Research Article

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Recurrent selection with reduced 2,4-D amine doses results in the rapid evolution of 2,4-D herbicide resistance in wild radish (*Raphanus raphanistrum* L.)

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Abstract

BACKGROUND: When used at effective doses, weed resistance to auxinic herbicides has been slow to evolve when compared with other modes of action. Here we report the evolutionary response of a herbicide-susceptible population of wild radish (*Raphanus raphanistrum* L.) and confirm that sublethal doses of 2,4-dichlorophenoxyacetic acid (2,4-D) amine can lead to the rapid evolution of 2,4-D resistance and cross-resistance to acetolactate synthase (ALS)-inhibiting herbicides.

RESULTS: Following four generations of 2,4-D selection, the progeny of a herbicide-susceptible wild radish population evolved 2,4-D resistance, increasing the LD_{50} from 16 to 138 g ha^{-1}. Along with 2,4-D resistance, cross-resistance to the ALS-inhibiting herbicides metosulam (4.0-fold) and chlorsulfuron (4.5-fold) was evident. Pretreatment of the 2,4-D-selected population with the cytochrome P450 inhibitor malathion restored chlorsulfuron to full efficacy, indicating that cross-resistance to chlorsulfuron was likely due to P450-catalysed enhanced rates of herbicide metabolism.

CONCLUSION: This study is the first to confirm the rapid evolution of auxinic herbicide resistance through the use of low doses of 2,4-D and serves as a reminder that 2,4-D must always be used at highly effective doses. With the introduction of transgenic auxinic-herbicide-resistant crops in the Americas, there will be a marked increase in auxinic herbicide use and therefore the risk of resistance evolution. Auxinic herbicides should be used only at effective doses and with diversity if resistance is to remain a minimal issue.

Supporting information may be found in the online version of this article.

Keywords: 2,4-D; low dose; recurrent selection; wild radish; *Raphanus raphanistrum*; evolution; herbicide resistance

1 INTRODUCTION

Synthetic auxin herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 3,6-dichloro-2-methoxybenzoic acid (dicamba) have been widely used for decades to control dicot weeds in crop and non-crop situations. Auxinic herbicides induce a sustained increase in auxin signalling, which leads to unregulated growth[1] and to the accumulation of ethylene, abscisic acid and reactive oxygen species, resulting in tissue necrosis and death.[2,3] Synthetic auxin herbicides are used annually on an estimated 200 million ha globally.[4] Despite over 60 years of use, the evolution of auxinic herbicide resistance in targeted weed species has been limited in impact. Auxinic herbicide-resistant biotypes have so far been reported in 31 weed species, which is low when compared with the 158 species that have evolved resistance to the acetolactate synthase (ALS)-inhibiting herbicides, following 30 years of use.[5–7] Currently, a significant change and likely major increase in the use of auxinic herbicides are occurring with transgenic 2,4-D- and dicamba-resistant crops being commercially grown, initially in North America.[8] This will increase the frequency of auxinic herbicide application in crop fields with a lengthy but sporadic history of use of auxinic herbicides.

This study concerns the evolution of auxinic herbicide-resistant weed populations. Most agricultural weeds display considerable genetic variation, including heritable variation in herbicide susceptibility.[9,10] When cross-pollinated species are recurrently selected at low herbicide doses (within the population's standing genetic variability for herbicide response), both weak and strong gene traits are selected and accumulated in the survivors

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Table 1. Recurrent selection with 2,4-D from basal wild radish population G0

Generation	2,4-D amine dose applied (g ha^{-1})	Population size	Herbicide efficiency (% control)[a]	Survivors selected for seed production (*N*)	Progeny name
Control 1 to 4	0	20	0	20	C1–C4
Selected 1	125	382	71	20	S1
Selected 2	250	396	88	20	S2
Selected 3	250	393	77	20	S3
Selected 4	750	379	76	20	S4

[a] Calculated as 100 – (% plant survival).

