In Vivo $^{31}$P-Nuclear Magnetic Resonance Studies of Glyphosate Uptake, Vacuolar Sequestration, and Tonoplast Pump Activity in Glyphosate-Resistant Horseweed$^{1,2}$

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Horseweed ($Conyza canadensis$) is considered a significant glyphosate-resistant (GR) weed in agriculture, spreading to 21 states in the United States and now found globally on five continents. This laboratory previously reported rapid vacuolar sequestration of glyphosate as the mechanism of resistance in GR horseweed. The observation of vacuole sequestration is consistent with the existence of a tonoplast-bound transporter. $^{31}$P-Nuclear magnetic resonance experiments performed in vivo with GR horseweed leaf tissue show that glyphosate entry into the plant cell (cytosolic compartment) is (1) first order in extracellular glyphosate concentration, independent of pH and dependent upon ATP; (2) competitively inhibited by alternative substrates (aminomethyl phosphonate [AMPA] and N-methyl glyphosate [NMG]), which themselves enter the plant cell; and (3) blocked by vanadate, a known inhibitor/blocker of ATP-dependent transporters. Vacuole sequestration of glyphosate is (1) first order in cytosolic glyphosate concentration and dependent upon ATP; (2) competitively inhibited by alternative substrates (AMPA and NMG), which themselves enter the plant vacuole; and (3) saturable. $^{31}$P-Nuclear magnetic resonance findings with GR horseweed are consistent with the active transport of glyphosate and alternative substrates (AMPA and NMG) across the plasma membrane and tonoplast in a manner characteristic of ATP-binding cassette transporters, similar to those that have been identified in mammalian cells.

Glyphosate is arguably the world’s most important herbicide (Duke and Powles, 2008). Environmental factors affecting its uptake and translocation in higher plants have been widely studied (Kells and Rieck, 1979; Coupland, 1983; Devine et al., 1983; Masiunas and Weller, 1988; Zhou et al., 2007). Notably, the role of light is important for effective uptake and translocation, suggesting that metabolic energy plays a role in this process (Simarmata et al., 2003; Shaner et al., 2005). Death of the whole plant requires effective glyphosate translocation from source to sink tissue, a process requiring ATP to maintain Suc gradients, which drive phloem movement (Bromilow et al., 1990; Dill et al., 2010).

Weed species have developed glyphosate-resistant (GR) biotypes during the past decade (Heap, 2014). This has spurred interest in factors that may contribute to resistant attribute(s) as well as methods that can be used to screen plants for herbicide toxicity (Shaner, 2010). Resistance mechanisms have been reported for horseweed ($Conyza canadensis$; Feng et al., 2004; Zelaya et al., 2004; Ge et al., 2010, 2011), Palmer amaranth ($Amaranthus palmeri$; Gaines et al., 2010), and ryegrass ($ Lolium rigidum$ and $Lolium multiflorum$; Powles et al., 1998; Perez et al., 2004; Ge et al., 2012).

Since glyphosate is foliar applied, glyphosate toxicity involves a multistep delivery process. Glyphosate must traverse the nonliving structures of the leaf cuticle and the cell walls of the epidermis, apospory, and mesophyll prior to accessing the phloem for transport to sink tissues (Bromilow et al., 1990; Bromilow and Chamberlain, 2000). Indeed, restriction of glyphosate delivery to the plant cell cytoplasm (and chloroplast) by any means is, in itself, a resistance mechanism (Shaner, 2009; Ge et al., 2013). Elucidation of key factors governing delivery to the intracellular milieu of plant source leaves is critical for developing a complete understanding of the mechanism(s) of resistance to glyphosate.

Glyphosate’s phosphonate group offers the opportunity to employ in vivo $^{31}$P-NMR spectroscopy to track glyphosate movement and metabolism, additionally including monitoring of cellular pH, and gradients therein, and ATP levels, both indicators of tissue viability (Roberts, 1984). This laboratory has extended the $^{31}$P-NMR approach initially used by Gout et al. (1992) with suspension-cultured sycamore ($Acer pseudoplatanus$) cells. The initial findings, that source and sink leaf tissue from GR horseweed rapidly and avidly...
sequestered glyphosate within the vacuole compartment and that leaf tissue from glyphosate-sensitive (GS) horseweed did not, led to the hypothesis that vacuole sequestration was a key, perhaps the dominant, component of the resistance mechanism (Ge et al., 2010). It was then shown that GR horseweed acclimated and maintained at cold temperature (approximately 10°C–12°C) did not rapidly and avidly sequester glyphosate within the vacuole. Importantly, under such conditions, GR horseweed succumbed to the toxic effects of glyphosate. In short, by preventing glyphosate sequestration, GR horseweed became glyphosate sensitive, a laboratory finding confirmed in the field (Ge et al., 2011).

The proposition that, by limiting the herbicide available for translocation, glyphosate vacuole sequestration could serve as an important if not dominant resistance mechanism was further strengthened by experiments that showed vacuolar glyphosate sequestration correlated with glyphosate resistance in ryegrass (Lolium spp.) from Australia, South America, and Europe (Ge et al., 2012). However, 31P-NMR studies of other weeds revealed that in some species, for example, Palmer amaranth, waterhemp (Amaranthus tuberculatus), and johnsongrass (Sorghum halepense), resistance correlated strongly with a lack of glyphosate uptake into the plant cell, a frontline resistance mechanism (Ge et al., 2013).

Throughout these previous 31P-NMR studies, the finding that plants could regulate the compartmental access of glyphosate led us to speculate that the apoplastic, tonoplast, and perhaps chloroplast possessed glyphosate-active transporters whose up-regulation or down-regulation and/or expression would confer resistance (Ge et al., 2010, 2011, 2012, 2013). This hypothesis motivated additional in vivo 31P-NMR experiments to further describe the determinants of glyphosate delivery in horseweed leaf tissue. Specifically, experiments with GR horseweed were designed with the goal of probing the transporter hypothesis.

