69)	–	0.530
F_1 SR_1	657 (116)	1.5 (0.8)	94 (5.9)	657 (116)	–	0.530
F_1 R_2S	490 (93)	0.90 (0.3)	100 (5.4)	490 (93)	–	0.614
F_1 SR_2	590 (209)	0.57 (1.9)	101 (5.3)	590 (209)	–	0.614

[a] The estimated LD_{50} was much greater than the highest tested dose and therefore the statistic appears not significant.

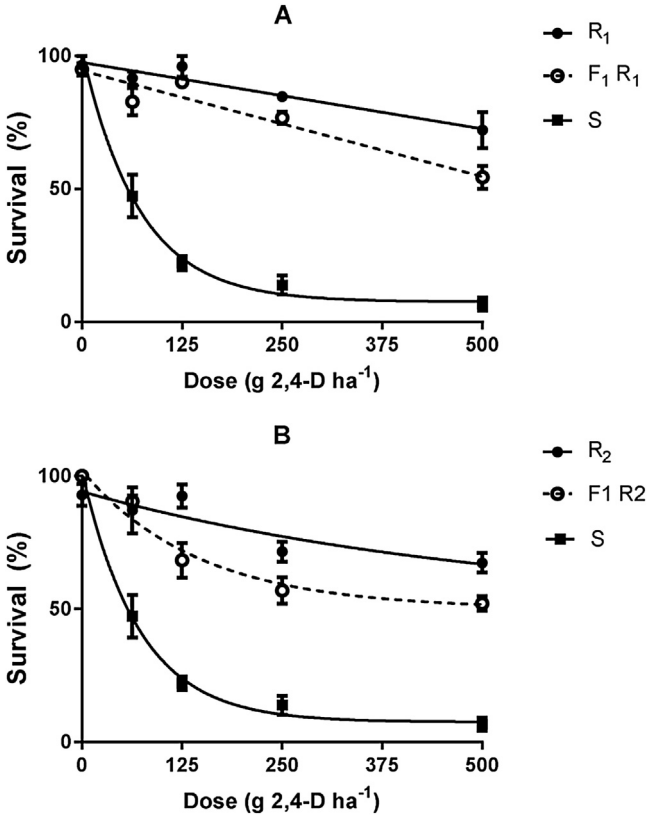

Fig. 1. Survival response to a range of doses of 2,4-D. A) Response of R_1 parental line (filled diamonds and continuous line), F_1R_1 (open diamonds and dashed line represent pooled data for values for F_1 R_1S and F_1 SR_1) B) Response of R_2 parental line (filled circles and continuous line), F_1R_2 (open circles and dashed line represent pooled data for or F_1 R_2S and F_1 SR_2) and herbicide-susceptible S population (solid squares and continuous line). Symbols for R_1, R_2 and S are mean ± SE ($n = 3$). Pooled data for F_1 R_1 and F_1 R_2 ($n = 6$). Lines are predicted values of the non-linear regression fit of Eq. (2).

achieved at the recommended label dose of 500 g ha^{-1}. In contrast, the parental R_1 and R_2 populations and F_1 families were clearly 2,4-D resistant (Fig. 1). The estimated LD_{50} values for F_1 R_1S and F_1 SR_1 or F_1 R_2S and F_1 SR_2, respectively, were not statistically different, indicating that in pair crosses 2,4-D resistance trait(s) was transferred from R_1 or R_2 into S plants *via* pollen (Table 2). Thus, 2,4-D resistance is nuclear inherited in these two R populations of *R. raphanistrum*. Pooled values of plant survival from dose-response studies was compared with the respective resistant parent. The analysis of LD_{50} values revealed no significant difference between F_1R_1 and the original parent R_1 ($P = 0.33$), indicating dominance of 2,4-D resistance in that population (Fig. 1A). Conversely, there was a significant difference between F_1R_2 and R_2 LD_{50} values ($P = 0.05$), suggesting incomplete dominance in population R_2 (Fig. 1B). In addition, at the recommended 2,4-D dose there was no difference (81% vs 82%) between the observed plant survival in F_1R_1 and parental R_1 plants ($\chi^2 = 0.0003$, $P = 0.99$), whereas 41% survival was evident in F_1R_2 and confirmed to be significantly different to the 68% survival observed in resistant parental R_2 plants ($\chi^2 = 6.97$, $P = 0.008$) (Fig. 1). Thus, in the F_1 pair crosses, we confirmed nuclear inheritance and either complete or incomplete 2,4-D resistance dominance for two parental *R. raphanistrum* populations.

