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Mechanisms of glyphosate resistance in common ragweed (*Ambrosia artemisiifolia*): patterns of absorption, translocation, and metabolism

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Abstract

Glyphosate's efficacy is influenced by the amount absorbed and translocated throughout the plant to inhibit 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS). Glyphosate resistance can be due to target-site (TS) or non–target site (NTS) resistance mechanisms. TS resistance includes an altered target site and gene overexpression, while NTS resistance includes reduced absorption, reduced translocation, enhanced metabolism, and exclusion/sequestration. The goal of this research was to elucidate the mechanism(s) of glyphosate resistance in common ragweed (*Ambrosia artemisiifolia* L.) from Ontario, Canada. The resistance factor for this glyphosate-resistant (GR) *A. artemisiifolia* biotype is 5.1. No amino acid substitutions were found at positions 102 or 106 of the EPSPS enzyme in this *A. artemisiifolia* biotype. Based on [^{14}C]glyphosate studies, there was no difference in glyphosate absorption or translocation between glyphosate-susceptible (GS) and GR *A. artemisiifolia* biotypes. Radio-labeled glyphosate metabolites were similar for GS and GR *A. artemisiifolia* 96 h after application. Glyphosate resistance in this *A. artemisiifolia* biotype is not due to an altered target site due to amino acid substitutions at positions 102 and 106 in the EPSPS and is not due to the NTS mechanisms of reduced absorption, reduced translocation, or enhanced metabolism.

Introduction

Glyphosate is a nonselective, broad-spectrum, herbicide widely used for weed management in glyphosate-resistant (GR) crops. Glyphosate's efficacy can be attributed to its rapid absorption, efficient translocation, slow degradation, and unique mode of action. Glyphosate is a systemic herbicide that moves throughout the plant and binds to 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS), stopping the production of aromatic amino acids resulting in plant death (Powles and Preston 2006). Glyphosate's target site, EPSPS, is located primarily in plastids, so glyphosate must enter the plant and be translocated to these highly active sites (Baylis 2000). Understanding glyphosate's movement and target site within the plant has also led to a better understanding of glyphosate's resistance mechanisms.

Following many years of widespread glyphosate use, resistance was first documented in 1996 in Australia (Powles et al. 1998) and has now been confirmed in 53 species worldwide (Heap 2021), including seven GR weeds in Canada, five of which are in Ontario. The resistance mechanisms for GR Canada fleabane [*Conyza canadensis* (L.) Cronquist], kochia [*Bassia scoparia* (L.) A.J. Scott], and waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] are well understood; however, the resistance mechanism for GR common ragweed (*Ambrosia artemisiifolia* L.) has not been characterized. Herbicide resistance can be due to target-site (TS) and non–target site (NTS) mechanisms (Gaines et al. 2020). TS resistance includes an altered target site and target-site gene overexpression, while NTS resistance includes reduced absorption, reduced translocation, enhanced metabolism, and herbicide exclusion/sequestration (Devine and Eberlein 1997; Nol et al. 2012; Powles and Preston 2006; Shaner 2009; Wakelin et al. 2004; Yu et al. 2009). In GR *C. canadensis*, Dinelli et al. (2006) found that reduced glyphosate translocation, higher levels of mRNA encoding for EPSPS, and production of new leaves all contributed to survival; however, as these experiments were conducted on different plants from the bulk

sample, it is unknown whether each plant possessed all three of these resistance mechanisms (Dinelli et al. 2006). In Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot], glyphosate resistance was due to either reduced translocation or an altered target site in two separate populations (Perez-Jones et al. 2007). As herbicide efficacy relies on its ability to interrupt plant function, any alterations in the plant that result in a decrease in herbicide movement to the target site or prevent binding to the target will allow for plant survival and poor weed control.

In most cases, herbicides inhibit the activity of key enzymes in a vital plant pathway required to produce an essential molecule or the neutralizing of threats to a plant. Therefore, plants can escape the activity of these herbicides by either preventing the herbicide from reaching the target enzyme at a lethal dose, by altering the target site so that the herbicide cannot bind, or by increasing the production of the target enzyme. An altered target site may decrease the efficiency of the plant pathway, resulting in a fitness cost such as reduced photosynthetic capability, leading to a decreased growth rate (Masabni and Zandstra 1999; McCloskey and Holt 1990). A mutation in the EPSPS gene has been reported as a mechanism of glyphosate resistance in goosegrass [*Eleusine indica* (L.) Gaertn.], *C. canadensis* (Page et al. 2018), and *L. perenne* ssp. *multiflorum* (Baerson et al. 2002; Beres et al. 2020; Gaines et al. 2020; Kaundun et al. 2008; Ng et al. 2003, 2004; Perez-Jones et al. 2007; Sammons and Gaines 2014). A mutation within the target site of the herbicide allows the plant pathway to escape inhibition by the herbicide.

Reduced absorption, reduced translocation, and enhanced glyphosate metabolism would allow plants to survive following glyphosate applications due to sublethal doses reaching the target site. Glyphosate may be prevented from reaching its target site at a lethal dose through a number of mechanisms. Reduced absorption occurs in species naturally tolerant to glyphosate and in GR johnsongrass [*Sorghum halepense* (L.) Pers.] (Nandula et al. 2008) and GR *L. perenne* ssp. *multiflorum* (Michitte et al. 2007). Glyphosate is sequestered in the vacuole of *C. canadensis*, preventing the herbicide from reaching the active site at a lethal dose (Ge et al. 2010). Transporters within the plant may also aid in excluding glyphosate from the cell or chloroplast, preventing glyphosate interaction with EPSPS (Pan et al. 2021). Another mechanism of resistance is enhanced metabolism. In an Australian population of junglerice [*Echinochloa colona* (L.) Link], a higher expression of aldo-keto reductase was observed in the GR biotype compared with the GS plants (Pan et al. 2019). The breakdown of the herbicide into its non-herbicidal metabolites before it reaches the target enzyme results in a lack of a complete molecule to bind to the target site.