Findings from these experiments are reported herein and are consistent with the existence of a tonoplast transporter that is responsible for glyphosate resistance via vacuole sequestration. As described here, vacuole sequestration requires ATP, is active for multiple substrates, and shows substrate competition. Furthermore, glyphosate entry into the cell can be markedly inhibited by vanadate pretreatment. These characteristics are similar to those of ATP-binding cassette transporters in plants (Hetherington et al., 1998; Rea, 2007; Verrier et al., 2008; Prosecka et al., 2009; Conte and Lloyd, 2011) and mammalian cells (van de Ven et al., 2009; Ernst et al., 2010).

RESULTS

Tissue Assessment

In vivo 31P NMR monitors tissue metabolism through the detection of ATP and other phosphorous-containing metabolites as well as the measurement of intracellular pH via the pH-dependent 31P-NMR chemical shift of inorganic phosphate (Pi; Wray et al., 1985; Ackerman et al., 1996; Kulichikhin et al., 2007). In the absence of herbicide treatment, mature GR and GS horseweed leaves are readily maintained for a period of 24 h in the NMR perfusion apparatus with circulating perfusion medium (hereafter buffer) equilibrated with 100% oxygen (Ge et al., 2010). Figure 1 illustrates the importance of employing buffer equilibrated with 100% oxygen for maintaining in vivo 31P-NMR-detectable ATP levels with leaf tissue in the dark (respiration). Resonance line shapes are also narrower at the higher oxygen conditions. This likely reflects enhanced homeostasis (increased uniformity of compartmental ionic environments, and thus uniformity of compartmental chemical shifts; Couldwell et al., 2009).

Glyphosate Delivery and Compartmentalization: Effect of Oxygen and Light

Table I provides the results of quantitative spectral analysis for fully expanded, freshly harvested, mature GR horseweed leaves incubated for 14 h in buffer containing glyphosate under three different conditions: (1) in the dark, immersed in buffer equilibrated with 100% oxygen; (2) in the dark, immersed in buffer equilibrated with air (approximately 20% oxygen); and (3) in the light (150 µE m⁻² s⁻¹), floating on the surface of buffer without air equilibration.

Total Glyphosate Uptake

Leaf tissue incubated in the dark, immersed in buffer equilibrated with 100% oxygen, showed 50% greater total glyphosate uptake compared with the other two incubation conditions, where uptake was the same.

Glyphosate Sequestration

Glyphosate vacuole sequestration accounts for only approximately 20% of the total glyphosate content for leaf tissue incubated in the dark, immersed in buffer equilibrated with air. This degree of sequestration is notably reduced compared with the other two incubation conditions, where over 90% of the total glyphosate content was found in the vacuole.

ATP Levels

ATP content in leaf tissue incubated in the dark, immersed in buffer equilibrated with air, was two-thirds that found under the other two incubation conditions, where ATP content was the same.

Shikimate-3-Phosphate Levels

Shikimate-3-phosphate (S3P) can build to millimolar concentrations in response to the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) by glyphosate. In leaf tissue incubated in the dark, immersed in buffer equilibrated with 100% oxygen, S3P reached two-thirds the level found in leaf tissue incubated in the
light, with leaves floating on the buffer surface. S3P was not detected by $^{31}$P NMR in leaf tissue incubated in the dark, immersed in buffer equilibrated with air.

**Time Course of Glyphosate Uptake: GR versus GS Horseweed**

Figure 2 shows the time course of cytosolic glyphosate content during a 16-h perfusion of mature GS and GR horseweed leaves with buffer containing 10 mM glyphosate. Because the vacuolar glyphosate signal has a chemical shift similar to that of the intense buffer glyphosate resonance, only the chemical shift-distinct cytosolic glyphosate is quantified in this protocol. Data represent means ± SE (n = 3 separate trials) and are normalized (ratio) to the resonance amplitude of the external reference, methylene diphosphonate (MDP). The buildup of cytosolic glyphosate follows a similar profile for GR and GS biotypes for the initial 6 h of exposure. In the GR biotype, cytosolic glyphosate content plateaus for a short period (hours 6–8) and then begins a slow decrease that continues for the rest

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**Table 1. $^{31}$P-NMR determined cytosolic and vacuolar glyphosate (in vivo analysis) content and ATP and S3P (extraction, in vitro analysis) content in mature GR horseweed leaves**

Leaf tissue was either (1) fully immersed for 14 h in the dark in buffer (20°C) containing 10 mM glyphosate, which was equilibrated (via bubbling) with air or with 100% oxygen; or (2) allowed to float for 14 h in the light (150 μE m$^{-2}$ s$^{-1}$) on top of buffer (20°C) containing 10 mM glyphosate, which was fully exposed to air (but in the absence of gas bubbling). Data are reported as means ± SE (n ≥ 3) and reflect the ratio of individual resonance amplitudes to that of the external reference (MDP). Extract results (ATP and S3P) are normalized to per gram leaf wet weight.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Air Saturation (Dark)</th>
<th>Oxygen Saturation (Dark)</th>
<th>Air Exposure (Light)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosolic glyphosate</td>
<td>0.31 (0.04)</td>
<td>0.03 (0.01)</td>
<td>0.03 (0.01)</td>
</tr>
<tr>
<td>Vacuolar glyphosate</td>
<td>0.09 (0.01)</td>
<td>0.58 (0.03)</td>
<td>0.39 (0.04)</td>
</tr>
<tr>
<td>Sum</td>
<td>0.40 (0.05)</td>
<td>0.61 (0.04)</td>
<td>0.42 (0.05)</td>
</tr>
<tr>
<td>Cytosolic/vacuolar glyphosate</td>
<td>3.40 (0.05)</td>
<td>0.05 (0.04)</td>
<td>0.08 (0.05)</td>
</tr>
<tr>
<td>Extract analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>0.18 (0.05)</td>
<td>0.28 (0.04)</td>
<td>0.30 (0.02)</td>
</tr>
<tr>
<td>S3P</td>
<td>Not detectable</td>
<td>1.38 (0.05)</td>
<td>2.06 (0.76)</td>
</tr>
</tbody>
</table>
of the time course. However, in the GS biotype, cytosolic glyphosate continues to increase until hour 8 and then plateaus during hours 8 to 16.