3.2. Segregation of 2,4-D resistance

Overall, the responses obtained in two *R. raphanistrum* populations with F_2 families treated with the recommended dose of 2,4-D strongly indicates 2,4-D resistance is conferred by a major gene segregating at one locus, as χ^2 test revealed that a one gene model best fitted the data. Segregation data indicates that 75% of F_2 families fit well with the one locus segregation model indicated above with Eq. (1). Deviations from the expected survival as calculated with a one locus genetic model was observed in nine of the 36 F_2 families (four families with lower and five families with higher than expected survival) (Table 3). When those F_2 families were removed from the analysis there was homogeneity among the remaining 75% of F_2 families ($P > 0.26$, data not shown). Other genetic models were used to fit the segregation of those F_2 families (Table S2) not explained by one-locus genetic model. A model implying the segregation of 2,4-D resistance at two quantitative loci resulted in no significant deviations for those four F_2 families which had lower than expected survival (Table 4). Similarly, a segregation model of two independent loci for 2,4-D resistance (classic Mendelian independent assortment 9:3:3:1) fitted the segregation observed in the F_2 families which had higher than expected survival when fitted with a one locus genetic model (Table 4; Table S2). There was no significant heterogeneity in segregation of 2,4-D resistance in F_2 families fitted with these two-locus models (Table 4).

3.3. Multiple-resistance in F_1 families of 2,4-D resistant R. raphanistrum

In addition to 2,4-D resistance, resistance to several other herbicide modes of action applied at doses near the recommended label rate was assessed in R_1, R_2 and F_1R_1, F_1R_2 and S plants. Yu et al. [34] reported two distinct ALS mutations endowing chlorsulfuron resistance in the R_1 (Asp/Glu-376) and R_2 (Pro/Thr-197) populations, respectively. Thus, as expected, substantial survival (>89%) in response to the ALS-inhibiting herbicide chlorsulfuron was observed in R_1, R_2 and F_1R_2, indicating high-level multiple-resistance to 2,4-D and chlorsulfuron in these populations and families (Fig. 2). Resistance to chlorsulfuron in F_1R_1

Table 3
One-locus phenotypic resistance segregation observed in F_2 families treated with 500 g 2,4-D ha^{-1}. Chi-square (χ^2) analysis for expected plant survival was performed, assuming segregation and control of 2,4-D resistance by one major locus. Survivors expected in (pseudo) F_2 were calculated by multiplying the number of plants treated with 2,4-D by the theoretical one gene segregation ratio for F_2 (*1R1:2F1:1S*) by the observed survival (%) in R, F_1 and S at 500 g 2,4-D ha^{-1}. Heterogeneity test was conducted for each group of F2 families.