GR *A. artemisiifolia* was first confirmed in the United States in 2004 in Missouri and Arkansas (Brewer and Oliver 2009; Pollard et al. 2004); it was subsequently confirmed in 2011 in Ontario, Canada (Van Wely et al. 2015). While some studies have been conducted on the Jackson county, AR, population to determine the mechanism of glyphosate resistance in *A. artemisiifolia* (Brewer and Oliver 2009), the mechanism has not been determined for the Ontario population. The studies by Brewer and Oliver (2009) found that resistance was not due to an insensitive target site, reduced absorption, or reduced translocation and that additional investigation was needed. Research completed on a Nebraska GR *A. artemisiifolia* biotype found that there were no mutations at the 102 or 106 residues in the *EPSPS* sequence, no variation in *EPSPS* copy number, and no difference in ^{14}C absorption between the susceptible and resistant biotypes (Ganie et al. 2017); however, there were differences in translocation that require further investigation (Ganie et al. 2017). The objective of this research was to explore both TS and NTS mechanisms of resistance in GR *A. artemisiifolia* from Ontario, Canada, including TS resistance due to alterations at positions 102 and 106 of the EPSPS enzyme, differential absorption, differential translocation, and enhanced metabolism using [^{14}C]-labeled glyphosate.

Materials and Methods

For all experiments, *A. artemisiifolia* seed was collected in 2012 from a site near Merlin, ON (glyphosate-susceptible [GS]) and a site with previously confirmed GR *A. artemisiifolia* near Windsor, ON (GR) (Van Wely et al. 2015). Seed for the resistant biotype in these experiments was collected from established herbicide trial plots in soybean [*Glycine max* (L.) Merr.], which had been sprayed with glyphosate at 900 g ae ha^{-1}. Seed was collected in bulk paper bags, with seeds from multiple plants in each bag to obtain a large number of seeds from the research site.

Seed was deposited into nylon mesh bags and then placed in wet sand in a refrigerator at 4 C for 2 mo to break dormancy. Greenhouse transplant trays were filled with soilless mixture (Sunshine Professional Growing Mix, Sun Gro Horticulture, Agawam, MA, USA) and watered. *Ambrosia artemisiifolia* seed was spread on the soil surface and covered with a thin layer of soilless mixture. Trays were watered and placed in a greenhouse with a 16-h photoperiod and day/night temperatures of 25/18 C. At 3 to 4 wk following germination, *A. artemisiifolia* seedlings at the 2-leaf stage were transplanted into individual 10-cm-diameter round pots.

Resistance Factor

When *A. artemisiifolia* plant height was an average of 10 cm, glyphosate was applied at 0, 14, 28, 56, 112, 225, 450, 900, 1,800, and 3,600 g ae ha^{-1} for the GS biotype and 0, 225, 450, 900, 1,800, 3,600, 7,200, 14,400, and 28,800 g ae ha^{-1} for the GR biotype. Applications were made in a 1.80-m spray-length spray chamber fit with a flat-fan nozzle calibrated to deliver 200 L ha^{-1} at 280 kPa while moving at 2.15 km h^{-1}. Following herbicide application, *A. artemisiifolia* was left to dry and then returned to the greenhouse. Aboveground biomass was harvested at 35 DAT, and dry weights were taken. Results were analyzed using SAS 9.2 PROC NLIN, using a sigmoidal log-logistic equation:

$$Y = C + (D - C)/\{1 + \exp[B(\ln(\text{dose}) - \ln(\text{GR}_{50}))]\} \quad [1]$$

where Y is % *A. artemisiifolia* biomass of the untreated control at 35 DAA, C is the lower limit, D is the upper limit, B is the slope at the inflection point, and GR_{50} is the dose at which there is a 50% response. The resistance factor was then calculated by dividing the GR_{50} dose of the resistant biotype by the GR_{50} dose for the susceptible biotype.

Altered Target Site

Glyphosate (900 g ha^{-1}) was applied to 10-cm-tall GR *A. artemisiifolia* to confirm that each individual plant was resistant to glyphosate. Applications were made as described for the dose response. Fresh plant tissue was collected from 10 GS and 10 GR *A. artemisiifolia* plants, and DNA was extracted using the FastDNA® SPIN Kit (MP Biomedicals, Solon, OH, USA) following the protocol provided. DNA was run on the Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) to assess DNA concentration and then

diluted to 5 ng μl^{-1}in water. Forward and reverse primers were designed based on primers used by Baerson et al. (2002). The sequence generated by using these primers with these *A. artemisiifolia* biotypes were then compared with known EPSPS sequences and were confirmed to cover positions 102 and 106 of the *EPSPS* gene. The final primers used were forward: 5′-AATTAAAAGGGCAGTTGTGGAA-3′; and reverse: 5′-GAA AGCAGGAGAATATATCAACATACC-3′.

A PCR master mix was made according to the number of samples with 2 µl 10X buffer, 14.4 µl water, 0.5 µl of deoxynucleotide triphosphates (dNTPS), 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), and 0.1 µl of Hot Taq per sample; 2 µl of diluted DNA (5 ng μl^{-1}) was then added to each sample for a total DNA quantity of 10 ng.

The PCR conditions started with a 15-min initialization period at 95 C, followed by 40 cycles of 30-s denaturation phase at 95 C/60-s annealing phase at 62 C/30-s extension phase at 72 C. This was followed by a final extension cycle for 7 min at 72 C, then the reaction was terminated and samples were held at 4 C. The samples were then run at 100 V for 1 h on a 2% agarose gel containing ethidium bromide to visually estimate the length of the PCR product and compare it with the expected product length based on the primer pairs.

