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Author for correspondence:
Eric Page, Agriculture and Agri-Food Canada, Harrow Research and Development Centre, 2585 County Road 20, Harrow, ON N0R 1G0, Canada. Email: Eric.Page@agr.gc.ca

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Cross-resistance to photosystem II inhibitors observed in target site–resistant but not in non–target site resistant common ragweed (*Ambrosia artemisiifolia*)

Martin Laforest[1], Marie-Josée Simard[1], Sydney Meloche[2], Lydia Maheux[3], François Tardif[4] and Eric Page[5]

[1]Research Scientist, Saint-Jean-sur-Richelieu Research and Development Center, Agriculture and Agri-Food Canada, Saint-Jean-sur-Richelieu, QC, Canada; [2]Research Technician, Agriculture and Agri-Food Canada, Harrow Research and Development Centre, Harrow, ON, Canada; [3]Research Technician, Saint-Jean-sur-Richelieu Research and Development Center, Agriculture and Agri-Food Canada, Saint-Jean-sur-Richelieu, QC, Canada; [4]Professor, Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada and [5]Research Scientist, Agriculture and Agri-Food Canada, Harrow Research and Development Centre, Harrow, ON, Canada

Abstract

The full spectrum of herbicide resistance in a weed can vary according to the mechanistic basis and cannot be implied from the selective pressure. Common ragweed (*Ambrosia artemisiifolia* L.) is an important weed species of horticultural crops that has developed resistance to linuron based on either target site– or non–target site resistance mechanisms. The objective of the study is to characterize the cross-resistance to metribuzin of linuron-selected biotypes of *A. artemisiifolia* with target site– and non–target site resistance and determine its genetic basis. Crosses were made between two types of linuron-resistant biotype and a linuron-susceptible biotype, and the progeny were further backcrossed with susceptible plants to the third backcross (BC3) generation to determine their responses to both herbicides compared with parental lines. The target site–based linuron-resistant biotype was cross-resistant to metribuzin, and resistance to both herbicides was maintained at the same level in the BC3 line. In contrast, the linuron-selected biotype with a non–target site resistance mechanism was not cross-resistant to metribuzin. In addition, the BC3 lines deriving from the non–target site resistant parents had very low-level resistance. While the target site–resistance trait is maintained through multiple crosses, non–target site based resistance would be lost over time when selection is absent or insufficient to retain all genes involved in resistance as a complex trait. This would imply *A. artemisiifolia* biotypes with different mechanisms would need to be managed differently over time.

Introduction

Common ragweed (*Ambrosia artemisiifolia* L.) is an outcrossing wind-pollinated annual in the Asteraceae family. The species is monoecious and exhibits phenotypic plasticity that includes habitat-driven variation in size, weight, and reproductive capacity (Molina-Montenegro et al. 2011). It is native to North America (Basset and Crompton 1975) and is widespread in eastern Canada, especially in southern Québec and Ontario (Basset and Crompton 1975; Lavoie 2019). *Ambrosia artemisiifolia* is an important weed of field and horticultural crops such as corn (*Zea mays* L.), soybean [*Glycine max* (L.) Merr.], onions (*Allium cepa* L.), and carrots (*Daucus carota* L.) (Bouchard 2006; Delabays et al. 2005; Simard and Benoit 2010; Tyr et al. 2009).

Many herbicides that inhibit photosynthesis at photosystem II (PSII) provide good control of *A. artemisiifolia* in many crops. Linuron is a substituted urea that inhibits photosynthesis by binding to the D1 protein of the PSII complex in the chloroplast at Site A (Q_B) (WSSA/HRAC group 5 [HRAC 2020], formerly group 7 [C2]) (Battaglino et al. 2021; Shaner 2014) and is registered in both Canada and the United States for use in a range of crops such as field corn, soybeans, carrots, potatoes (*Solanum tuberosum* L.), asparagus (*Asparagus officinalis* L.), and parsnips (*Pastinaca sativa* L.) (Anonymous 2019; Ministry of Agriculture and Rural Affairs 2021a, 2021b). Metribuzin, a triazinone herbicide, also inhibits photosynthesis by binding on the D1 protein at Site A (WSSA/HRAC Group 5, formerly C1) but occupies a different region of the receptor (Gardner 1989). Substituted urea and triazinone herbicides were historically classified in different groups based on the frequent lack of cross-resistance between the two groups (Shaner 2014). Metribuzin is registered for use in soybean, potatoes, field corn, transplanted tomatoes (*Solanum lycopersicum* L.), processing carrots, asparagus, lupins (*Lupinus*