until resistance is evident (reviewed by Yu and Powles[11] and investigated by Busi *et al.*[12]). Such rapid (three-generation) evolution of polygenic herbicide resistance through recurrent low herbicide dose selection has been repeatedly demonstrated in the cross-pollinated monocot species annual ryegrass (*Lolium rigidum*).[13–16] Wild radish (*Raphanus raphanistrum*) is a cross-pollinated, genetically diverse dicot weed of global crops.[17] Herbicidal control of wild radish has resulted in the evolution of resistance to several herbicide modes of action, including ALS-[18] and phytoene desaturase (PDS)-inhibiting[19] herbicides, triazine herbicides,[20] glyphosate[21] and auxinic herbicides.[19] Wild radish is at risk of rapid herbicide resistance evolution owing to its high genetic variability and obligate cross-pollination.[22] This study investigated the potential of a herbicide-susceptible wild radish population to evolve 2,4-D resistance when recurrently selected at low 2,4-D amine doses. The evolution of cross-resistance to chemically dissimilar herbicides was also assessed.

2 MATERIALS AND METHODS

2.1 Plant material

This selection study was conducted using the known herbicide-susceptible wild radish biotype WARR7 (referred to hereafter as G0) originally collected in 1999 from Yuna, Western Australia (28.34° S, 115.01° E).[19] Since collection, seed stocks of this population have been maintained without herbicide selection and multiplied in isolation, preventing the ingression of any herbicide resistance genes. Using this population, four successive generations were recurrently selected at low 2,4-D amine doses in June 2011 (S1), April 2012 (S2), September 2012 (S3) and January 2013 (S4) using the general procedure detailed below (see also Table 1).

2.2 General procedure for population selection

This study was conducted by recurrently selecting one population (G0) over four consecutive generations from a herbicide dose–response study, as described by Neve and Powles.[13] Seeds (400 per dose) were planted approximately 1 cm deep into four replicate polystyrene foam trays (400 mm wide × 500 mm long × 150 mm deep) containing standard potting mixture (25% peat moss, 25% sand and 50% mulched pine bark). After planting, seedlings were grown in the outdoor growth facility at the University of Western Australia, being watered as required and fertilised weekly with 2 g of Scotts PolyFeed™ soluble fertiliser [N 19% (urea 15%, ammonium 1.9%, nitrate 2.1%), P 8%, K 16%, Mg 1.2%, S 3.8%, Fe 400 mg kg^{-1}, Mn 200 mg kg^{-1}, Zn 200 mg kg^{-1}, Cu 100 mg kg^{-1}, B 10 mg kg^{-1}, Mo 10 mg kg^{-1}). At the two-true-leaf stage, seedlings were treated with Amicide 625 (625 g 2,4-D amine L^{-1}; Nufarm, Laverton North, Vic., Australia). Herbicide treatments were applied using a twin-nozzle laboratory sprayer fitted with 110° 01 flat-fan spray jets (Tee jet™) delivering herbicide in 118 L ha^{-1} of water at 210 kPa, travelling at a speed of 3.6 km h^{-1}. After treatment, plants were returned to the outdoor area. Survival was assessed 42 days after treatment (DAT). Plants with new healthy leaf tissue were considered to be survivors. The 2,4-D dose used for each selection was based upon the rate providing 20% survival. From the selected dose, 20 plants were subsampled based on maximum regrowth (S1 to S4) (Table 1). Prior to flowering, the selected survivors were isolated to prevent ingress of foreign pollen. Once all plants were actively flowering, plants were crossed manually using the 'bee-stick method' as outlined by Williams,[23] ensuring a random pattern of cross-pollination (panmixia). At maturity, all siliques were collected, and the seed, representing the next generation for 2,4-D selection, were threshed using a modified 'grist mill'. During each selection, a separate population of 20 untreated plants was maintained, isolated and crossed using the general procedure to produce four successive generations of unselected controls (C1 to C4) (Table 1).