DNA from 10 GS and 10 GR *A. artemisiifolia* plants was run in the PCR master mix using the reaction conditions described earlier. PCR products were then added to a sequencing reaction and sequenced at Agriculture and Agri-Food Canada. GS *A. artemisiifolia* sequences were then compared with GR *A. artemisiifolia* sequences, as well as the GS and GR *E. indica* sequences where the nucleotide substitution resulting in an amino acid change and resistance were previously identified.

Absorption and Translocation Experiments

GS and GR *A. artemisiifolia* plants were transplanted into a Turface soilless mix (Turface Athletics, Buffalo Grove, IL, USA) in a growth chamber with a 16:8-h photoperiod of and a day/night temperature regime of 25/20 C. At the 6-leaf stage, the oldest pair of leaves were removed 2 d before treatment. Plants were sprayed over the top with glyphosate (900 g ha^{-1}; Roundup WeatherMax®, Bayer Crop Science, St. Louis, MO, USA) using a single-nozzle track sprayer (DeVries Manufacturing, Hollandale, MN, USA) equipped with a 11002 EV nozzle (TeeJet® Technologies, Rockford, MI, USA) calibrated to deliver 180 L ha^{-1}. The second-youngest, fully expanded leaf was covered with aluminum foil to avoid double treating that leaf. Immediately following the whole-plant glyphosate application, the aluminum foil was removed and the covered leaf was treated with ten 1-µl droplets of radiolabeled glyphosate (specific activity 50 mCi mM^{-1}). The radiolabeled treatment solution was achieved by combining 1,850 MBq with cold glyphosate. Each plant was treated with 3,666 Bq of radiolabeled glyphosate.

Plants were harvested at 6, 12, 24, and 48 h after treatment (HAT). Each plant was divided into the treated leaf, apex, opposite leaf, below treated leaf, stem, and roots. The treated leaf was washed in a 10% (v/v) ethanol and 0.5% (v/v) Tween 20 mixture and then dried. Ecolite liquid scintillation cocktail (ICN Biomedicals Inc., Irvine, CA, USA) was added to determine non-absorbed radioactivity by liquid scintillation spectrometry (LSS) using a Beckman LS6K-SC scintillation counter (Packard Tri-Carb, Model 2500 TR, Packard Instrument Co., Meriden, CT, USA) to determine radioactivity. The dried treated leaf and the rest of the plant parts were then wrapped in tissue, and radioactivity was determined using a biological sample oxidizer (Harvey Instruments OX-300, R.J. Harvey Instrument Co., Tappan, NY, USA). The experiment was repeated three times, with four repetitions per time point per biotype. Accumulation and distribution of radioactivity over the 48-h time course was analyzed using SAS 9.4, PROC NLMIXED, using the exponential to a maximum model:

$$Y = a^{*}[1 - \exp(-b(x - x_0))] \quad [2]$$

where a is the maximum rate, b is the regression coefficient of the nonlinear equation, and x is the number of hours.

Metabolism Experiments

Greenhouse-grown plants of similar size at the 4-leaf stage were selected for the metabolism experiment and transferred to a growth chamber. Plants were treated with 3,333 Bq of [^{14}C]glyphosate in 10 µl applied as five 1-µl droplets on the second set of fully expanded leaves. Plants were harvested at 96 HAT. The treated leaf was washed and dried, and the whole plants were stored in a freezer until the extraction phase, as previously described.

Plant tissue was transferred to 50-ml glass test tubes and extracted with 10 ml of 90% methanol solution by homogenization for 30 s (Fischer Scientific Power Gen 125, Life Technologies Corporation, CA, USA). Tubes were placed on a shaker for 24 h. The extraction solution was quantitatively transferred to 50-ml centrifuge tubes with 0.45-µm filters and centrifuged for 10 min at 1,500 × g (Whatman, VectaSpin 20™, Cytiva, Marlborough, MA, USA)A 1-ml subsample of the extraction solution that passed through the filter was subjected to LSS to establish the total amount of soluble radioactivity. The extraction solution was concentrated under vacuum for 6 to 8 h at a vortex speed of 22 rpm and a temperature of 35 C (Labconco RapidVac, Kansas City, MO, USA). When approximately 5 ml of extraction solution remained, it was passed through a 0.2-µM filter. Samples were allowed to evaporate to dryness and resuspended in 500 µl of 100 µM phosphoric acid. Samples were passed through another 0.2-µM filter. To determine total radioactivity, 100 µl was analyzed by LSS, while 200 µl was analyzed by reverse-phase high-performance liquid chromatography (HPLC) (Hitachi 7000 Series HPLC system, Hamilton PRP-100, 15 cm by 2.2 mm column, Hamilton Company, Reno, NV, USA) combined with flow-through radioactivity detection (Beta Ram radioactive detector, LabLogic, Tampa, FL). HPLC analysis was HPLC-grade water adjusted to pH 2.2 using phosphoric acid isocratically for 7 min at a flow rate of 0.3 ml min^{-1}, followed by 30% methanol with 100 mM phosphoric acid at 0.5 ml min^{-1} for 4 min. The column was allowed to re-equilibrate for 9 min.