Family	Seeds treated	Survivors (observed)	Survival ratio	Survivors (expected)	*Segregation ratio*	χ2	*P*
R_1	37	21	0.568				
S	76	2	0.026				
F_1 R_1S/SR_1	411	207	0.504				
F_2 1	11	6	0.55	4.4	*1R_1:2F_1:1S*	1.0	0.33
F_2 2	34	6	0.18	13.6	*1R_1:2F_1:1S*	7.1	0.01[a]
F_2 3	20	6	0.30	8.0	*1R_1:2F_1:1S*	0.8	0.36
F_2 4	30	6	0.20	12.0	*1R_1:2F_1:1S*	5.0	0.03[a]
F_2 5	33	17	0.52	13.2	*1R_1:2F_1:1S*	1.8	0.18
F_2 6	29	20	0.69	11.6	*1R_1:2F_1:1S*	10.1	<0.001[a]
F_2 7	24	3	0.13	9.6	*1R_1:2F_1:1S*	7.6	0.01[a]
F_2 8	24	8	0.33	9.6	*1R_1:2F_1:1S*	0.5	0.50
F_2 10	25	12	0.48	10.0	*1R_1:2F_1:1S*	0.7	0.42
F_2 11	35	18	0.51	14.0	*1R_1:2F_1:1S*	1.9	0.17
F_2 13	25	13	0.52	10.0	*1R_1:2F_1:1S*	1.5	0.22
F_2 14	23	16	0.70	9.2	*1R_1:2F_1:1S*	8.3	<0.001[a]
F_2 3b	27	10	0.37	10.8	*1R_1:2F_1:1S*	0.1	0.75
F_2 4b	15	10	0.67	6.0	*1R_1:2F_1:1S*	4.4	0.04[a]
F_2 5b	6	4	0.67	2.4	*1R_1:2F_1:1S*	1.8	0.18
F_2 6b	35	21	0.60	14.0	*1R_1:2F_1:1S*	5.8	0.02[a]
F_2 7b	17	4	0.24	6.8	*1R_1:2F_1:1S*	1.9	0.16
F_2 8b	29	12	0.41	11.6	*1R_1:2F_1:1S*	0.0	0.88
F_2 9b	18	6	0.33	7.2	*1R_1:2F_1:1S*	0.3	0.56
F_2 10b	16	7	0.44	6.4	*1R_1:2F_1:1S*	0.1	0.76
F_2 11b	12	8	0.67	4.8	*1R_1:2F_1:1S*	3.5	0.06
F_2 12b	34	15	0.44	13.6	*1R_1:2F_1:1S*	0.2	0.63
F_2 14b	36	21	0.58	14.4	*1R_1:2F_1:1S*	5.0	0.03[a]
Heterogeneity						65.8	<0.001
R_2	65	43	0.662				
S	76	2	0.026				
F_1 R_2S/SR_2	402	214	0.532				
F_2 19	28	14	0.50	12.3	*1R_2:2F_1:1S*	0.4	0.51
F_2 20	26	16	0.62	11.4	*1R_2:2F_1:1S*	3.3	0.07
F_2 21	32	10	0.31	14.0	*1R_2:2F_1:1S*	2.0	0.15
F_2 22	29	12	0.41	12.7	*1R_2:2F_1:1S*	0.1	0.79
F_2 23	10	2	0.20	4.4	*1R_2:2F_1:1S*	2.3	0.13
F_2 24	40	6	0.15	17.5	*1R_2:2F_1:1S*	13.5	0.00[a]
F_2 25	36	12	0.33	15.8	*1R_2:2F_1:1S*	1.6	0.21
F_2 26	6	2	0.33	2.6	*1R_2:2F_1:1S*	0.3	0.61
F_2 27	31	16	0.52	13.6	*1R_2:2F_1:1S*	0.8	0.38
F_2 28	29	17	0.59	12.7	*1R_2:2F_1:1S*	2.6	0.11
F_2 20b	21	11	0.52	9.2	*1R_2:2F_1:1S*	0.6	0.43
F_2 21b	38	12	0.32	16.6	*1R_2:2F_1:1S*	2.3	0.13
F_2 27b	29	14	0.48	12.7	*1R_2:2F_1:1S*	0.2	0.63
Heterogeneity						29.1	0.004

[a] For those F_2 families in which there was a significant deviation from the one locus segregation model we recalculated the segregation according to two-locus models (see Table 4).

Table 4
Polygenic (two loci) phenotypic resistance segregation observed in F_2 families treated with 500 g 2,4-D ha^{-1}. Chi-square (χ^2) analysis for expected plant survival assuming segregation and control of 2,4-D resistance by two loci was performed. Survivors expected in (pseudo) F_2 was calculated by multiplying the number of plants treated with 2,4-D by the theoretical two additive loci segregation ratio (*5R:6F1:5S*) or two independent loci (*9R:6F1:1S*) multiplied by the observed survival (%) in R, F_1 and S at 500 g 2,4-D ha^{-1}. Heterogeneity test was conducted for each group of F_2 families.

Family	Seeds treated	Survivors (observed)	Survival ratio	Survivors (expected)[a]	Segregation ratio	χ2	*P*
No. survivors < than expected							
F_2 2	34	6	0.18	9.4	*5R:6F1:5S*	1.1	0.20
F_2 4	30	6	0.20	8.3	*5R:6F1:5S*	0.0	0.36
F_2 7	24	3	0.13	6.6	*5R:6F1:5S*	2.6	0.10
F_2 24	40	6	0.15	11	*5R:6F1:5S*	3.1	0.08
Heterogeneity						0.7	0.88
No. survivors > than expected							
tF_2 4b	15	10	0.67	7.7	*9R:6F1:1S*	4.4	0.23
F_2 6	29	20	0.69	14.8	*9R:6F1:1S*	3.7	0.05
tF_2 6b	35	21	0.60	17.9	*9R:6F1:1S*	1.1	0.29
tF_2 14b	36	21	0.58	18.4	*9R:6F1:1S*	0.8	0.38
F_2 14	23	16	0.70	11.7	*9R:6F1:1S*	3.2	0.08
Heterogeneity						1.4	0.84

[a] Additional details are provided in Table S2.