This same trend can be seen from our earlier study (Ge et al., 2010; Fig. 2) under slightly different pulse-chase conditions. In that case, the total (cytosol plus vacuole) glyphosate measured after 8 h of infusion was always greater in the GR compared with the GS biotype. However, the content of glyphosate in the cytosol was greater in the GS biotype, as there was essentially no vacuole sequestration. Other metabolic changes were also observed at later stages of the time course with the GS biotype (Ge et al., 2010), including (1) decrease of ATP content, (2) decrease of the cytosolic pH, and (3) decrease of Pi from cellular leakage to the buffer. Such effects were either not observed or were greatly muted with the GR biotype (Ge et al., 2010; Supplemental Fig. S1). Note that in Supplemental Figure S1G, a decrease in vacuolar Pi over time in both GR and GS horseweed was evident. However, cytosol Pi remained a small pool (approximately 5%) compared with the content of vacuole Pi.

Dependence of Glyphosate Uptake Rate on Buffer Glyphosate Concentration with GR Horseweed

Figure 3A presents GR horseweed data from seven separate pulse-chase protocols in which the buffer glyphosate concentration was varied from 2 to 20 mM; specifically, for a given pulse-chase protocol, one of seven different, fixed glyphosate concentrations was employed during the entire 10-h pulse phase. The cytosolic glyphosate content can be quantified during the pulse phase because it is chemical-shift resolved from other $^3$P resonances. The initial rate of cytosolic glyphosate accumulation was monitored over the first 6 h of the pulse phase, where vacuole sequestration and the export of cytosolic glyphosate (to the buffer) are assumed to be negligible. A linear cytosolic glyphosate content-versus-time relationship is expected under initial
rate conditions. (Consistent with initial rate conditions, the buffer glyphosate concentration remains essentially constant during the pulse phase because the total buffer volume is much greater than that of the total intracellular volume.)

The dependency of plant tissue cytosolic glyphosate content versus glyphosate exposure time was well approximated as linear over the first 6 h of the pulse phase (data not shown), and the slope determined by linear least-squares analysis was taken as the rate of uptake ($\Delta$-content/$\Delta$-time) at the given glyphosate concentration ($r^2$ ranged from 0.89 to 0.98). The plot (Fig. 3A) of uptake rate as a function of buffer glyphosate concentration, $x$, is well modeled as linear, $\ln(x) = 0.010x$ ($r^2 = 0.74$). Thus, the rate of cytosolic glyphosate accumulation is first order in buffer glyphosate concentration.

At the end of the 12-h chase with glyphosate-free buffer (hours 22–24), the vacuole glyphosate occupancy was determined (Fig. 3B) and showed a nearly linear increase with pulse-phase buffer glyphosate concentration up to saturation at 8 mM and above.

Aminomethyl Phosphonate, N-Methyl Glyphosate, and Glyphosate: Compartmentalization and Competition

Compartmentalization

Mature GR horseweed leaves were exposed to 10 mM N-methyl glyphosate (NMG), aminomethyl phosphonate (AMPA), or glyphosate (Ge et al., 2010) during the standard pulse-chase protocol described earlier. $^{31}$P-NMR monitoring during the chase phase assessed cytosolic and vacuole occupancies (Table II). GR horseweed effectively sequesters multiple substrates within the vacuole (i.e. the putative tonoplast pump is promiscuous). Uptake of NMG and glyphosate each occurs to a similar extent during the pulse phase, with roughly two-thirds of the total tissue content of either substrate found sequestered in the vacuole late in the chase phase. Uptake of AMPA during the pulse phase occurs to an approximately 5- to 6-fold greater extent than that of either NMG or glyphosate. AMPA vacuole sequestration is also more extensive, approaching 84% of the total tissue AMPA content late in the chase phase.

Competition

Pulse-chase protocols in which either 10 mM glyphosate and 10 mM AMPA or 10 mM glyphosate and 10 mM NMG are presented to leaf tissue simultaneously serve to test for the competitive inhibition of glyphosate transport via a putative transmembrane pump(s). During the substrate-free buffer chase period, the relative concentration and compartmentalization of substrates can be quantified via their differing $^{31}$P-NMR chemical shifts (Fig. 4, A and B).

In the presence of the competing substrate AMPA, total leaf tissue glyphosate content (vacuole and cytosol) at the start of the chase phase is only approximately 25% of that found in the absence of competing AMPA (Fig. 4C); the total leaf tissue AMPA content is estimated by analyses of the data shown in Figure 4A to be approximately 5.5-fold greater than the glyphosate content. In the presence of the competing substrate NMG, total leaf tissue glyphosate content (vacuole and cytosol) at the start of the chase phase is also only approximately 25% of that found in the absence of competing NMG (Fig. 4C); the total leaf tissue NMG content estimated by analyses of the data shown in Figure 4B is approximately 2.5-fold greater than the glyphosate content. These observations argue for the preferential transport of AMPA and NMG versus glyphosate into the plant cell when these competing substrates are simultaneously present. The normalized rate of glyphosate sequestration into the vacuole (Fig. 4D) is also reduced in the presence of either AMPA or NMG, further suggesting competition via the same tonoplast pathway. The discrimination of plasmalemma transporters(s) for substrates appears not to be based solely on molecular size. Both AMPA and NMG, being smaller and larger, respectively, than glyphosate, enter the plant cell in greater amounts than simultaneously applied glyphosate. Curiously, we find that when glyphosate and NMG are separately delivered in identical concentration to the plant tissue, glyphosate enters the cell at a concentration roughly 30% greater than NMG (Table II). It is interesting, and not understood, that when NMG and glyphosate are delivered simultaneously at the same concentration, NMG is found to enter the cell at roughly 2.5 times that of glyphosate (Fig. 4B). Further complicating the comparison, the slightly more lipophilic NMG may have more than one mechanism of uptake, although evidence for this is lacking.