2.3 Dose–response analysis

In May 2013, the commencing (G0) and the selected (S1 to S4) as well as the control (C1 to C4) progenies were all concomitantly evaluated in a 2,4-D dose–response study conducted under field conditions. For each 2,4-D dose, 20 seeds per pot were planted into four replicate 180 mm diameter plastic pots containing standard potting mixture and maintained outdoors, simulating field conditions in the normal growing season for this species. At the two-true-leaf stage, the commencing (G0) and control generations (C1 to C4) were sprayed with 2,4-D amine at 0, 45, 90, 125, 250, 500, 750 and 1000 g ha^{-1} (field dose 500 g ha^{-1}), with the selected generations (S1 to S4) treated at 0, 90, 180, 250, 500, 750, 1000, 1500 and 2000 g ha^{-1} as described above. Plant survival was assessed 42 DAT, which was based on the development of further leaves. At this time, above-ground shoot biomass was harvested and dried at 65 °C for 7 days before weighing.

2.4 Cross-resistance profiles of commencing susceptible (G0) and fourth-generation 2,4-D-amine-selected progeny (S4)

To determine the extent of any cross-resistance to dissimilar herbicide modes of action, the G0 and S4 populations were treated at the recommended dose with a range of chemically dissimilar herbicides with known activity against wild radish (Table 2). In April 2013, 20 seeds of each population were established in four replicate 180 mm plastic pots containing standard potting mixture. At the two-leaf stage, the recommended dose of each herbicide (Table 2) was applied. Plant survival was assessed 35 DAT. Survivors

Table 2. Screening of populations G0 and S4 for cross-resistance to chemically dissimilar herbicides

Herbicide mode of action	Herbicide active	Dose(g ha^{-1})	Adjuvant	Mean plant survival (%)[a]			
				Commencing population (G0)	Fourth generation selected (S4)	G0 progeny	S4 progeny
ALS inhibitor	Chlorsulfuron	15	0.1% BS1000	0	58 (2)	0	80 (3)
ALS inhibitor	Metosulam	5	0.1% BS1000	0	30 (8)	0	59 (2)
ALS inhibitor	Sulfometuron-methyl	7.5	0.1% BS1000	0	0	nd[b]	nd
ALS inhibitor	Imazamox	32	0.1% BS1000	0	5 (3)	nd	nd
PDS inhibitor	Diflufenican	100	–	3 (1)	1 (1)	nd	nd
PSII inhibitor	Bromoxynil	400	–	0	0	nd	nd
PSII inhibitor	Diuron	1000	–	0	0	nd	nd
PSII inhibitor	Metribuzin	280	–	0	0	nd	nd
PSII inhibitor	Atrazine	1000	2% Hasten	0	0	nd	nd
EPSPS inhibitor	Glyphosate	540	–	0	0	nd	nd
PSI inhibitor	Diquat	100	–	0	0	nd	nd

[a] Each result is the mean of four replicates. Standard errors of the means are in parentheses.
[b] nd: not determined.

from the chlorsulfuron and metosulam treatments were isolated and crossed as described above to produce progeny. This progeny was herbicide treated as previously described and assessed to confirm the heritability of the cross-resistance trait (Table 2).

To quantify the strength of the cross-resistance to the ALS-inhibiting herbicides, dose–response studies were conducted using the S4 generation. For each dose, 20 seeds per pot were planted into four replicate 180 mm diameter plastic pots containing standard potting mixture and maintained outdoors, simulating field conditions in the normal growing season for this species. Chlorsulfuron (750 g kg^{-1}; Nufarm) was applied at 0, 3.75, 7.5, 15, 30 and 60 g ha^{-1}, metosulam (100 g kg^{-1}; Bayer Cropscience, Hawthorn East, Vic., Australia) at 0, 1.2, 2.5, 5, 10 and 20 g ha^{-1}, imazamox (700 g kg^{-1}, Crop Care, Murarrie, Qld, Australia) at 0, 8, 16, 32, 64 and 128 g ha^{-1} and sulfometuron-methyl (750 g kg^{-1}; Nufarm) at 0, 1.5, 3, 6, 12 and 24 g ha^{-1} (all corresponding to 0.25×, 0.5×, 1×, 2× and 4× the recommended dose). Plants that were alive 35 DAT were considered to have survived, and their biomass was assessed as previously described.