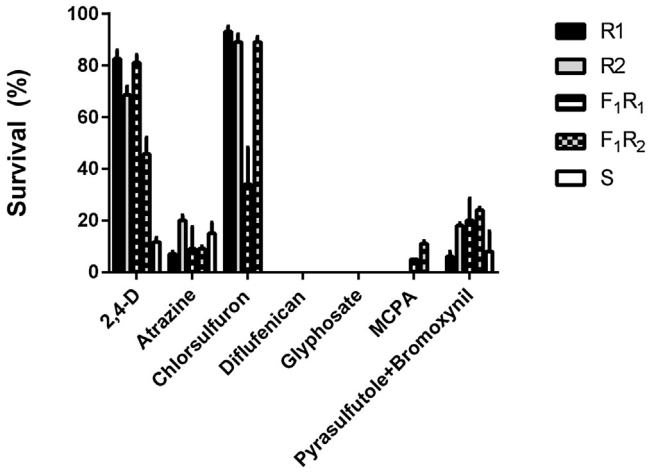

Fig. 2. Multiple-resistance in *Raphanus raphanistrum* based on plant survival in parental 2,4-D resistant R_1 and R_2 populations, F_1 families F_1R_1, F_1R_2 and S (susceptible control parent) in response to a range of herbicides. Bars are mean ± SE ($n > 2$).

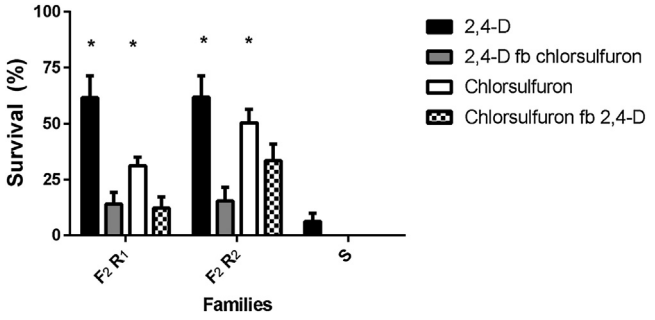

Fig. 3. Association between 2,4-D and chlorsulfuron resistance traits measured as survival of single plants in F_2R_1, F_2R_2 and S (susceptible control parent) in response to 500 g 2,4-D ha^{-1} alone (2,4-D, black bars) or followed by 15 g chlorsulfuron ha^{-1} applied 21 days after first herbicide treatment on 2,4-D survivors (2,4-D fb chlorsulfuron, grey bars); and 15 g chlorsulfuron ha^{-1} alone (white bars) or followed by 500 g 2,4-D ha^{-1} applied 21 days after first herbicide treatment on chlorsulfuron survivors (Chlorsulfuron fb 2,4-D, black-and-white bars). Survival proportions (total number of survivors/total number of plants treated with herbicide) after the first herbicide treatment and after the second sequential herbicide treatment were assessed by a χ^2 test. The asterisk symbol (*) indicates significant differences ($\chi^2 > 12.8$; $P < 0.01$) between the two proportions of plant survival to one herbicide treatment (2,4-D or chlorsulfuron) vs two sequential herbicide treatments (2,4-D fb chlorsulfuron or chlorsulfuron fb 2,4-D, respectively). Minimum number of herbicide-treated plants = 85. Bars are mean ± SE ($n > 5$).

families was significantly lower (34% survival, $\chi^2 > 13.8$, $P < 0.001$), likely indicating a high frequency of heterozygous plants in the parental population R_1 (Fig. 3) and no linkage with 2,4-D resistance traits. There was no resistance to other herbicide modes of action, as survival at the recommended label dose of atrazine (PSII inhibitor), glyphosate (EPSPS inhibitor), diflufenican (PDS inhibitor), MCPA (synthetic auxin) or pyrasulfotole + bromoxynil (HPPD + PSII inhibitor) was low and not significantly higher than that of the S population ($\chi^2 < 0.92$; $P > 0.33$) (Fig. 2).