Results and Discussion

Resistance Factor

The use of a dose–response curve using the putative resistant and known susceptible biotypes provides information on the resistance level plus it provides insight into the discriminating dose for determining resistant and susceptible populations within that species for that particular herbicide (Burgos et al. 2013). The resistant factor for the GR *A. artemisiifolia* in Ontario was similar to the previously reported resistance factor for GR weeds from other jurisdictions. For the two Ontario, Canada, *A. artemisiifolia* populations, the glyphosate GR_{50} doses for the GS and GR populations

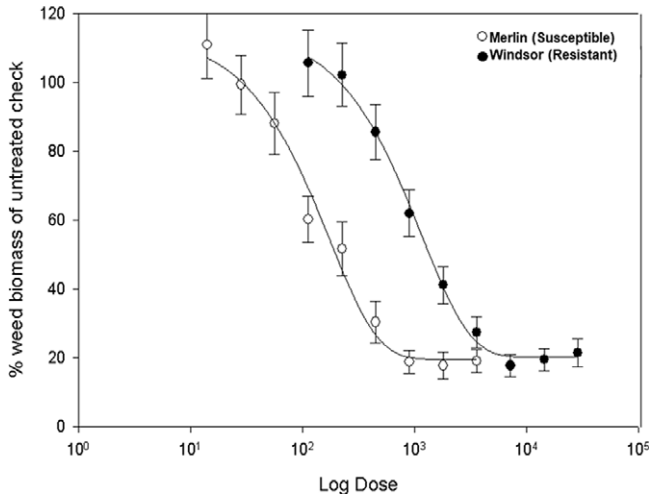

Figure 1. Dose–response curves of greenhouse studies in Ridgetown, ON, Canada, comparing susceptible (Merlin, ON, glyphosate-susceptible [GS]; $n = 16$) and resistant (Windsor, ON, glyphosate-resistant [GR]; $n = 27$) *Ambrosia artemisiifolia*. Weed biomass is expressed as a percentage of the untreated control.

Population	C^a	D^b	B^c	$GR_{50}50^d$
Merlin	15.0 (0.30)	82.5 (0.61)	1.8 (0.71)	201 (46.5)
Windsor	12.8 (0.18)	83.6 (0.72)	1.5 (0.45)	1018 (232.6)

[a]*C* is the lower limit.
[b]*D* is the upper limit.
[c]*B* is the slope of the line.
[d]GR_{50} is the dose at which there is a 50% response.

were 201 (±46.5) g and 1,018 (±232.6) g ae ha^{-1}, which yield a resistance factor of 5.1 (Figure 1). In a study completed by Nandula et al. (2013), GS and GR *A. tuberculatus* had GR_{50} values of 280 and 1,280 g ha^{-1}; none of the GS plants survived glyphosate at 840 g ha^{-1}, resulting in a 5-fold resistance factor similar to this study. Brewer and Oliver (2009) reported a resistance factor of 3.7 in Arkansas *A. artemisiifolia* populations, while a Missouri GR *A. artemisiifolia* population had a resistance factor of 9.6 (Pollard et al. 2004).

This level of resistance is similar to what has been reported with other GR species. In *C. canadensis*, hairy fleabane [*Conyza bonariensis* (L.) Cronquist], rigid ryegrass (*Lolium rigidum* Gaudin), and *L. perenne* ssp. *multiflorum*, populations with reduced translocation as the resistance mechanism reported ranges in resistance factors from 3- to 10-fold when comparing GR and GS biotypes (Wakelin et al. 2004; reviewed by Shaner 2009). Similarly, GR populations of *E. indica*, *L. perenne* ssp. *multiflorum*, and *L. rigidum* with an altered target site as the mechanism of resistance had a range in resistance factorsfrom 2- to 4-fold (Baerson et al. 2002; Wakelin and Preston 2006). While resistance factors can be reflective of a resistance mechanism for some herbicides, this is not always the case.

Altered Target Site

The most common mutations that have been reported are at positions 102 and 106 of the EPSPS enzyme (Gaines et al. 2020). Therefore, this area was the focus in sequencing the *EPSPS* gene in the Ontario *A. artemisiifolia* populations. GS and GR *A. artemisiifolia* had the same amino acid sequence, and any changes in the third nucleotide position of the codon did not result in a change in amino acid sequence. Because the complete *EPSPS* gene was not sequenced, we cannot be certain that there is not a nucleotide substitution at another location that may result in resistance, and further studies should be completed in the future to look at the full *EPSPS* gene, as well as its expression levels.

GR *E. indica* was the first species to be identified with a nucleotide substitution from proline at position 106 to serine within *EPSPS* (Baerson et al. 2002). Since then, additional species have been identified with amino acid substitutions at this position resulting in proline-106 to alanine, leucine, serine, or threonine amino acid substitutions (Powles and Preston 2006). While substitutions in nucleotide sequences do not always result in resistance, due to the wobble position of codons providing amino acid redundancy, changes from proline to alanine, leucine, serine, or threonine result in altered binding ability of glyphosate to the target due to the changes caused by the type and size of amino acid (Bostamam et al. 2012). A nucleotide substitution within *EPSPS* may result in variable levels of glyphosate resistance, or no resistance at all. In studies by Baerson et al. (2002), EPSPS in the resistant biotype was less sensitive to glyphosate, which reflects the four single-nucleotide differences observed from the cDNA sequencing analysis; however, two of these substitutions were silent mutations, which did not confer resistance, while the other two resulted in an amino acid change, including Pro-106-Ser and Pro-381-Leu (Baerson et al. 2002). In these cases, the substitutions are significant and alter the target binding site, as proline is a helix-destabilizing nonpolar residue, while serine is a polar residue, and leucine is a hydrophobic residue (Baerson et al. 2002). Populations of GR *L. rigidum* with the same mechanisms of resistance (altered target site or reduced translocation, depending on the population) had different resistance factors. GR populations of *E. indica*, *L. rigidum*, and *L. perenne* ssp. *multiflorum* with an altered target site as the mechanism of resistance had a range in resistance factors from 2- to 4-fold (Baerson et al. 2002; Wakelin and Preston 2006), which is similar to what has been observed for the resistance factor in *A. artemisiifolia*. In *E. indica*, TS resistance may be due to a substitution at proline-106 to either serine (Baerson et al. 2002) or threonine (Ng et al. 2003) or two mutations (Pro-106-Ser and Thr-102-Ile) (Yu et al. 2015). When considering changes in nucleotides, it is important to determine both the nucleotide change and the resultant change in the amino acid sequence and the change in enzyme configuration.