Glyphosate Delivery and Compartmentalization: Effect of pH and Ammonia

When glyphosate has entered the cell and is undergoing vacuole sequestration, the dramatic effect of a rapid intracellular pH change can be readily evaluated. These data are presented in Figure 5, where GR horseweed leaf tissue was monitored under a variety of conditions after incubation with buffer containing 10 mM glyphosate for a 14-h period in the dark followed by a quick wash with glyphosate-free buffer. During the first phase, a 3-h exposure to circulating glyphosate-free buffer (Fig. 5A), glyphosate migrates from the higher pH cytosol (approximately 7.9 ppm, pH approximately 6.8) to the lower pH vacuole (approximately 8.4 ppm, pH approximately 5.5). Under these conditions, the major Pi pool is associated with the vacuole compartment. During the second phase, a 3-h exposure to 20 mM ammonium sulfate at pH 9 (Fig. 5B), vacuole pH increases (shift of vacuole Pi to higher chemical shift, from 0.96 to 1.43 ppm, with a resulting merger of the vacuolar and cytosolic glycosphosphate signals) and either Pi migrates from the vacuole to the

Studies of Glyphosate-Resistant Weed Species


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cytosol and/or some vacuoles alkalinize more quickly than others, leading to a situation in which some of the vacuoles have a sufficiently high pH for their Pi signal to overlap the cytosolic signal. During the third phase, a repeat 3-h exposure to circulating glyphosate-free buffer (Fig. 5C), normal cellular pH gradients and Pi compartmentalization are reestablished as well as continuation of the vacuole sequestration of glyphosate. Notably, in spite of the severity of this ammonia challenge, glyphosate is retained in the plant cell and does not efflux to the buffer. For the data shown in Figure 5, the ratio of cytosolic glyphosate to total cellular glyphosate (cytosolic glyphosate plus vacuolar glyphosate) decreased from 16% at the end of the control phase (hour 3) to 11% at the end of the recovery phase (hour 9). Vacuole glyphosate, obviously, mirrored this decrease, increasing from 84% to
Glyphosate Delivery: Effect of Vanadate

In a cursory series of exploratory experiments, the effect of GR horseweed leaf tissue perfusion with buffer containing orthovanadate (2–50 mM) was monitored by in vivo $^{31}$P NMR (Supplemental Fig. S2). The ATP level was reduced but vacuole/cytosol/buffer pH gradients were maintained, suggesting continued tissue viability. (The decrease in ATP levels is in contrast to findings with maize [$Zea mays$] roots exposed to 0.5 mM vanadate [Skenar et al., 1994], where, while pH did not change, ATP levels increased.) Incubation of GR horseweed leaf tissue in 20 mM orthovanadate for 6 h, followed by vanadate-free perfusion with buffer containing 10 mM glyphosate for a period of 14 h, in turn followed by wash and chase periods employing glyphosate-free buffer, revealed only trace amounts of cellular glyphosate compared with leaves treated similarly but in the absence of vanadate during the initial 6-h incubation period (Fig. 6).

DISCUSSION

Energy-dependent transmembrane substrate pumps are common in plants and may enable glyphosate to gain entry into the cell and, separately, into organelles. It is attractive to speculate that a putative tonoplast pump in GR horseweed is responsible for the rapid vacuole sequestration of glyphosate, thus shielding the chloroplast and limiting phloem translocation to sink tissues (Ge et al., 2010, 2011, 2012). As reported previously (Ge et al., 2010), glyphosate entry into the GR horseweed vacuole is a unidirectional process as long as the tissue is viable. Thus, in the experiments reported here, which are consistent with prior reports of the irreversibility of vacuole sequestration at least up to several days following exposure (Ge et al., 2010, 2011), there is minimal efflux of glyphosate out of the plant cell into the flowing buffer during the chase period. Hetherington et al. (1998) found a slow efflux time course for cultured soybean (Glycine max) cells defined by three separate rate constants. A fast efflux rate constant was ascribed to adventitiously adherent glyphosate, apparently outside the cell’s plasma membrane. A slower efflux rate constant was assigned to the cytoplasmic glyphosate effusing out of the cell. A very slow efflux rate constant was identified with glyphosate hypothesized to be in the vacuole. In total, these observations argue against the passive diffusion of glyphosate into the cell but for transporters that facilitate effectively unidirectional entry across the cell membrane (and tonoplast). Considering the structure of glyphosate, amino acid transporters may be involved in the movement of glyphosate into the cell and within the cell. Unpublished $^{31}$P-NMR data from our laboratory (X.G., D.A.d’A., J.H.A., and R.D.S.) have shown that Gly competes effectively for the uptake of glyphosate into horseweed, suggesting that they both use the same channel.

The data in Table I support the hypothesis that vacuole sequestration is ATP dependent. The level of ATP generated in the dark with buffer equilibrated with 100% oxygen (respiration) is equal to that produced under photosynthetic conditions and is but only one and a half times that produced under photosynthetic conditions and is but only one and a half times that produced in the dark with buffer equilibrated with 20% oxygen. Lower ATP levels correlate with lower vacuole sequestration as well as lower cellular uptake of glyphosate under respiratory (dark) conditions. The data in Table I are consistent with reports of glyphosate uptake by whole plants that have been treated in full sun, in shade, and in the dark (Kells and Rieck, 1979; Coupland, 1983; Devine et al., 1983); uptake is greatest under conditions where ATP levels are anticipated to be maximal.