angustifolius L.), and fruit trees (Ministry of Agriculture and Rural Affairs 2021a, 2021b) as well as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), lentils (*Lens culinaris* Medik.), peas (*Pisum sativum* L.), chick peas (*Cicer arietinum* L.), and faba beans (*Vicia faba* L.) in North America (Anonymous 2016). The application of either herbicide to actively growing plant tissue results in excess photo-oxidation and plant death (Caverzan et al. 2019).

In Canada, *A. artemisiifolia* resistant to linuron was first reported in carrots in 1999 in the province of Québec (Saint-Louis et al. 2005). A recent survey of carrot fields in southern Quebec documented the widespread occurrence of linuron-resistant *A. artemisiifolia* and the genetic analysis of these populations revealed the presence of both target site (TSR) and non–target site resistant (NTSR) biotypes (Simard et al. 2017). Target-site resistance to PSII inhibitors is conferred by mutations encoded in chloroplast DNA and are maternally inherited (Gronwald 1994). The mutation located in the *psbA* gene that confers resistance to linuron in *A. artemisiifolia* is Val-219-Ile (Simard et al. 2017). Non–target site resistance to PSII inhibitors has been associated with detoxification of herbicides by cytochrome P450 monooxygenases (Ma et al. 2020) or glutathione *S*-transferases (Beckie and Tardif 2012). In addition to linuron, both TSR and NTSR biotypes can be cross-resistant to other PSII inhibitors (Beckie and Tardif 2012; Dumont et al. 2016). The TSR linuron-resistant Powell's amaranth (*Amaranthus powellii* S. Watson) and common purslane (*Portulaca oleracea* L.) biotypes are cross-resistant to metribuzin and the triazine herbicide atrazine, respectively (Dumont et al. 2016; Masabni and Zandstra 1999). Moreover, depending on which *psbA* mutation they have, various linuron-selected resistant biotypes of redroot pigweed (*Amaranthus retroflexus* L.) may also be cross-resistant to other PSII inhibitors such as metribuzin, atrazine, and prometryn (Davis 2014). In addition, a biotype of linuron-resistant *A. powellii*, with the Val-219-Ile D1 substitution is cross-resistant to basically all other PSII inhibitors (Dumont et al. 2016). These observations would therefore suggest that a linuron-selected *A. artemisiifolia* biotype with the same mutation (Val-219-Ile) would also be cross-resistant to metribuzin. Many studies (Battaglino et al. 2021; Gardner 1989; Huppatz 1996; Shipman 1981; Zharmukhamedov and Allakhverdiev 2021) have been and are still being done to better understand the binding nature of the different PSII inhibitors and how they compete with plastoquinone. It is currently still difficult to predict cross-resistance among various chemically unrelated PSII inhibitors, because it would be strongly dependent on particular resistance mechanisms occurring in each biotype. Predictions could be confounded by the presence of uncharacterized non–target site resistance mechanisms in addition to identified target-site mutations causing resistance. Appropriate crosses, segregating populations, and the creation of near-isogenic inbred lines can be used to separate and characterize various resistance mechanisms.

The goal of the present study was to generate inbred lines of *A. artemisiifolia* biotypes with target–site and non–target site resistance to linuron and evaluate resistance levels as well as cross-resistance to metribuzin in both parental and backcrossed lines. We hypothesized that TSR biotypes would be cross-resistant to other PSII inhibitors. Non–target site resistance mechanisms are considered more prone to cross-resistance, because it is a more generalist process thought to involve multiple genes (Yu and Powles 2014). To test these hypotheses, we characterized both TSR and NTSR biotypes for resistance to linuron and tested for cross-resistance to another PSII inhibitor from group 5, metribuzin.