Absorption and Translocation

Efficacy of glyphosate is dependent on its systemic movement to its target site, the EPSPS enzyme in the chloroplast. Preventing glyphosate from reaching its target site at a lethal dose through reduced absorption and/or translocation may result in plant survival (Vila-Aiub et al. 2012). Weaver and Herrmann (1997) reported that *EPSPS* gene expression is greatest in the meristems, followed by the flowers and the stem, and lowest in mature leaves and cotyledons. Similarly, studies by Feng et al. (2004) found that the meristems of the plant are the most sensitive to glyphosate. Reduced glyphosate absorption, reduced translocation, vacuolar sequestration, and chloroplast exclusion are all effective mechanisms for preventing the interaction of glyphosate with EPSPS. Reduced translocation has been reported as the mechanism of glyphosate resistance in multiple weed species, including GR *L. rigidum*, the first GR weed worldwide (Lorraine-Colwill et al. 1999). In *C. canadensis*, *C. bonariensis*, *L. rigidum*, and *L. perenne* ssp. *multiflorum*, populations with this resistance mechanism were

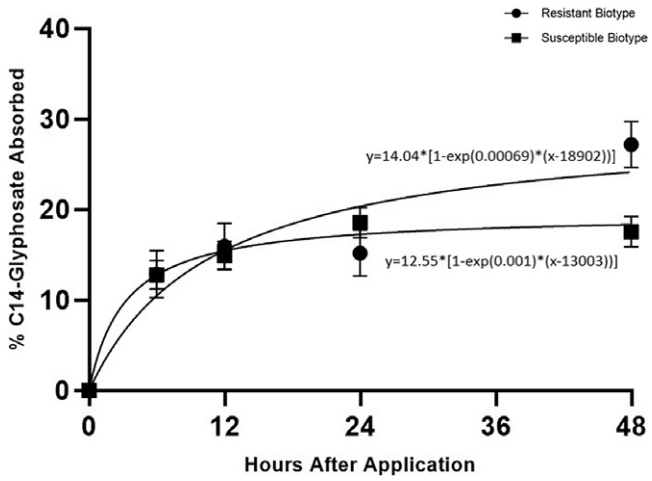

Figure 2. Percent [^{14}C]glyphosate absorption between glyphosate-susceptible (GS) and glyphosate-resistant (GR) *Ambrosia artemisiifolia* 6, 12, 24, and 48 h after application (HAA). Studies completed at University of Guelph, Guelph, ON, Canada, and Colorado State University, Fort Collins, CO, USA.

Absorption susceptible population						
	Estimate	SE	*t*-value	Pr > *t*	95% confidence limits	
a	12.55	1.14	11.04	<0.0001	10.27	14.83
b	−0.001	0.062	−0.02	0.99	−0.13	0.12
x_0	13,003	0	Infty	<0.0001	−Infty	Infty
s2e	68.45	13.3	5.15	<0.0001	41.78	95.13
Absorption resistant population						
	Estimate	SE	*t*-value	Pr > *t*	95% confidence limits	
a	14.04	1.51	9.28	<0.0001	11	17.08
b	−0.00069	0.05	−0.01	0.9891	−0.1	0.1
x_0	18,902	0	Infty	<0.0001	−Infty	Infty
s2e	114.4	22.88	5	<0.0001	68.45	160.35

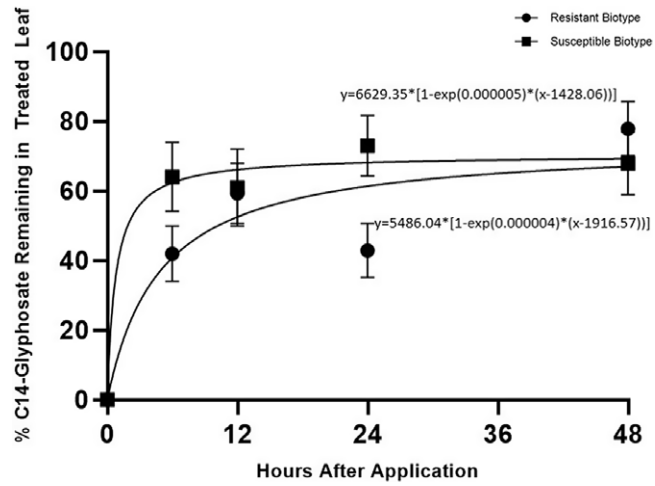

Figure 3. Percent of absorbed/translocated [^{14}C]-labeled glyphosate in treated leaf of glyphosate-susceptible (GS) and glyphosate-resistant (GR) *Ambrosia artemisiifolia* 6, 12, 24, and 48 h after application (HAA). Means and SEs were calculated as a percent of total radioactivity. Studies completed at University of Guelph, Guelph, ON, Canada, and Colorado State University, Fort Collins, CO, USA.