$S_3P$ was not detectable by $^{31}$P NMR for GR horseweed leaf tissue incubated in the dark with buffer equilibrated with air. This may reflect the lower ATP content, as ATP is a participant in $S_3P$ synthesis, or

### Table II. Chase-phase quantification of substrate compartmentalization in GR horseweed leaf tissue following a 10-h pulse phase and 2-h wash phase

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Compartmentalization</th>
<th>$^{31}$P-NMR Chemical Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early Chase Phase (Hours 12–14)</td>
<td>Late Chase Phase (Hours 18–20)</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>0.36 (0.03)</td>
<td>0.41 (0.03)</td>
</tr>
<tr>
<td>AMPA</td>
<td>0.86 (0.07)</td>
<td>3.15 (0.38)</td>
</tr>
<tr>
<td>NMG</td>
<td>0.36 (0.03)</td>
<td>0.25 (0.02)</td>
</tr>
</tbody>
</table>

89%. Thus, while glyphosate compartmentalization was not resolvable during the ammonia challenge phase, it appears that vacuole sequestration continued. Total cellular glyphosate remained unchanged (see Fig. 5 legend).
alternatively decreased performance of an ATP-dependent transporter responsible for glyphosate entry into the chloroplast, which would further increase the resistance level (less S3P) by decreasing glyphosate entry into the chloroplast (Shaner et al., 2005).

In healthy horseweed plant tissue, the initial uptake of glyphosate into GR and GS biotypes under continuous perfusion with 10 mM glyphosate is similar (shown in Fig. 2, where the cytosolic component in GR and GS tissue is plotted). However, the handling of glyphosate after 6 h of perfusion differs for GR and GS tissue. Vacuole sequestration becomes a dominant process for GR horseweed, shunting the cytosolic pool to the vacuole at a rate faster than cellular uptake from continued perfusion can occur, thus limiting the maximum concentration achievable in the cytosolic (and phloem translocatable) pool (Fig. 2; Feng et al., 2004). Following the initial 6 h of glyphosate perfusion, the cytosolic component decreases, reflecting ongoing vacuole sequestration. GS horseweed tissue, in contrast, shows no evidence for vacuole sequestration; thus, the cytosolic glyphosate pool increases under

Figure 5. In vivo $^{31}$P-NMR spectra of GR horseweed leaf tissue preloaded with glyphosate showing the response to treatment with 20 mM ammonium sulfate at pH 9 and recovery following return to normal recirculating buffer. The leaf tissue was pretreated by incubation with buffer containing 10 mM glyphosate for 14 h (100% oxygen, 20°C, and dark) followed by a quick wash with glyphosate-free buffer. All spectra result from successive longitudinal 1-h data collection increments in glyphosate-free perfusion under three different conditions. A, Control. Spectra were collected during the first phase following pretreatment, a 3-h glyphosate-free buffer perfusion. The inset shows cytosolic glyphosate (CG) and vacuolar glyphosate (VG) compartmentalization at the start and end of the control phase. Note the increase of the vacuolar component at the expense of the cytosolic component. For the 3-h data shown, the ratio of vacuole glyphosate signal amplitude to the total glyphosate signal amplitude (VG + CG) is 84% and total glyphosate signal amplitude relative to the MDP external reference [(VG + CG)/MDP] is 0.77, with an estimated uncertainty of 0.05. B, Ammonia challenge. Spectra were collected during the second phase, a 3-h perfusion with glyphosate-free 10 mM ammonium sulfate-containing buffer adjusted to pH 9. Note the cellular response, specifically the changes in pH and Pi compartmental occupancy. C, Recovery. Spectra were collected during the third phase, in which the perfusion medium is returned to the normal glyphosate-free (and ammonium-free) buffer. Note the return to a spectral pattern (CG, VG, cytoplasmic Pi [CPi], and vacuolar Pi [VPi]) very close to the pattern seen at the end of the first (control) phase (A, 3 h). The inset shows cytosolic and vacuolar glyphosate compartmentalization at the start and end of recovery with evidence that vacuolar sequestration has continued at the expense of cytosolic glyphosate content. For the 3-h data shown, the ratio of vacuole glyphosate signal amplitude to the total glyphosate signal amplitude (VG + CG) is 89% and total glyphosate signal amplitude relative to the MDP external reference [(VG + CG)/MDP] is 0.85, with an estimated uncertainty of 0.05, not statistically different from that at the end of the control phase. Abbreviations regarding resonance assignments are as follows: PEP, phosphoenolpyruvate; and Sugar-P, sugar phosphates.
continued perfusion, reaching a maximum at roughly 8 to 10 h.

For perfusion with buffer containing 10 mM glyphosate for times longer than 12 to 14 h, GS and to a lesser extent GR horseweed experience some decrease of cytosolic pH and an eventual decrease of ATP consistent with cell death (Ge et al., 2010; Supplemental Fig. S1). Efflux of glyphosate out of the cell also can be observed for times beyond 12 to 14 h, where pH gradients become compromised (Supplemental Fig. S1). These observations from continuous glyphosate perfusion, where only the cytosolic glyphosate signal is resolvable (i.e. the intense signal from glyphosate in the perfusion medium overlaps that from glyphosate in the vacuole), are fully consistent with the vacuole sequestration reported previously during hours 12 to 24 of the chase phase of a pulse-chase protocol, where both cytosolic and vacuolar glyphosate could be monitored (Ge et al., 2010). The near equivalence of cytosolic glyphosate content for GR and GS horseweed leaf tissue during the initial 6 h of perfusion with buffer containing glyphosate suggests that the vacuole sequestration process may include an induction or turn-on period, only becoming significant several hours after glyphosate has entered the cell.