A separate dose–response study was conducted to investigate the possibility for cytochrome P450 monooxygenase (P450)-catalysed herbicide metabolism to be the basis for the chlorsulfuron cross-resistance in the S4 population, using the design previously described. Malathion has been demonstrated effectively to synergise chlorsulfuron activity in maize (*Zea mays*)[24] and annual ryegrass (*Lolium rigidum*), likely by inactivating certain P450 enzymes.[25–27] At the two-true-leaf stage, 1000 g ha^{-1} of malathion[26] (Maldison™ 500 g kg^{-1}; Nufarm) was applied to plants from the commencing (G0) and S4 populations. Thirty minutes later, chlorsulfuron was applied at 0, 7.5, 15, 30 and 60 g ha^{-1} to these plants and to malathion-untreated controls. A malathion-only treatment was also included. Plants that were alive 35 DAT were considered to have survived, and their biomass was assessed as previously described.

2.5 Data analysis

Survival and biomass data were analysed using non-linear regression analyses with the DRC package v.2.5-12[28] in R v.3.3.0 (R Development Core Team, 2011; http://www.R-project.org). Biomass was modelled under the assumption of normally distributed errors, as the residuals from the fitted log-logistic model were approximately normally distributed. Survival data were analysed assuming a binomial distribution. Plant survival and biomass were fitted to the three-parameter log-logistic model

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

where Y denotes plant survival (%) or biomass (g plant^{-1}) in response to herbicide dose x, a is the upper and c is the lower asymptotic value of Y and b is the slope of the curve around e, which is the dose causing 50% mortality (LD50) or biomass reduction (GR50). LD_{50} and GR_{50} parameters of the selected (S1 to S4) and unselected progeny (C1 to C4) were compared with the commencing (G0) population using the selectivity indices (SI) function (R v.3.0.0) to determine whether the ratios between these values were significantly different ($P < 0.05$). A ratio of recurrently selected and commencing populations (R/S) at the estimated LD_{50} and GR_{50} level was used to highlight the change in population susceptibility to 2,4-D. Survival data were plotted using SigmaPlot v.12 (Systat Software Inc., 2011).

3 RESULTS

3.1 Effect of low-dose 2,4-D amine selections on the commencing population G0

Commencing with the known herbicide-susceptible wild radish population (G0), four generations of low-dose 2,4-D amine selection were applied to produce four selected generations (S1 to S4) which were compared with four concurrently grown generations without 2,4-D selection (C1 to C4). Dose–response screening of the commencing G0 population confirmed susceptibility to 2,4-D amine at the recommended application rate and an LD_{50} of 16 g ha^{-1} (Fig. 1 and Table 3). Four generations of low-dose 2,4-D amine selection resulted in heritable increases in plant survival, increasing the 2,4-D LD_{50} dose from 16 g ha^{-1} (G0) to 41 g ha^{-1} (S1), 55 g ha^{-1} (S2), 62 g ha^{-1} (S3) and, in the fourth generation, 138 g ha^{-1} (S4). This equated to a 8.6-fold (S4: LD_{50}) level of 2,4-D amine resistance (Table 3). Resistance was also reflected in increased plant growth of survivors, with the G0 population and