Treated leaf susceptible population						
	Estimate	SE	*t*-value	Pr > *t*	95% confidence limits	
a	6,629.35	8,749.55	0.76	0.4531	−11,054	24,313
b	-5.02×10^{-6}	0	Infty	<0.0001	−Infty	Infty
x_0	1,428.06	1,856.66	0.77	0.4463	−2,324.39	5,180.52
s2e	1,235.91	362.65	3.41	0.0015	502.96	1,968.86
Treated leaf resistant population						
	Estimate	SE	*t*-value	Pr > *t*	95% confidence limits	
a	5,486.04	6,451.37	0.85	0.3993	−7,485.31	18,457
b	-4.17×10^{-6}	0	Infty	<0.0001	−Infty	Infty
x_0	1,916.57	2,227.1	0.86	0.3938	−2,561.32	6,394.46
s2e	1,146.78	333.62	3.44	0.0012	475.99	1,817.58

reported to have a range in resistance factors from 3- to 10-fold (Shaner 2009; Wakelin et al. 2004). Glyphosate must enter the plant and be moved to sink tissues with high EPSPS expression for it to be effective.

GS and GR *A. artemisiifolia* were treated with [^{14}C]glyphosate and glyphosate and harvested at 6, 12, 24, and 48 HAT. The overall absorption and accumulation of glyphosate in each of these tissues was measured over time and compared between the GS and GR biotypes (Figure 2). There was no difference in glyphosate absorption between the GS and GR biotypes, with absorption ranging from 12.77% to 17.56% in the susceptible and 12.84% to 27.17% in the resistant biotype. When both biotypes were modeled using nonlinear models (exponential to a maximum), their confidence intervals overlapped, indicating no difference between the GS and GR biotypes. This was confirmed by looking at the treated leaf (Figure 3) over time in the GS and GR biotypes. The percent accumulation in the apex increased from 1.97% at 6 HAA to 6.23% at 48 HAA in the susceptible and 2.51% at 6 HAA to 7.55% at 48 HAA in the resistant, which was not significantly different between the biotypes (Figure 4).

Glyphosate accumulation did not change over time in the leaf below the treated leaf, the leaf opposite the treated leaf, or in the stem. This was expected, as glyphosate is translocated more rapidly to growing parts of the plant, the meristem portions of the plant, rather than the older tissues. The meristems, flowers, and stems are most sensitive to glyphosate, while the mature leaves and cotyledons have least sensitivity (Feng et al. 2004; Weaver and Herrmann 1997). Glyphosate accumulation in the roots in the GS biotype changed over time (5.72% at 6 HAA to 16.76% at 48 HAA) and similarly changed over time in the GR biotype (6.77% at 6 HAA to 14.19% at 48 HAA). These models were not significantly different between biotypes, as shown by the overlapping confidence intervals, as well as the curves on the graph (Figure 5).

In GR biotypes with reduced translocation as the mechanism of resistance, glyphosate accumulates in the treated leaf and does not reach highly metabolically active tissues in sufficient quantities for plant control. In a study by Koger and Reddy (2005) using radioactively labeled glyphosate, translocation was reduced by 28% to 47% in the GR *C. canadensis* compared with the GS biotypes. Radioactivity was measured from the highest to lowest concentration in the treated leaf, roots, other leaves, and finally the crown in both GS and GR *C. canadensis* biotypes; radioactivity was greater in the treated leaf and lower in the roots in the GR compared with the GS biotype (Koger and Reddy 2005). GS *C. canadensis* plants had approximately twice the amount of glyphosate in the culm and roots than GR plants (Dinelli et al. 2006). In GR *S. halepense*, translocation from the treated leaf to the roots was three times less

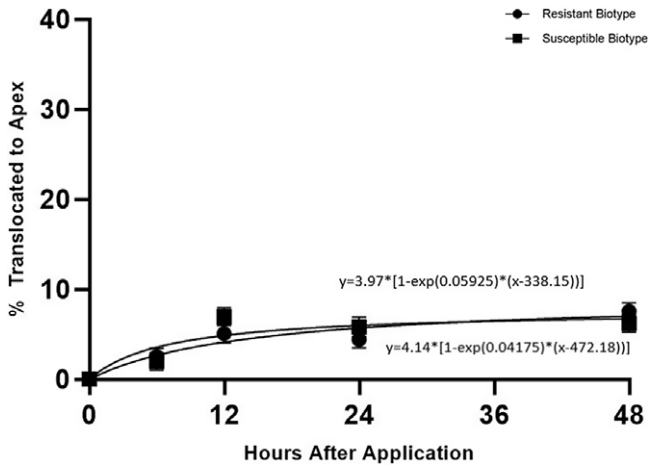

Figure 4. Percent accumulation of absorbed [^{14}C]-labeled glyphosate in apex of glyphosate-susceptible (GS) and glyphosate-resistant (GR) *Ambrosia artemisiifolia* 6, 12, 24, and 48 h after application (HAA). Means and SEs were calculated as a percent of total radioactivity. Studies completed at University of Guelph, Guelph, ON, Canada, and Colorado State University, Fort Collins, CO, USA.

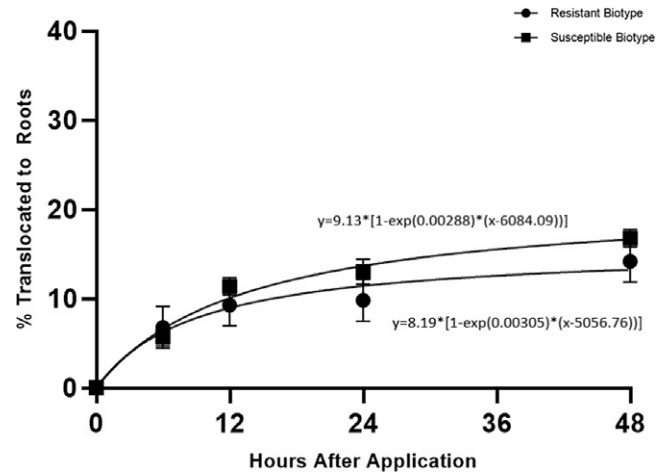

Figure 5. Percent accumulation of absorbed [^{14}C]-labeled glyphosate in roots of glyphosate-susceptible (GS) and glyphosate-resistant (GR) *Ambrosia artemisiifolia* 6, 12, 24, and 48 h after application (HAA). Means and SEs were calculated as a percent of total radioactivity. Studies completed at University of Guelph, Guelph, ON, Canada, and Colorado State University, Fort Collins, CO, USA.