Materials and Methods

Seed Collection and Storage

Ambrosia artemisiifolia involucral achenes (hereafter referred to as "seeds") of biotypes known to be resistant to linuron were collected in southern Québec, Canada, as a part of a previous study (Simard et al. 2017). These resistant biotypes were previously characterized using Lorox® L (Tessenderlo Kerley, Phoenix, AZ, USA) as TSR and NTSR as described by Simard et al. (2017). The TSR biotype contains a valine to isoleucine substitution at position 219. A linuron-susceptible biotype was collected in September 2013 from a soybean field in Ridgetown, ON, Canada (42.27°N, 81.52°W). The susceptibility of this biotype was confirmed by screening with a discrimination dose of linuron of 1,000 g ai ha^{-1} (Lorox®, Tessenderlo Kerley). Collectively, these biotypes were considered the parental germplasm for this study and were referred to as the parental susceptible (PS), parental TSR (PR_{TSR}) and parental NTSR (PR_{NTSR}) biotypes. Before the start of the experiment, a greenhouse seed increase was performed on the PS, PR_{TSR}, and PR_{NTSR} biotypes. In brief, seedlings of resistant biotypes (i.e., PR_{TSR}, PR_{NTSR}) were screened with 1,000 g ai ha^{-1} of linuron, and 4 to 6 surviving individuals of each biotype were selected for a seed increase; a similar number of individuals were also selected for the increase of the PS biotype. Biotypes were placed in separate greenhouse compartments, and all individuals of each biotype were genotyped before anthesis for the presence or absence of Val-219-Ile, as described in the following section. The seeds produced from these increases were stored dry in a controlled seed storage room at 5 C and 50% relative humidity (RH) until needed.

Plant Propagation and Introgression of Linuron-Resistance Traits

Seeds of PS, PR_{TSR}, and PR_{NTSR} were cold stratified to break dormancy and facilitate germination. One hundred seeds of each biotype were placed in a petri dish lined with blotter paper (steel-blue germination blotters, Anchor Paper, St Paul, MN, USA) and moistened with 10 ml of deionized water. Petri dishes were then stored within an aluminum canister at 4 C for 6 wk. Following stratification, 25 seeds of each biotype were transferred to new petri dishes lined with blotter paper moistened with 10 ml of deionized water. The dishes were then placed into a germination cabinet (model G1000, Conviron, Controlled Environment Canada, Winnipeg, MB, Canada) with a 14-h photoperiod, 60% RH, and an alternating temperature of 25/15 C (day/night).

Germinated seeds of the parental biotypes were planted in 4-cm-diameter by 6-cm-tall pots filled with high-porosity potting media (BM6, Berger, Saint-Modeste, QC, Canada). The pots were placed on a bench in a growth cabinet (model TPC-15, Biochambers, Winnipeg, MB, Canada) with a thermoperiod of 25/15 C and a photoperiod of 14 h. The plants were watered daily and fertilized twice a week with 250 ml of a fertilizer blend previously described by Page et al. (2011). When plants reached the 2- to 3-node stage (~10-cm tall), the PR_{TSR} and PR_{NTSR} seedlings were treated with linuron at a dose of 1,080 g ai ha^{-1}. Linuron was applied to the plants using an automated spray chamber (DeVries Manufacturing, Hollandale, MN, USA) equipped with an even-spray nozzle (TeeJet® TP8002E-SS, TeeJet Technologies, Wheaton, IL, USA) set to apply a water volume of 333.3 L ha^{-1} at a pressure of 207 kPA.

Three weeks after linuron application, three surviving PR_{TSR} and PR_{NTSR} plants and six similarly staged PS plants were

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Table 1. Parameters of the dose–response curves for target site–resistant (TSR) and non–target site resistant (NTSR) parental lines (PR), backcrossed lines (BC3), and susceptible (PS) *Ambrosia artemisiifolia* biotypes sprayed with linuron and metribuzin.[a]