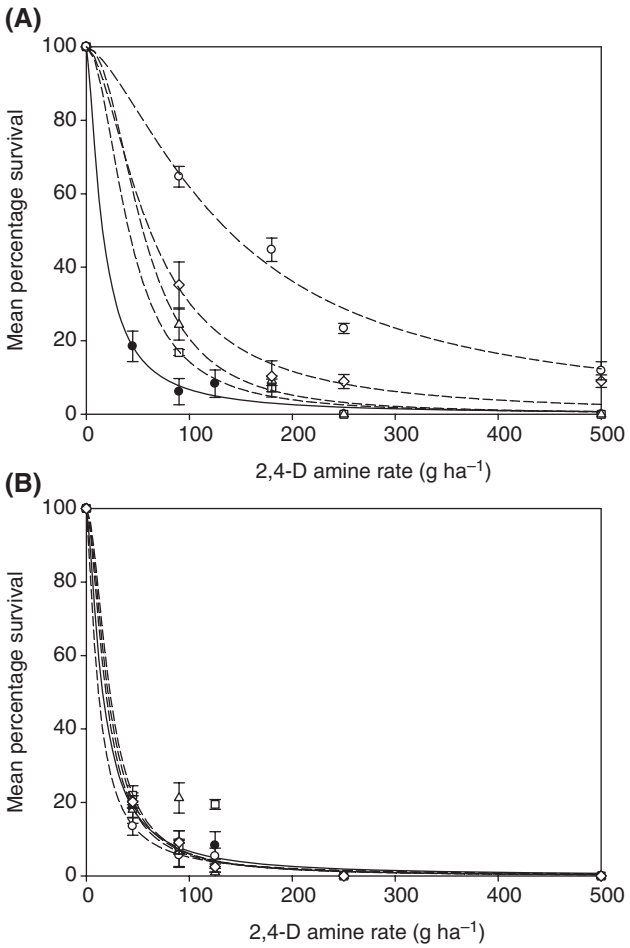

Figure 1. Dose–response curves for (A) recurrent low-dose 2,4-D-amine-selected generations and (B) recurrently grown generations in the absence of 2,4-D selection. The plotted lines are predicted percentage survival using the three-parameter log-logistic model [equation (1)]. The commencing wild radish population is denoted as G0 (- ● -), with the recurrent generations denoted as generations 1 (– – □ – –), 2 (– – △ – –), 3 (– – ◇ – –) and 4 (– – ○ – –). Each symbol represents the mean of nine dose treatments. Vertical bars represent SE of the mean.

S4 generation displaying a 2,4-D GR_{50} of 60 and 234 g ha^{-1} respectively, equating to an estimated 3.9-fold increase in GR_{50} between the G0 and S4 generations (Table 4). Four generations of growth in the absence of 2,4-D (C1 to C4) did not alter the population susceptibility to 2,4-D (survival or biomass), indicating that environmental factors did not contribute to the 2,4-D resistance shifts evident in the 2,4-D-selected populations (S1 to S4) (Fig. 1).

3.2 Cross-resistance to chlorsulfuron and metosulam

In addition to the evolution of 2,4-D amine resistance (Fig. 1), there was evidence of cross-resistance to the chemically dissimilar ALS-inhibiting herbicides chlorsulfuron and metosulam (metabolisable), but not to imazamox or sulfometuron (non-metabolisable) (Table 5). Initial screening with the recommended field dose of 15 g ha^{-1} of chlorsulfuron or 5 g ha^{-1} of metosulam gave zero survival of the G0 population but 58 and 30% survival, respectively, in the S4 generation (Table 2). Screening of the cross-resistant progeny found these traits to be heritable, with, respectively, 80 and 59% survival following chlorsulfuron and metosulam treatment (Table 2). Full dose–response screening of the 2,4-D-selected S4 population quantified a 4.5-fold modest level of chlorsulfuron resistance and a fourfold level of metosulam resistance, based on LD_{50} values (Table 5). Resistance was also evident with respect to plant growth (GR_{50}), with S4 biomass minimally reduced by chlorsulfuron (4.8-fold resistance) or metosulam (5.8-fold resistance) application (Table 5). The genetic and biochemical basis of this chlorsulfuron and metosulam cross-resistance is yet to be investigated. However, based on the fact that there was no detectable resistance to the non-metabolisable herbicides, it is possible that enhanced metabolism (detoxification) of chlorsulfuron and metosulam is responsible for resistance. To test this hypothesis, the S4 generation was pretreated with the known P450 inhibitor malathion.[26] This resulted in the complete reversal of chlorsulfuron cross-resistance in the S4 generation (Table 6). In the absence of chlorsulfuron, malathion pretreatment had no effect on survival or biomass in the G0 and S4 generations.

4 DISCUSSION

4.1 Wild radish response to recurrent low-dose 2,4-D amine selection

This study demonstrates that, commencing with only 382 individuals of a herbicide-susceptible wild radish population (G0), there is sufficient genetic variability to evolve an almost ninefold level of 2,4-D resistance following four generations of recurrent low-dose 2,4-D selection (Fig. 1). Given the consistent increases in both the survival and biomass in this study, 2,4-D selection past the fourth generation is considered likely to result in a further increase in 2,4-D resistance.