Translocation to apex susceptible population						
	Estimate	SE	*t*-value	Pr > *t*	95% confidence limits	
a	4.14	0.58	7.12	<0.0001	2.97	5.31
b	−0.042	6.71	−0.01	0.99	−13.51	13.43
x_0	472.18	0.00063	750,158	<0.0001	472.18	472.18
s2e	17.6	3.45	5.1	<0.0001	10.68	24.55
Translocation to apex resistant population						
	Estimate	SE	*t*-value	Pr > *t*	95% confidence limits	
a	3.97	0.4886	8.13	<0.0001	2.99	4.95
b	−0.059	5.3	−0.01	0.99	−10.70	10.57
x_0	338.15	0.001	328,725	<0.0001	338.15	338.16
s2e	12.41	2.43	5.1	<0.0001	7.53	17.3

Translocation to roots susceptible population						
	Estimate	SE	*t*-value	Pr > *t*	95% confidence limits	
a	9.13	1.09	8.38	<0.0001	6.95	11.32
b	−0.0029	0.75	0	0.99	−1.51	1.51
x_0	6,084.09	0	Infty	<0.0001	−Infty	Infty
s2e	61.74	12.11	5.1	<0.0001	86.04	86.04
Translocation to roots resistant population						
	Estimate	SE	*t*-value	Pr > *t*	95% confidence limits	
a	8.19	1.05	7.78	<0.0001	6.08	10.3
b	−0.0031	0.36	−0.01	0.99	−0.73	0.72
x_0	5,056.76	0	Infty	<0.0001	−Infty	Infty
s2e	60.93	11.62	5.24	<0.0001	37.65	84.22

compared with the GS biotype (Vila-Aiub et al. 2012). Similarly, in this study, the greatest radioactivity was measured in the treated leaf, then the roots and stem, and then the apex of GR *A. artemisiifolia*; there was minimal radioactivity measured in the stem, below treated leaf, and opposite treated leaf. Glyphosate accumulation was greatest in the treated leaf in all the GR *L. rigidum* populations, except for the population in which an altered target site was the mechanism of resistance (Bostamam et al. 2012), although an increase in glyphosate in the treated leaf of *L. perenne* ssp. *multiflorum* remained high in the GS biotypes due to the phytotoxic effects of glyphosate causing the plant to shut down and therefore not translocate glyphosate (Perez-Jones et al. 2007). Lorentz et al. (2011) similarly stated that survival of GR weeds following glyphosate application may be due to a block in phloem loading. Translocation studies using [^{14}C]glyphosate in *A. tuberculatus* showed that absorption was similar between GS and GR biotypes up to 24 HAA, following which the GS biotype continued to absorb glyphosate, but glyphosate absorption in the GR biotype plateaued (Nandula et al. 2013). A greater percentage of the absorbed glyphosate remained in the treated leaf of the GR (77% to 80%) compared with the GS (68% to 69%) biotype up to 48 HAT (Nandula et al. 2013). This was supported by phospho-imaging autoradiographs, which showed that most of the radioactive glyphosate was in the treated leaf of the GR biotypes; translocated glyphosate accumulated in the primary growing point (Nandula et al. 2013).

Metabolism

In this experiment, [^{14}C]glyphosate was applied to GS and GR *A. artemisiifolia*. Plants were harvested at 96 HAT and treated as described earlier. Samples were then run on the HPLC system, producing graphs that could compare the GS and GR biotypes, as well as known retention times between the various metabolites (Figure 6). All samples produced similar curves, indicating that enhanced metabolism is not the mechanism of glyphosate resistance in *A. artemisiifolia* from Ontario, Canada.

Enhanced metabolism can be evaluated as a mechanism of resistance using [^{14}C]glyphosate. In original metabolism studies by Sandberg et al. (1980), a 5-µl drop of 0.125 µCi [^{14}C]glyphosate was applied to two leaves. Plants were dissected into treated leaves, above treated leaves, below treated leaves, and roots at preplanned harvest intervals, with three harvests occurring up to 30 d after treatment (Sandberg et al. 1980). In Feng et al. (2004), treated tissue was rinsed with water, frozen, and ground for extraction and analysis of metabolites using high-performance liquid chromatography (HPLC). The tissue was then analyzed for glyphosate