Herbicide	Biotype	Slope	Lower[b]	Upper[b]	ED_{50} (SE)[c]	RF[d]
					—g ai ha^{-1}—	
Linuron	PR_{TSR}	2.249^{++}	0.119^{++}	1.342***	971.7*** (264.3)	4.2
	$BC3_{TSR}$	1.68^{++}	0.056^{++}	1.086***	719.6*** (90.7)	3.1
	PR_{NTSR}	3.15^{++}	0.139^{++}	1.074***	944.9*** (193.1)	4.1
	$BC3_{NTSR}$	2.311**	0.092**	0.850***	276.2*** (49.0)	1.2
	PS[e]	5.786^{+}	0.070***	0.808***	230.7*** (30.9)	1.0
Metribuzin	PR_{TSR}	1.535*	0.047^{++}	1.594***	245.1* (110.3)	3.6
	$BC3_{TSR}$	2.497***	0.059	1.014***	333.5*** (29.5)	5.5
	PR_{NTSR}[e]	0.095^{+}	-0.001^{++}	0.850^{+}	0.6^{++} (2.2)	0.1
	$BC3_{NTSR}$[e]	3.580***	0.085***	1.568***	67.9*** (5,2)	1.1
	PS	7.201***	0.062**	1.538***	61.1*** (4.9)	1.0

[a]P-value significance codes: ***, P ≤ .001; **, .001 < P ≤ .01; *, .01 < P ≤ .05; +, .05 < P ≤ .1; ++, .1 < P ≤ 1.
[b]“Lower” and “upper” refer to calculated concentrations (g ai ha^{-1}) for the high and low asymptotes, respectively.
[c]ED_{50}, dose that generates a 50% reduction in biomass.
[d]RF, resistance factor.
[e]Type 1 Weibull (W1.4) model fit; log-logistic (LL.4) model fit when not indicated.

randomly selected for crossing (i.e., three plants to pair up with each parental resistant biotype) and transplanted into medium-sized 1.4-L plastic pots (16.5-cm diameter) filled with BM6 potting mix. The PS plants were used as pollen donors, whereas PR_{TSR} and PR_{NTSR} biotypes were pollen recipients and were emasculated daily. No other *A. artemisiifolia* plants were in the growth chambers at the time crosses were made. Plants were grown under the previously described conditions. Before each cross, leaf tissue was sampled from each individual for genotyping. Lyophilized tissue was ground in a commercial bead mill (SpeedMILL Plus, Analytik Jena AG, Jena, Germany) and genomic DNA was extracted using the NucleoSpin Plant II kit (Machery-Nagel, Düren, Germany) following the supplied protocol. Eluted DNA was amplified by polymerase chain reaction (PCR) for sequencing. A 767-bp fragment was amplified using the following primers: forward 5′-AGCTGCGACTGCTGTTT-3′, and reverse 5′-ACACGCAAATCGAACCAAAC-3′. Reaction conditions were as follows: an initial denaturation at 95 C for 1 min, 35 cycles of 95 C for 15 s, 56 C for 15 s for annealing, 72 C for 30 s, followed by a final extension at 72 C for 7 min. Following PCR, the samples were cleaned for sequencing using GenepHlow Gel/PCR kit (FroggaBio, Concord, ON, Canada) according to the provided protocol. Sanger sequencing of the PCR products was carried out by the London Regional Genomics Centre (Robarts Research Institute, London, ON, Canada) using the same primers from the PCR amplification. Alignment to the *A. artemisiifolia psbA* reference sequence (GenBank accession number: AB427162.1) was performed using Sequencher software (Gene Codes, Ann Arbor, MI, USA) and analyzed for the presence of the Val-219-Ile target-site mutation. When combined, results from Sanger sequencing and the initial screening with linuron ensured that the individuals used in all crosses were representative of their initial parental biotypes (e.g., lack of Val-219-Ile in PR_{NTSR} but survival at discriminating dose).

This screening, genotyping, and crossing procedure was repeated for both the TSR and NTSR lines during the creation of each generation. For example, following the original cross of the PS and PR_{TSR} and PR_{NTSR} biotypes, seeds of the progeny lines (i.e., the $F1_{TSR}$ and $F1_{NTSR}$, respectively) were germinated, grown, and screened with linuron as described earlier. Six individual of PS were once again propagated and matched up with three individuals of each of the $F1_{TSR}$ and $F1_{NTSR}$, and all were genotyped for the presence or absence of Val-219-Ile using Sanger sequencing. As *psbA* is maternally inherited, the PS individuals were used as pollen donors, and crosses were carried out to generate the $BC1_{TSR}$ and $BC1_{NTSR}$ progeny, respectively. This process was repeated with the $BC1_{TSR}$ and $BC1_{NTSR}$ and the PS to create the $BC2_{TSR}$ and $BC2_{NTSR}$, and similarly repeated again to create the $BC3_{TSR}$ and $BC3_{NTSR}$ lines, which were used in the dose–response assays described in the following section.