The evolutionary shifts in 2,4-D resistance evident in this study with the dicot species wild radish concur with previous reports of low-dose herbicide selection in the genetically diverse grass species annual ryegrass.[13–16] Low-dose selection of annual ryegrass with the metabolisable herbicide diclofop-methyl resulted in metabolic herbicide resistance, likely P450 endowed.[29–31] The genetic basis of the 2,4-D resistance in this study is yet to be determined. It is, however, unlikely that a rare, highly resistant, monogenic 2,4-D-resistance-endowing gene trait would pre-exist in the small (382 plants) commencing unselected population used in this study.[32] The progressive increases in resistance observed in the selected populations in this study (Fig. 1) are characteristic of the progressive accumulation and enrichment of minor gene traits, which collectively endow a level of 2,4-D resistance.[13]

The biochemical basis of auxinic herbicide resistance in plants has been notoriously difficult to identify. In an Australian population of 2,4-D-resistant wild radish (WARR20[33]) it has been found that 2,4-D resistance is conferred by the inability of 2,4-D to translocate, with no difference in 2,4-D metabolism measured between resistant and susceptible biotypes.[34] Even though enhanced auxinic herbicide metabolism has not been measured in field-evolved 2,4-D-resistant populations, it is plausible that an outcrossing dicot species such as wild radish could evolve metabolic 2,4-D resistance through the accumulation of minor genes. Conjugates of 2,4-D and MCPA have been identified in several crop species, including: peas (*Pisum sativum*);[35,36] soybean (*Glycine max*),[37] tobacco (*Nicotiana tobacum*)[38] and sunflowers (*Helianthus annum*).[38] However, the amino acid and carboxylic glucoside conjugates of 2,4-D, which predominate in dicots, retain some herbicidal activity and are readily hydrolysed back to parent 2,4-D.[39] Therefore, only dicots that could develop the capacity to form non-hydrolysable phenolic glycosides from 2,4-D would be likely to evolve metabolic resistance

Table 3. Parameter estimates for percentage survival from recurrent low-dose 2,4-D-amine-selected generations, calculated by non-linear regression analysis using the three-parameter log-logistic model [equation (1)]

	Progeny	Selection dose (g ha^{-1})	Population size	Survival(%)	Plants selected	a[a]	b	e (LD_{50}) (g ha^{-1})	P-value[b]	R/S[c]
	G0	–	–	–	–	99.98 (2.26)	1.42 (0.32)	16 (5)		–
2,4-D selected	S1	125	382	71	20	99.99 (0.99)	2.02 (0.24)	41 (5)	<0.05	2.6
	S2	250	396	88	20	99.98 (1.52)	2.24 (0.29)	55 (5)	<0.05	3.4
	S3	250	393	77	20	99.99 (2.72)	1.71 (0.25)	62 (7)	<0.05	3.8
	S4	750	379	76	20	99.93 (2.31)	1.52 (0.09)	138 (7)	<0.05	8.6
Unselected	C1	0	20	–	20	99.99 (1.14)	1.85 (0.19)	22 (2)	0.44	1.4
	C2	0	20	–	20	99.99 (1.77)	1.69 (0.31)	19 (4)	0.50	1.2
	C3	0	20	–	20	99.99 (1.35)	1.76 (0.22)	19 (2)	0.51	1.2
	C4	0	20	–	20	99.99 (1.55)	1.35 (0.28)	14 (3)	0.64	0.9

[a] Standard errors for parameter estimates are in parentheses.
[b] LD_{50} P-value comparing the difference between selected and commencing populations assessed by the SI function in DRC package v.2.5.12 in R v.3.3.0.
[c] R/S values were calculated as the ratio of LD_{50} for the respective 2,4-D-amine-selected (S1 to S4) or control (C1 to C4) populations and the commencing population (G0).