The GR horseweed data shown in Figure 3A, where the pulse-chase protocol was employed over a 10-fold concentration range, demonstrates that the rate of glyphosate entry into the plant leaf is linear in glyphosate concentration, consistent with first order kinetics. The advantage of the pulse-chase protocol is that both the cytosol and vacuole components can be determined. That vacuole sequestration is saturable (Fig. 3B)

Figure 6. $^{31}$P-NMR spectra of GR horseweed leaf tissue pretreated for 6 h with either perfusion buffer containing 20 mM vanadate or vanadate-free buffer prior to exposure, in either case, to buffer containing 10 mM glyphosate for 14 h. A 4-h wash phase followed the 14-h glyphosate pulse phase. Leaf tissue was maintained throughout the entire protocol at 20°C, in the dark, in buffer equilibrated with 100% oxygen. A, $^{31}$P-NMR spectra obtained during three 2-h, chase-phase, signal-averaging time blocks, hours 18 to 24 (of glyphosate perfusion), with leaves that were not exposed to vanadate prior to glyphosate exposure. B, $^{31}$P-NMR spectra obtained during three 2-h, chase-phase, signal-averaging time blocks, hours 18 to 24, with leaves that were exposed to vanadate prior to glyphosate exposure. Time labels indicate the end of each 2-h signal-averaging increment. Abbreviations regarding resonance assignments are as follows: CG, cytosolic glyphosate; CPI, cytosolic Pi; MEnPP, 2-C-methyl-d-erythritol-2,4-cyclopyrophosphate; Sugar-P, sugar phosphates; UDPG, uridine 5-diphosphoglucose; VG, vacuolar glyphosate; and VPI, vacuolar Pi. $\alpha$, $\beta$, and $\gamma$-ATP refer to corresponding phosphate groups of ATP, and asterisks indicate residual buffer glyphosate. MDP, an external chemical-shift and resonance-amplitude reference, is assigned a $^{31}$P chemical shift of 18.2 ppm relative to the $^{31}$P chemical shift of 85% phosphoric acid, which is taken as 0.00 ppm.
This, coupled with the lack of cellular evidence for nonpassive, transporter-mediated entry. glyphosate for cell entry (Fig. 4, A and B) provides either glyphosate or NMG. AMPA has a lower AMPA was markedly greater than that observed for similar for all substrates but (2) the vacuole content of (Table II) reveals that (1) cytosolic content was rather glyphosate, NMG, and AMPA when delivered alone into the vacuole. The inhibition shown in Fig. 4) with respect to AMPA and NMG adds further support to the existence of a tonoplast pump. AMPA and NMG are zwitterionic structural analogs of glyphosate. Horseweed biotypes have also been found to be resistant to five other herbicide classes (Heap, 2014). Plausibly, these herbicides may be sequestered in the vacuole in a fashion similar to glyphosate, thus accounting for their resistance (Powles and Yu, 2010; Yu et al., 2010).

That vacuole sequestration is both structurally promiscuous (Table II) and competitive (with regard to the inhibition shown in Fig. 4) with respect to AMPA and NMG adds further support to the existence of a tonoplast pump. AMPA and NMG are zwitterionic structural analogs of glyphosate. Horseweed biotypes have also been found to be resistant to five other herbicide classes (Heap, 2014). Plausibly, these herbicides may be sequestered in the vacuole in a fashion similar to glyphosate, thus accounting for their resistance (Powles and Yu, 2010; Yu et al., 2010).

The finding that AMPA and NMG compete with glyphosate for cell entry (Fig. 4, A and B) provides evidence for nonpassive, transporter-mediated entry. This, coupled with the lack of cellular efflux of glyphosate when tissue is maintained as viable, suggests that the putative plasma transporter is a one-way entry portal into the cell. Likewise, studies of GR horseweed at 44 h after treatment (Ge et al., 2010) or at longer time periods (up to 3 weeks) show that glyphosate remains in the vacuole, consistent with the putative tonoplast transporter being a one-way portal into the vacuole.

A comparison of the transport and sequestration of glyphosate, NMG, and AMPA when delivered alone (Table II) reveals that (1) cytosolic content was rather similar for all substrates but (2) the vacuole content of AMPA was markedly greater than that observed for either glyphosate or NMG. AMPA has a lower $M_t$ than glyphosate and NMG. Furthermore, both glyphosate and NMG carry an additional negative charge due to their carboxyl groups (the pH of the substrate perfusate is above their respective acid dissociation constants). Their larger size and the presence of a carboxyl group may limit the transport/sequestration of glyphosate and NMG relative to AMPA.

Acid or ionic trapping is a common theory to explain both the cellular delivery and cellular retention of ionic herbicides. The herbicide bentazon, for example, was reported to experience a 10-fold improved uptake at pH 4.6 versus 6.6 (Sterling et al., 1990). This was attributed to increased passive transport across the plant cell membrane for the uncharged molecule present at pH 4.6, followed by deprotonation of bentazon at the higher intracellular pH. The deprotonated state effectively traps the anionic herbicide, as passive transport across a membrane is less favored for a charged species.