Dose–Response Curves

The initial experiment started in October 2020. Seeds from the five lines (PS, PR_{TSR}, $BC3_{TSR}$, PR_{NTSR}, and $BC3_{NTSR}$) generated earlier were placed in moist potting media in trays in a refrigerator under constant darkness for 6 wk at a temperature of 5 C to break dormancy. On December 2, seeds were planted in multicelled trays and placed in a growth chamber under a 14-h day at 25 C and a 10-h night at 10 C for 2 wk, with RH kept at 75% and no fertilizer added before being transferred to the greenhouse. The experiment included the five lines, two herbicides (linuron and metribuzin), seven doses (0X, 0.25X, 0,5, 1X, 2X, 4X, and 12X) and four replicates of five plants. The 1X dose was set at 1,080 g ai ha^{-1} of linuron based on postemergence single doses in carrots, and 375 g ai ha^{-1} of metribuzin (TriCor 75 DF, United Phosphorus, King of Prussia, PA, USA) based on the early postemergent dose in carrots and compared with the untreated control. Based on the results of the initial experiment, a second trial was done to generate data at lower doses allowing an improved evaluation of the resistance factor. This second trial started in April 2021. Plants from only three lines (PS, $BC3_{TSR}$, and $BC3_{NTSR}$) were grown, as there were not enough seeds from the resistant parental lines left. The second experiment included three biotypes with the same herbicides (linuron and metribuzin), six doses (0X, 0.0625X, 0.125X, 0.25X, 0.5X, and 1X), and four replicates of five plants.

For both experiments, seedlings were grown in the greenhouse under a 14-h day at 22 C and a 10-h night at 12 C. Plants were watered daily and were fertilized with a solution of 150 ppm of 20:8:20 (N:P:K) (4 d wk^{-1}) and 14:0:14 (N:P:K) (1 d wk^{-1}). Trays were distributed in a completely randomized design and re-randomized every week to eliminate positional

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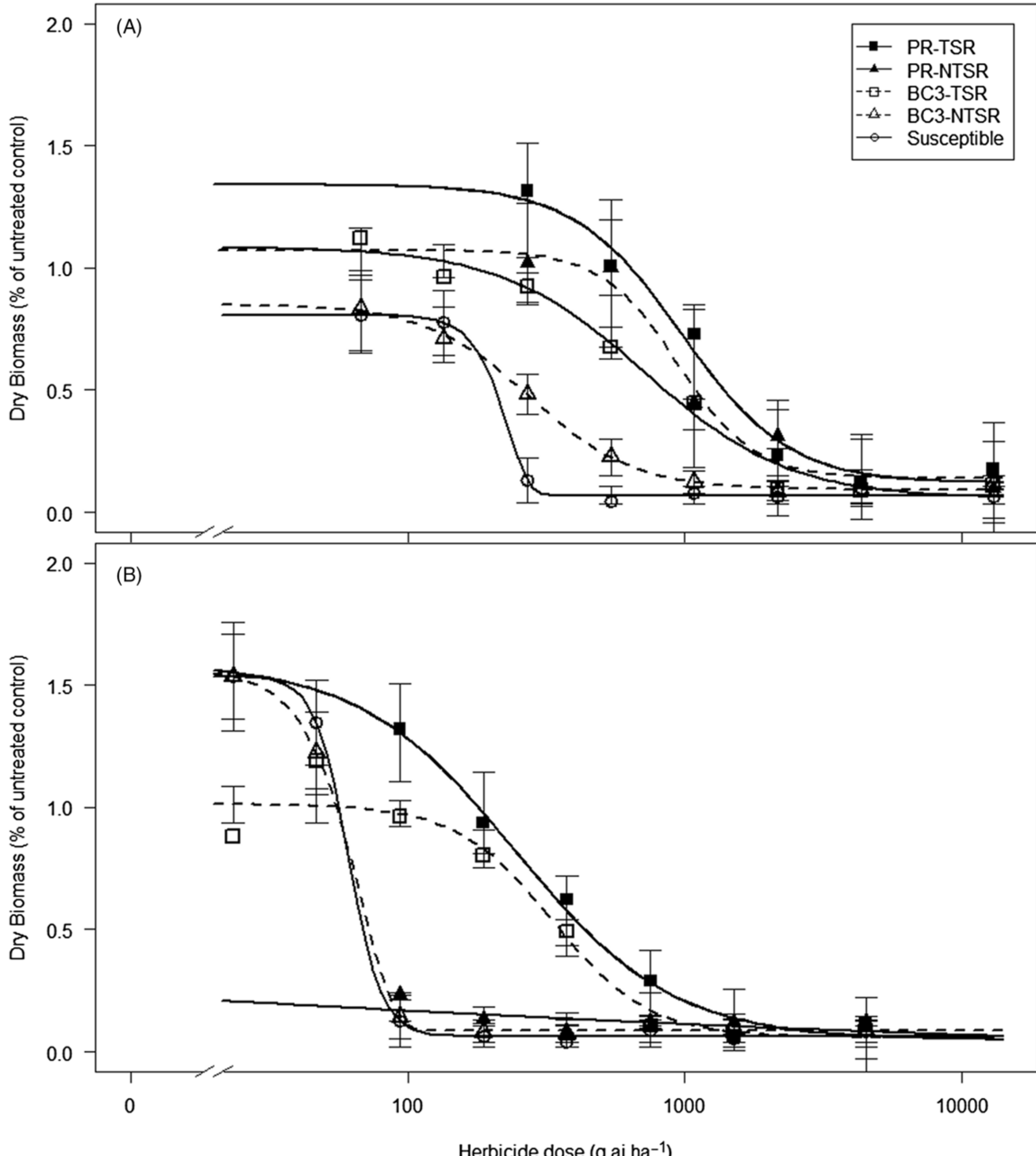