Table 4. Parameter estimates for biomass (g plant^{-1}) from recurrent low-dose 2,4-D-amine-selected generations, calculated using non-linear regression analysis using the three-parameter log-logistic model [equation (1)]

	Population	a[a]	b	e (GR_{50}) (g ha^{-1})	P-value[b]	R/S[c]
	G0	2.76 (0.19)	4.54 (1.01)	60 (6)	–	–
2,4-D selected	S1	2.48 (0.17)	4.05 (0.95)	111 (9)	<0.05	1.8
	S2	2.89 (0.15)	3.76 (0.67)	111 (8)	<0.05	1.8
	S3	2.68 (0.15)	1.92 (0.31)	165 (17)	<0.05	2.8
	S4	2.27 (0.11)	1.07 (0.13)	234 (33)	<0.05	3.9
Unselected	C1	2.30 (0.14)	2.48 (0.46)	64 (7)	0.59	1.1
	C2	2.31 (0.14)	1.86 (0.46)	51 (7)	0.24	0.8
	C3	2.52 (0.15)	3.55 (0.74)	67 (6)	0.58	1.1
	C4	2.12 (0.24)	3.36 (1.17)	60 (9)	0.96	1.0

[a] Standard errors for parameter estimates are in parentheses.
[b] LD_{50} P-value comparing the difference between selected and commencing populations assessed by the SI function in DRC package v.2.5.12 in R v.3.3.0.
[c] R/S values were calculated as the ratio of GR_{50} for the respective 2,4-D-amine-selected (S1 to S4) or control (C1 to C4) populations and the commencing population (G0).

to this herbicide. While resistance to multiple auxinic herbicides has been previously identified (reviewed by Beckie and Tardif[40]), the evolution of cross-resistance between auxinic herbicides as a result of recurrent selection was not investigated in this study.

4.2 Cross-resistance

Along with 2,4-D resistance, there was concomitant evolution of cross-resistance to the chemically dissimilar, metabolisable herbicides chlorsulfuron and metosulam but not the non-metabolisable sulfometuron[8] (Tables 2 and 5). This is consistent with the hypothesis that cross-resistance evolution to certain ALS herbicides from low-dose 2,4-D selection is due to enhanced capacity to metabolise certain herbicides and, understandably, not due to any target-site change in ALS.[41,42] Furthermore, the elimination of chlorsulfuron cross-resistance with the known P450 inhibitor malathion implies the involvement of a P450 enzyme.[27] Malathion is an organophosphate insecticide that has been found effectively to synergise chlorsulfuron activity by inactivating certain P450 enzymes in corn and annual ryegrass.[24,26] The restoration of chlorsulfuron efficacy in the S4 population following malathion pretreatment (1000 g ha^{-1}) (Table 6) indicates the involvement of P450s in catalysing the enhanced metabolism of chlorsulfuron in this study.

4.3 Implication of auxinic herbicide dose for herbicide resistance evolution

When a herbicide is applied at high dose, targeted plant mortality is high. Only very rare individuals with resistance traits outside the population's natural genetic variability for herbicide response will survive.[5,13] However, unlike high doses, reduced herbicide doses (doses that kill most plants but many still survive) result in many survivors, possessing any possible resistance-endowing genes, both weak and strong,[5,43] with weak traits predicted to exist at far higher frequencies in a population.[44] Here we demonstrate that recurrent use of reduced 2,4-D doses for four generations led to the rapid evolution of 2,4-D resistance and cross-resistance to dissimilar, metabolisable ALS herbicides. The evolution of non-target-site, metabolism-based herbicide resistance poses a significant threat to the management of herbicide resistance worldwide, as resistance can rapidly evolve in small populations,

Table 5. Parameter estimates for (A) plant survival (%) and (B) biomass (g plant) following treatment of the G0 and S4 populations with chlorsulfuron, metosulam, imazamox and sulfometuron-methyl. Parameters calculated by non-linear regression analysis using the three-parameter log-logistic model [equation (1)]

(A) Survival

	Biotype	*a*[a]	*b*	*e* (LD_{50}) (g ha^{-1})	*P*-value[b]	R/S[c]
Chlorsulfuron	G0	99.99 (1.14)	6.04 (0.47)	4 (0)		–
	S4	100.41 (4.01)	1.17 (0.13)	18 (2)	<0.05	4.5
Metosulam	G0	99.97 (1.86)	1.95 (0.25)	1 (0)		–
	S4	100.52 (3.34)	1.33 (0.12)	4 (0)	<0.05	4.0
Imazamox	G0	99.99 (1.24)	2.15 (0.21)	5 (0)		–
	S4	99.99 (1.47)	2.34 (0.31)	5 (0)	0.83	1.0
Sulfometuron-methyl	G0	100.47 (2.45)	2.51 (0.19)	2 (0)		–
	S4	100.47 (2.89)	3.02 (0.28)	2 (0)	0.72	1.0