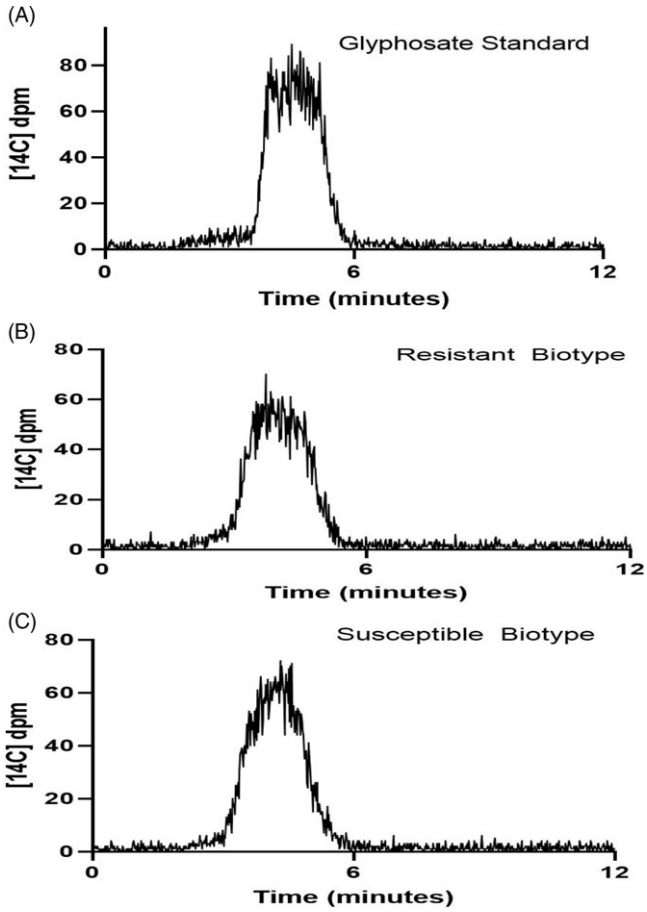

Figure 6. A single peak from running on an HPLC column, representing glyphosate for the glyphosate standard (A), glyphosate-susceptible (GS) *Ambrosia artemisiifolia* (B), and glyphosate-resistant (GR) *Ambrosia artemisiifolia* (C) at 96 h after application of [^{14}C]glyphosate shows no difference in glyphosate metabolism between GR and GS. Studies completed at Colorado State University, Fort Collins, CO, USA.

metabolites AMPA, glycine, and sarcosine following the application of [^{14}C]-glyphosate.

Though enhanced metabolism has been ruled out as a mechanism of glyphosate resistance in *L. rigidum* (Feng et al. 1999; Lorraine-Colwill et al. 2003), *E. indica* (Tran et al. 1999), and some *C. bonariensis* populations (Feng et al. 2004), González-Torralva et al. (2012) reported enhanced metabolism as the mechanism of resistance for a GR *C. bonariensis* population in Spain. Glyoxylate, sarcosine, and aminomethylphosphoate (AMPA) were detected in place of glyphosate at 96 HAA (González-Torralva et al. 2012). This is similar to what is found in glyphosate-tolerant plants such as tall morningglory [*Ipomoea purpurea* (L.) Roth], field bindweed (*Convolvulus arvensis* L.), butterfly-pea (*Clitoria ternatea* L.), and perennial soybean [*Neonotonia wightii* (Wight & Arn.) Lackey], which have been reported to metabolize glyphosate to AMPA, sarcosine, and glycine (Cruz-Hipolito et al. 2011; Rojano-Delgado et al. 2012; Sandberg et al. 1980; Sprankle et al. 1978). Studies by Castle et al. (2004) found that glyphosate *N*-acetyltransferase isolated from bacteria could metabolize glyphosate to *N*-acetylglyphosate, which does not effectively inhibit EPSPS and therefore has reduced herbicidal activity. These enzymes could provide greater levels of glyphosate tolerance in crops such as tobacco (*Nicotiana tabacum* L.) and maize (*Zea mays* L.) by metabolizing glyphosate into less phytotoxic metabolites (Castle et al. 2004).

Enhanced herbicide metabolism that confers herbicide resistance has been mainly linked to three enzymes: aryl acylamidase (AAA), glutathione *S*-transferase (GST), and cytochrome P450 monooxygenase (CYP450) (Preston 2003). AAAs catalyze the hydrolysis of certain acylamides, including the herbicide propanil, which allows for its selectivity in rice (*Oryza sativa* L.) (Preston 2003). Enhanced metabolism by CYP450 in some species, such as *L. rigidum*, confers resistance to up to four herbicide modes of action (Preston 2003). This provides an example of why understanding the mechanism of resistance is important for managing herbicide resistance (Preston 2003). Knowing the resistance mechanism allows a science-based recommendation for alternative herbicide options to be made without having to wait on research results.

This study concludes that the mechanism of glyphosate resistance in *A. artemisiifolia* from Ontario, Canada, is not due to an altered target site (at positions 102 or 106 of the EPSPS enzyme), reduced absorption, reduced translocation, or enhanced metabolism. The resistance factor in this biotype is 5.1, compared with a known local susceptible biotype. While this GR *A. artemisiifolia* biotype is not widespread in Ontario, better understanding of its mechanism of resistance of as well as how to control it in the field are important for controlling its spread. Further studies on this *A. artemisiifolia* biotype are needed to elucidate the mechanism of glyphosate resistance, including looking at a wider sequence of the *EPSPS* sequence, *EPSPS* gene overexpression studies, and further [^{14}C]glyphosate tracing between the susceptible and resistant biotypes to look at chloroplast exclusion of glyphosate. The development of genomic resources for this species would also provide greater power for determining the basis of glyphosate resistance in this species.

The confirmation and distribution of GR weeds provides important information for developing management strategies of these biotypes and alerts growers that current weed management practices are no longer appropriate. Yuan et al. (2010) stated that understanding the evolution of resistant weeds is important for their management, with common evolutionary ancestry leading to a focus on controlling seed output and movement. Conversely, in multiple independently evolved populations, different mechanisms may evolve across the resistant populations, resulting in a focus on the mechanism of resistance for developing management solutions (Yuan et al. 2010). Monitoring the development of herbicide-resistant weed biotypes enables identification of areas where weed management changes should be implemented.

With herbicide resistance being present in such a wide number of weed species across a wide geographic area, weed management practitioners must develop diversified, integrated weed management programs to ensure long-term sustainable weed management programs. This is particularly true for the use of glyphosate in GR crops. Weed surveys should be conducted to identify herbicide-resistant biotypes, and the mechanism(s) of resistance should be elucidated so that science-based weed management programs can be implemented.

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