Figure 1. Dose–response curves for the susceptible and four biotypes of original and backcrossed progeny of *Ambrosia artemisiifolia* treated with different doses of linuron (A) and metribuzin (B). Each point is the average of four replicates and two runs. Bars represent 95% confidence intervals. PR-TSR, parental target site–resistant biotype; PR-NTSR, parental non–target site resistant biotype; BC3-TSR, target site–resistant backcrossed line; BC3-NTSR, non–target site resistant backcrossed line.

bias. At the 1- to 2-node stage, control plants (0X) were left unsprayed, while the other plants were sprayed with one of six rates of linuron or metribuzin. Herbicide applications were done using a track sprayer (DeVries Manufacturing) calibrated to deliver 280 L ha^{-1} of herbicide solution at 207 kPa using the building's air-pressure system (for laboratories) and a TeeJet® TP8001E spray tip. Visual assessments were made 14 and 28 d after application, and the percentage of visible damage was based on a scale of 0 (identical to untreated control plants) to 100 (completely dead) (Brown and Farmer 1991). The aboveground biomass of all plants was collected at 28 d after application, dried for 5 d at 70 C, and weighed.

Statistical Analysis

Dry biomass weight relative to the mass of untreated controls was used to create dose–response curves with DRC (Ritz et al. 2015) in R (R Core Team 2020). The four-parameter log-logistic model [$f(x) = c + (d - c)/(1 + \exp\{b[\log(x) - \log(e)]\})$]was used, except when the data could not be fit. In these cases, the four-parameter Weibull [type 1 (W1.4); $f(x) = c + (d - c)\exp(-\exp\{b[\log(x) - \log(e)]\})$] was used (indicated in Table 1). In these equations, c and d are the upper and lower asymptotes, respectively; b is the slope; and e is the effective dose (ED_{50}). Resistance factors were calculated as the ratio of the ED_{50} (dose that generates a 50% reduction in