(B) Biomass

	Biotype	*a*[a]	*b*	*e* (GR_{50}) (g ha^{-1})	*P*-value[b]	R/S[c]
Chlorsulfuron	G0	1.14 (0.03)	1.66 (0.83)	8 (2)		–
	S4	1.23 (0.63)	0.58 (0.06)	38 (8)	<0.05	4.8
Metosulam	G0	2.38 (0.08)	2.47 (0.57)	1 (1)		–
	S4	1.48 (0.09)	0.58 (0.15)	7 (2)	<0.05	5.8
Imazamox	G0	2.16 (0.09)	1.33 (0.28)	5 (1)		–
	S4	2.22 (0.06)	1.27 (0.17)	7 (1)	0.53	1.3
Sulfometuron-methyl	G0	1.41 (0.13)	1.95 (0.43)	7 (1)		–
	S4	1.73 (0.05)	2.22 (1.18)	7 (1)	0.87	1.1

[a] Standard errors for parameter estimates are in parentheses.
[b] LD_{50} *P*-value comparing the difference between selected and commencing populations using the SI function in DRC package v.2.5.12 in R v.3.3.0.
[c] R/S values were calculated as the ratio of LD_{50} or GR_{50} parameters for S4 and G0.

Table 6. Parameter estimates for (A) survival (%) and (B) biomass (g $plant^{-1}$) of G0 and S4 plants exposed to a malathion pretreatment prior to chlorsulfuron application. Parameter estimates were calculated by non-linear regression analysis using the three-parameter log-logistic model [equation (1)]

(A) Survival

Biotype	Malathion pretreatment (g ha^{-1})	*a*[a]	*b*	*e* (LD_{50}) (g ha^{-1})	*P*-value[b]	Pretreatment ratio[c]
G0	0	99.99 (1.10)	2.36 (0.66)	3 (1)	–	–
G0	1000	100 (0.29)	2.45 (0.87)	2 (1)	<0.05	0.5
S4	0	100.10 (4.94)	1.02 (0.17)	21 (3)	–	–
S4	1000	99.99 (1.59)	1.42 (0.33)	2 (1)	<0.05	0.1

(B) Biomass

Biotype	Malathion pretreatment (g ha^{-1})	*a*[a]	*b*	*e* (GR_{50}) (g ha^{-1})	*P*-value[b]	Pretreatment ratio[c]
G0	0	0.63 (0.01)	1.69 (0.64)	2 (1)	–	–
G0	1000	0.59 (0.04)	1.61 (0.94)	1 (1)	<0.05	0.7
S4	0	0.97 (0.54)	0.54 (0.19)	41 (8)	–	–
S4	1000	0.86 (0.32)	0.68 (0.63)	4 (1)	<0.05	0.1

[a] Standard errors for parameter estimates are in parentheses.
[b] LD_{50} and GR_{50} *P*-values comparing the difference between selected and commencing populations assessed by the SI function in DRC package v.2.5.12 in R v.3.3.0.
[c] Pretreatment ratios were calculated as the ratio of the LD_{50} or GR_{50} parameters between the malathion-pretreated and non-pretreated plants within both the G0 and S4 generations.

conferring an unpredictable array of cross-resistance traits.[11] This can confound the effectiveness of resistance management strategies such as herbicide rotation or the use of herbicide mixtures.[45,46] This report provides a stark evolutionary warning regarding the consequences of using low herbicide doses on genetically diverse, outcrossing species such as wild radish. With auxinic herbicide-resistant transgenic crops being introduced in North America and elsewhere for the control of rapidly increasing glyphosate-resistant weeds in the Americas,[47,48] it is important that these new technologies are accompanied with appropriate stewardship practices that continually promote the use of high, infrequently applied 2,4-D doses.[16,49,50] A high herbicide dose at the right growth stage and environmental conditions to ensure high mortality, combined with diversity in agroecosystems and weed control tactics, are necessary to preserve the effectiveness of finite herbicide resources such as the auxinic herbicides.[5,51]

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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