We reported previously that glyphosate uptake was not pH dependent in GS horseweed between pH 5 and 8, arguing against glyphosate entry into the plant cell by passive diffusion followed by acid/ionic trapping in the cytosol (Ge et al., 2013). However, vacuole sequestration may result from acid/ionic trapping, as the vacuole pH differs significantly from that of the cytosol. To determine if acid/ionic trapping of glyphosate in the vacuole is important, glyphosate-treated horseweed was exposed to buffer with basic pH. The question was whether a strongly basic extracellular pH would lead to an increase in cytosol and vacuole pH, via ammonia infiltration into the cell, and facilitate glyphosate efflux from the vacuole or possibly from the plant cell altogether. GR horseweed leaves were exposed to a 3-h, 20 mM ammonium sulfate perfusion at pH 9 in a fashion consistent with a prior report (Gerendás and Ratcliffe, 2000). Notably, the cellular response was: (1) either a massive outflow of Pi from the cell vacuole to the cytosol, presumably to maintain nearly physiological pH (6.8) of the cytosol in response to the influx of ammonia, or alternatively a pH increase of some vacuoles or vacuole subcompartments to that of the cytosol; (2) an increase in the majority vacuole pH to 6.2 from 5.5, as evidenced by the change in chemical shift (Gerendás and Ratcliffe, 2000; Ge et al., 2010); (3) a merging of the glyphosate vacuole and cytosol pH-dependent chemical shifts; and (4) no apparent loss of glyphosate from either the vacuole or the plant cell. With regard to observation 4, the maintenance of glyphosate levels in the vacuole during the pH jump phase was inferred, but not directly monitored (see observation 3), from the observations that by the end of the 3-h recovery phase (phase C, where cytosolic and vacuolar glyphosate are resolvable) the flow of glyphosate into the vacuole was found to have continued and the total cellular glyphosate signal amplitude (cytosol plus vacuole) was not different from that prior to treatment with basic medium. Importantly, during the recovery phase, there is a nearly complete reversal of the pH and Pi compartmental changes that occurred in response to the cellular ammonia influx that occurred during the pH 9, 3-h time period.

Figure 7. Chemical structures.

![Chemical structures](Image)

**AMP A**

**Glyphosate (PMG)**

**N-methyl glyphosate (NMG)**
The ammonia challenge to GR horseweed demonstrates the remarkable ability of the plant cell to metabolically adjust to the alkaline challenge and thereby maintain nearly normal cellular pH. Consistent with other reports (Roso and Vidal, 2010; Staub et al., 2012), these observations also provide evidence that the tonoplast phosphate portal is not used by glyphosate as vacuole sequestration resumes during the recovery phase (Fig. 5C), where it left off at the end of the control phase (Fig. 5A). Finally, this experiment provides no support for acid/ionic trapping of glyphosate in the vacuole.

Vanadate has been reported to alter function in membrane channels as well as to form ATP/ADP-like molecules where VO$_4^{3-}$ replaces PO$_4^{3-}$, thus modifying reactivity (Urbatsch et al., 1995a, 1995b). Vanadate has a history of being linked to the inhibition of transmembrane pumps that utilize ATP (Klein et al., 2006; Oldham et al., 2008). Of relevance herein, Hetherington et al. (1998) found that 1 μM vanadate inhibited glyphosate uptake by 22%. In contrast, Gosset et al. (2008) reported that the passive permeation of structurally related but highly hydrophobic aminomethylphosphonate esters, such as diethyl(2-methylpyrrolidin-2-yl) phosphonate, into the Dictyostelium discoideum cell membrane was not inhibited by vanadate. Thus, vanadate would be expected to inhibit ATP-dependent transmembrane pumps but not passive diffusion across membranes. That markedly reduced amounts of glyphosate are detected in GR leaf tissue pretreated with vanadate (Fig. 6) supports and is consistent with the hypothesis that glyphosate entry into the plant cell is an active ATP-dependent process involving a transporter.

Prior low-temperature studies (Ge et al., 2011) and the vanadate findings reported here are consistent with the proposition that interfering with transmembrane pump activity reduces glyphosate transport. In the case of vanadate exposure, the hypothesis is that vanadate interferes with transmembrane pump-specific dephosphorylation of ATP (ATP → ADP + Pi + energy), presumably inhibiting a leaf plasma membrane-bound ATPase or GTPase, and thus prevents glyphosate from entering the cell. In the case of our prior low-temperature 31P-NMR studies (Ge et al., 2011), the hypothesis is that low temperature reduces the activity (kinetic rate) of transtonoplast pumps and, thus, markedly reduces the vacuole sequestration of glyphosate. In both cases, reduced transmembrane pump activity results in reduced glyphosate transport: blocked entry into the cell in the case of vanadate and diminished entry into the vacuole in the case of lower temperature.

CONCLUSION

Evidence is presented from in vivo (and extract) 31P-NMR analysis for putative GR horseweed apoplast and tonoplast glyphosate transporters. Specifically, data are shown supporting (1) ATP-dependent transport, (2) vacuole sequestration of multiple substrates, (3) competitive inhibition of cell entry and vacuole sequestration, and (4) strong suppression of glyphosate cell entry by vanadate. Such behavior is consistent with active (ATP-dependent) membrane glyphosate transport. It is anticipated that in vivo NMR studies of whole-plant tissue will continue to play an important role in understanding herbicide action in weeds and genetically modified crops (Dayan et al., 2010; Grossmann et al., 2012; Délye et al., 2013).

MATERIALS AND METHODS

Horseweed Plants

GS and GR horseweed (Conyza canadensis) plants (Vangsessel, 2001) were grown in a greenhouse in 7.5 × 7.5 × 7.5-cm$^3$ pots with Read-Earth (Osmocote 14-14-14) soil under conditions of 30°C/22°C day/night and a 14-h photoperiod at 700 to 900 μE. The selection and maintenance of this genetic line of plants have been described (Ge et al., 2011).