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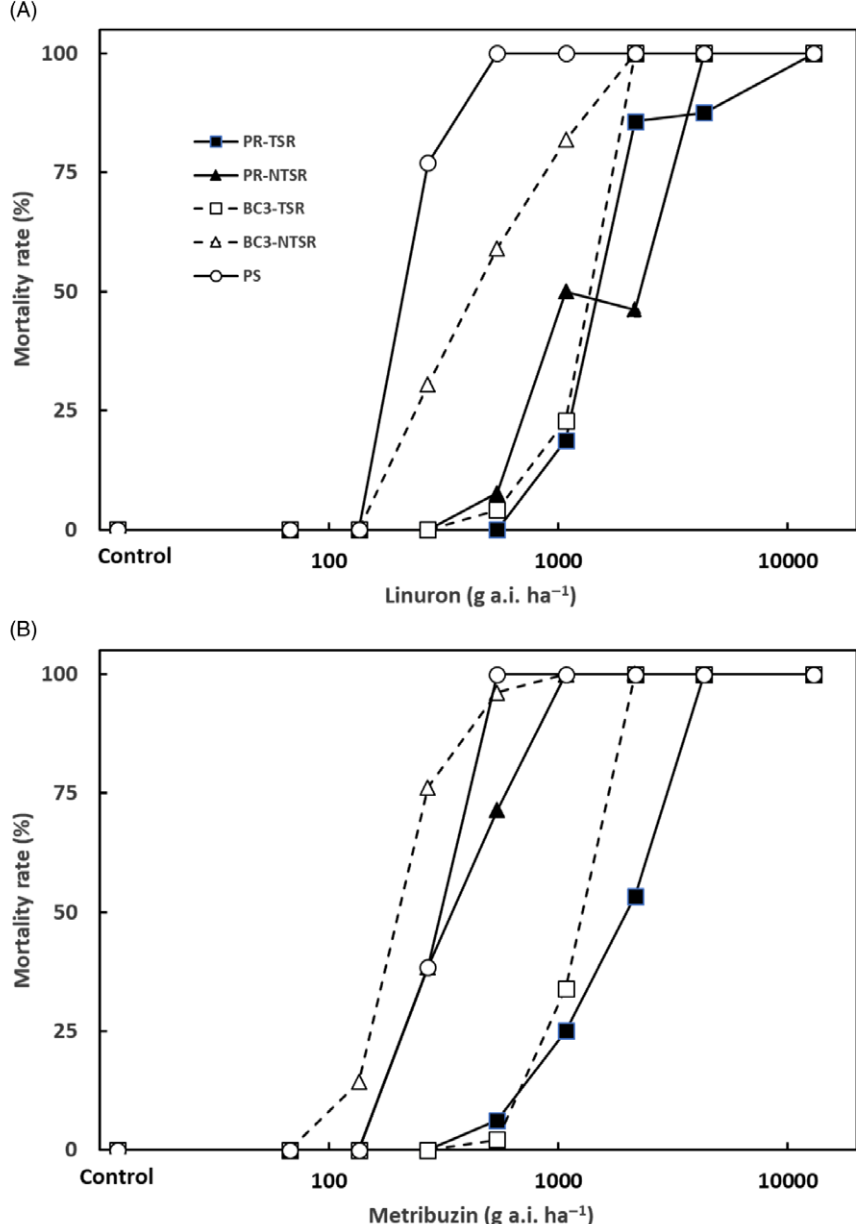

Figure 2. Herbicide-induced mortality rate (visible injury ≥80%) for the five biotypes after treatment with linuron (A) and metribuzin (B). Each point is the average of four replicates and two runs. PR-TSR, parental target site–resistant biotype; PR-NTSR, parental non–target site resistant biotype; BC3-TSR, target site–resistant backcrossed line; BC3-NTSR, non–target site resistant backcrossed line; PS, parental susceptible.

biomass) of resistant over susceptible biotypes (R/S) (Knezevic et al. 2007).

Results and Discussion

Resistance in the Parental Lines

Based on the dose–response curves and the resistance factors (RFs), both parental resistant lines were resistant to linuron (Figure 1A), as expected, and the percent survival (damage rating ≥80%) at the labeled rate in carrots was close to 100% and 92.31% for TSR and NTSR biotypes, respectively (Figure 2A). These two biotypes were initially selected on the basis of their resistance to this herbicide, which was determined using a single labeled rate and molecular markers (for the TSR biotype) (Simard et al. 2017). Levels of resistance were low, as defined by Beckie and Tardif (2012), and equivalent (PR_{TSR}: 4.2; PR_{NTSR}: 4.1) between both parental biotypes (Table 1). It is generally assumed that target-site resistance confers higher levels of resistance (Sammons and Gaines 2014; Yu and Powles 2014), but this is not always the case, and as more NTSR biotypes are discovered, equal or even higher levels of resistance are being observed (Deng et al. 2021).