3.4. Association of 2,4-D and chlorsulfuron resistance in single plants from F_2 families obtained under 2,4-D selection

To establish the association between traits conferring phenotypic resistance to 2,4-D and the ALS inhibitor herbicide chlorsulfuron, the response of a number of individual plants from F_2 families was monitored after sequential applications of 2,4-D followed by chlorsulfuron, or chlorsulfuron followed by 2,4-D. There was a significant decrease in plant survival from pooled data across 11 F_2R_1 and eight F_2R_2 families following the sequential application of 2,4-D followed by chlorsulfuron and vice versa ($\chi^2 > 0.92$; $P < 0.001$). Mean survival to 2,4-D in both F_2R_1 and F_2R_2 population was approximately 62% versus 14% and 15% survival to the subsequent chlorsulfuron treatment (Fig. 3). Likewise, mean survival to chlorsulfuron in 11 F_2R_1 and eight F_2R_2 was 31% and 50%, respectively, whereas this was much and significantly reduced to 12 and 33%, respectively by a subsequent 2,4-D treatment. These results suggest that the null hypothesis of genetic association of traits conferring resistance to the auxinic herbicide 2,4-D or the ALS inhibitor chlorsulfuron should be rejected. Plant survival of *R. raphanistrum* individuals subjected to two sequential herbicide treatments significantly decreased after the second herbicide treatment. This indicates that the selection with 2,4-D of parental R_1 and R_2 plants to produce F_1 and then F_2 families does not co-select for resistance-endowing *ALS* gene mutations (Fig. 3). Thus, ALS resistance traits carried by parental R_1 and R_2 plants could fluctuate or be randomly inherited by subsequent generations.

4. Discussion

4.1. Major genes explain most cases of auxinic herbicide resistance

Cases of resistance to auxinic herbicides have been reported in their 70 years of use for weed control [3]. This study reports that despite the complexity associated to the 2,4-D mode of herbicide action, 2,4-D resistance in *Raphanus raphanistrum* populations is mostly conferred by nuclear-inherited highly dominant trait(s) segregating at one single locus. Two-locus genetic models explained the segregation of 2,4-D resistance in the 25% of F_2 families not fitted to the one-locus model. The genetic traits endowing multiple-resistance to 2,4-D and chlorsulfuron are not closely associated as they appear to independently segregate in F_2 individual plants.

A recent recurrent selection study with the 2,4-D-susceptible parental S population of *R. raphanistrum* reported heritable variation for 2,4-D resistance at reduced doses of the herbicide [35]. As we observed incomplete control with 2,4-D in the same susceptible parental line S at the recommended dose of 2,4-D (2.6% survival), we suggest that minor genes endowing low-level 2,4-D resistance might have been introduced in the pair-crosses between R_1, R_2 and S parental plants originating the F_1 and F_2 families. We speculate, these minor genes could have contributed to some of the observed heterogeneity of 2,4-D resistance segregation values with deviations from the predominant one-locus resistance segregation in F_2 families following 2,4-D treatments.

Similarly to the revealed monogenic inheritance of traits conferring herbicide resistance, monogenic responses of plants exposed to selection with highly toxic chemicals such as heavy metals have often been reported [36,37]. In this study, the observed segregation of 2,4-D resistance is consistent with several other inheritance studies of evolved resistance to auxinic herbicides in dicotyledonous weeds, which show single dominant alleles for resistance to 2,4-D in *Brassica kaber* [38] and *Sisymbrium orientale* [39], MCPA in *R. raphanistrum* [40], dicamba resistance in *Kochia scoparia* [41] and *Brassica kaber* [42,43] or two additive genes for MCPA resistance in *Galium tetrahit* [44]. Yet, there are also reports of recessive gene mutations endowing 2,4-D resistance in *Arabidopsis thaliana* [45].

The inheritance patterns observed in the two *R. raphanistrum* populations studied are consistent with the reported biochemical and physiological basis of 2,4-D resistance in this species. In the population R_2 Goggin et al. [23] discovered that reduced translocation of parent 2,4-D is the major trait conferring resistance due to a loss of function of a plasma membrane auxin transporter

together with another minor mechanism involving a systemic change in auxin transport, perception, or signalling expressed in the roots. In the same species MCPA resistance was associated to an incompletely-dominant single gene [40]. Similar results of a semi-dominant single resistance genetic trait endowing reduced translocation of 2,4-D were reported in *Lactuca serriola*, [22]. Conversely, a semi-dominant mutation conferring a gain of function of the specific PDR9 transporter endowed 2,4-D resistance to in *Arabidopsis thaliana* plants capable of remove 2,4-D from cells without affecting endogenous auxin transport [46].