In the present study, PR_{TSR} was observed to possess cross-resistance to metribuzin (RF = 3.6; Table 1) and survival at the labeled dose was equivalent to that observed for linuron (93.75%) (Table 1; Figure 2A). In contrast, the NTSR parental line (PR_{NTSR}) was susceptible to metribuzin, with an ED_{50} value similar to PS and no survival at the labeled dose (0% for both) (Figures 1B and 2B). These results are not unexpected, as the same mutation (Val-219-Ile) confers cross-resistance to multiple PSII inhibitors in

A. powellii (Dumont et al. 2016), *A. retroflexus* (Davis 2014), kochia [*Bassia scoparia* (L.) A.J. Scott] (Mengistu et al. 2005), and annual bluegrass (*Poa annua* L.) (Mengistu et al. 2000). Until the most recent HRAC update (HRAC 2020), metribuzin and linuron were classified into separate groups (e.g., Groups 5 and 7, or C1 and C2, respectively). Our results support this reclassification based on the cross-resistance conferred by the Val-219-Ile mutation. Interestingly, cross-resistance could also have been expected for PR_{NTSR} biotype, because this type of resistance is usually conferred by a more generalist mechanism, such as enhanced herbicide metabolisms that can provide resistance to multiple herbicides with very different sites of action (Dimaano et al. 2020; Yu and Powles 2014). The *A. artemisiifolia* case reported herein does not display cross-resistance to metribuzin, suggesting that cross-resistance can equally be endowed by target site– or non–target site resistance mechanisms and that it is the nature of the interaction between the active ingredient and the conformation of the protein providing resistance, either the target site or proteins involved in other detoxifying mechanisms, that will determine cross-resistance. The observed cross-resistance in this case is related to the Val-219-Ile mutation. The unknown mechanism involved in the non–target site resistance of the PR_{NTSR} biotype appears to be specific to linuron, though it should be acknowledged that we tested only two herbicides. It would be interesting to evaluate whether the mechanism confers resistance to other Ser-264 binders (Group 5: ureas, amides, triazines, triazinones, phenylcarbamates, pyridazinones, and uracils) and the His-215 binders (Group 6: benzothiadiazinones, nitriles, and phenyl-pyridazines) (Battaglino et al. 2021).

Resistance in the Third Generation of Backcrosses

Results of our study indicate that resistance to linuron was relatively stable over generations for TSR biotypes but not for NTSR biotypes. For TSR biotypes, the RF declined slightly from 4.2 in PR_{TSR} to 3.1 in the $BC3_{TSR}$ biotype (Table 1). This result was expected, as the mutation conferring linuron resistance is maternally inherited and was tracked through Sanger sequencing during the creation of each generation. In contrast, the RF of 1.2 for the $BC3_{NTSR}$ was notably less than the 4.1 observed for the PR_{NTSR}. Such a result could be explained by a multigenic resistance mechanism, as observed in multiple studies (Preston 2004; Preston et al. 1996; Yuan et al. 2007). At each generation and depending on the herbicide dose used for discriminating susceptible from resistant offspring, alleles from genes contributing to the full extent of resistance of the parental line may be lost. This observation stresses the importance of using the appropriate dose for selection of resistant plants during the several generations of backcrossing, running the risk of losing genes responsible for minor effects if selection is not strong enough. Depending on the number of genes responsible for resistance, an exponential number of offspring may also need to be screened to find candidates with the optimal combination of alleles that results in the maximum level of resistance, keeping in mind that contributing alleles could also come from the susceptible parent. These results suggest that a similar decline in the level of resistance could occur over time in natural populations in which resistance is multigenic and susceptible alleles are present at high frequency, but the speed of this potential decline is likely to depend on other factors.

Cross-resistance to metribuzin and linuron is conferred by a target-site mechanism for the *A. artemisiifolia* biotype reported herein. A second biotype, also resistant to linuron but with no mutations in the target site and with an undefined non–target site resistance mechanism, is not cross-resistant to metribuzin. In both cases, the resistance level is rather low, requiring below a fifth of the label rate to reduce biomass by 50%. Interestingly, both mechanisms initially share approximately the same level of resistance. While resistance development depends on the number of individuals in the population and its genetic diversity, the level of resistance gained in such a process could also be in part attributed to the intensity of selection pressure in addition to chance.

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