4.2. Future research and management of auxinic-herbicide resistant weeds

In the last two decades the incidence of the agricultural weed species *R. raphanistrum* has significantly increased in cropping regions of Western Australia and its presence causes significant economic damage in cereal and legume crops [47]. Thus far, this species has evolved resistance to five different herbicide modes of action [24]. In particular, the levels of resistance to sulfonylureas, imidazolinones or the auxinic herbicide 2,4-D have dramatically increased, with at least 50% of randomly-sampled *R. raphanistrum* populations containing resistant plants to these herbicide modes of action [24].

Raphanus raphanistrum can grow vigorously in infested cropped fields and herbicide-resistant plants can potentially establish substantial soil-seed banks and disperse pollen at distance *via* pollinators [48]. Thus, traits such as 2,4-D or ALS resistance, with a high degree of dominance and monogenic nuclear inheritance, can be selected by repeated herbicide use and easily spread in field populations, resulting in plants that carry multiple genetic traits for resistance to multiple herbicide modes of action. This study provides some information on the biological and genetic features of *R. raphanistrum* that can help explain the rapid increase in resistance frequency as reported by Owen, Martinez and Powles [24]. However, we report that in *R. raphanistrum* single plants major genes for auxinic and ALS-inhibiting herbicide resistance are not closely associated and segregate independently in several F_2 families. This implies that in a population the expected frequency of single plants carrying a stack of two independent heterozygous traits for resistance to two distinct herbicide modes of action, is only 25% due to independent genetic segregation. Thus, the use of herbicide mixtures could still provide acceptable levels of weed control in multiple-resistant *R. raphanistrum* populations with relatively high levels of resistance to single herbicides.

This study reports that two *R. raphanistrum* populations resistant to 2,4-D and ALS-inhibiting herbicides remain susceptible to several other modes of action and finds an unexpected high level of control of 2,4-D-resistant parent and F_1 families by MCPA, which, like 2,4-D, is a phenoxyacetic acid. Goggin et al. [23] report cross-resistance between 2,4-D and MCPA based on a root elongation response assay and the variability found in *R. raphanistrum* populations. However, the complexities associated to transporters, receptors, signal transduction of putative traits conferring auxin resistance in the roots may only partially explain the response of whole plants treated with foliar applications of 2,4-D versus MCPA. For example, the loss of function of a transporter mediating reduced translocation of 2,4-D as documented by Goggin et al. [23] could only partially restrict MCPA translocation and therefore not endow sufficient resistance. Thus, the current use of a diverse range of herbicide modes of action including PDS inhibitors (i.e. diflufenican, picolinafen), PSII inhibitors (i.e. atrazine, bromoxynil), HPPD + PSII inhibitors (i.e. pyrasulfotole + bromoxynil)] in combinations with the auxinic herbicides 2,4-D and MCPA to control *R. raphanistrum* populations should be maintained, carefully monitored and complemented with non-herbicidal strategies to maximize their sustainability. For example, one study conducted on the two populations examined here has shown that herbicide use in combination with crop competition can cause strong reductions in above-ground biomass production and therefore may have significant effects on 2,4-D-resistant plant fitness [18].

There are several studies [21] suggesting that auxinic herbicide resistance has evolved at a slower rate than resistance to other herbicide modes of action such as ALS- or ACCase-inhibitors [49,50]. Thus far, the relatively low incidence of auxinic herbicide resistance has been attributed to typical factors such as rarity of resistance alleles, possible presence of fitness penalties in resistant phenotypes and complexities related to the mode of action or evolved resistance mechanism(s). However, thus far no study has reported on the expression of fitness costs in plants with resistance to auxinic herbicides. While the unravelling of the molecular complexities associated with 2,4-D resistance in *R. raphanistrum* is underway, future research on possible pleiotropic effects associated with reduced 2,4-D translocation in *R. raphanistrum* in the absence of the herbicide is warranted. This specific knowledge could provide some insight into population dynamics and life history traits to be exploited for developing successful resistance management practices. For example, it would be important to know whether herbicide discontinuity could be an effective option to reduce the frequency of non-target-site-based 2,4-D resistance.

Auxinic herbicides such as 2,4-D and dicamba are predicted to significantly increase in usage due to the adoption of transgenic auxinic-herbicide resistant crops in world agriculture. Thus, their sustainability will require pro-active management to constrain excessive herbicide usage and achieve greater herbicide and non-herbicidal weed management diversity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2017.01.003.

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