Chemical Reagents

All chemicals used were reagent quality and identical to those employed previously (Ge et al., 2010), unless specified otherwise. For studies on whole-leaf tissue, the aqueous infiltration and perfusion medium/buffer solution (hereafter buffer) was composed of 12 mM MES and 50 mM Suc. The buffer pH was adjusted to 5 with 2 M KOH, if necessary, and was used for each trial and discarded if older than 7 d. The buffer contains 0.1% (v/v) D$_2$O (Cambridge Isotope Laboratories) to maintain a field-frequency lock during the duration of each NMR experiment. Glyphosate [N(phosphono)methylglycine] and NMG were obtained from Monsanto. AMPA and sodium orthovanadate were obtained from Sigma-Aldrich. These reagents, when used in plant tissue perfusion protocols, were added to the buffer prior to pH adjustment. Chemical structures of glyphosate, AMPA, and NMG are shown in Figure 7.

Leaf Tissue Perfusion Apparatus and in Vivo 31P-NMR Analysis

Leaf Preparation and NMR Perfusion Apparatus

The leaf tissue perfusion protocol and 31P-NMR monitoring have been described in detail previously (Ge et al., 2010). Briefly, leaves are harvested, vacuum infiltrated with buffer, aligned parallel, and packed loosely in a vertical orientation within a 10-mm (i.d.) glass NMR tube (Wilmad-Labglass). A capillary tube containing MDPS is included in the tube with the plant tissue and serves as an external 31P-NMR concentration and chemical-shift reference. The sample tube is coupled to a perfusion apparatus, which is driven by a peristaltic pump circulating buffer (4–6 mL min$^{-1}$) to which various substrates can be added. Unless noted otherwise, the buffer is maintained at 20°C, equilibrated with 100% (v/v) oxygen, and adjusted to pH 5. Samples within the magnet of the NMR spectrometer are in the dark.

Pulse-Chase Protocol

A pulse-chase protocol, and variations thereof, are employed in many of the NMR leaf tissue perfusion experiments. In the pulse phase, leaf tissue is perfused with buffer containing a substrate (e.g. glyphosate, AMPA, or NMG) for the purpose of exposing the leaf tissue to the substrate. The pulse phase typically lasts for 10 h. The pulse phase is followed by a wash phase in which substrate-free buffer is used to wash out residual, adventitiously bound substrate(s) from the perfusion system, leaf surface, and apoplast (i.e. any substrate not residing in the intracellular compartments). Importantly, the wash phase eliminates what would otherwise be an interfering, intense, buffer-born substrate not residing in the intracellular compartments). Importantly, the wash phase eliminates what would otherwise be an interfering, intense, buffer-born substrate not residing in the intracellular compartments). Importantly, the wash phase eliminates what would otherwise be an interfering, intense, buffer-born substrate not residing in the intracellular compartments).

31P-NMR Data Acquisition and Analysis

31P-NMR data are generally acquired throughout the perfusion time course in 2-h signal-averaging periods on a 500-MHz DirectDrive (Agilent/Varian)
Glyphosate Delivery and Compartmentalization: Effect of Oxygen and Light

**In Vivo Analysis of Cytosolic and Vacuolar Glyphosate**

Mature leaves (five to six pieces, approximately 1–2 g wet weight) from healthy GR horseweed plants (12-cm rosette with 20–30 leaves total) were harvested, immersed in buffer containing 10 mM glyphosate, vacuum infiltrated, and then incubated in the same glyphosate-containing buffer for 14 h at 20°C. Variable incubation conditions included (1) maintenance in the dark (respiration) with buffer equilibrated with 100% oxygen via gas bubbling, (2) maintenance in the dark (respiration) without buffer equilibrated with air (approximately 20% [v/v] oxygen) via gas bubbling, and (3) maintenance in the light (150 μE m⁻² s⁻¹; photosynthesis) in the absence of gas equilibration (no oxygen or air bubbling) of the buffer with pH adjusted to 6.5 (sufficient dissolved CO₂ is maintained at a pH of 6.5). For the light-exposure condition, leaf tissue floated on the surface of the medium and dissolved CO₂ in the buffer solution was maintained by the addition of 5 mM potassium bicarbonate (Macdonald, 1975; Callies et al., 1990). Following all incubations, the wet leaves were washed quickly with glyphosate-free buffer to remove adventitiously adhering glyphosate, placed in the perfusion apparatus with circulating glyphosate-free buffer, and 31P-NMR data were acquired.

**Extract Analysis of ATP and S3P**

Mature leaves (approximately 2 g) from healthy GR horseweed plants were harvested and exposed to the incubation conditions described above. Following incubation, leaves were rinsed with deionized water to remove adventitiously adhering glyphosate and immediately flash frozen with liquid nitrogen. Ice-cold 3.5% (v/v) HClO₄ (6 mL) was added, and the leaf samples were ground to paste in a mortar. Samples were freeze thawed twice and centrifuged (Sorvall rc-5b refrigerated superspeed centrifuge) at 10,000 × g for 20 min, and the supernatant was collected. Leaf debris was extracted again with 3 mL of ice-cold 3.5% (v/v) HClO₄. Supernatant volumes were dissolved CO₂ is maintained at a pH of 6.5. For the light-exposure condition, leaf tissue floated on the surface of the medium and dissolved CO₂ in the buffer solution was maintained by the addition of 5 mM potassium bicarbonate (Macdonald, 1975; Callies et al., 1990).

Following all incubations, the wet leaves were washed quickly with glyphosate-free buffer to remove adventitiously adhering glyphosate, placed in the perfusion apparatus with circulating glyphosate-free buffer, and 31P-NMR data were acquired.

**Glyphosate Delivery and Compartmentalization: Effect of pH and Ammonia**

Mature leaves (approximately 1 g) from healthy GR horseweed plants were harvested and immersed in buffer containing 10 mM glyphosate, vacuum infiltrated, and then incubated in the glyphosate-containing buffer with bubbling of 100% oxygen in the dark for 14 h at 20°C. Following the incubation period, the leaves were quickly washed with glyphosate-free buffer and placed in the perfusion apparatus for the in vivo ³¹P-NMR assay. Data were then collected longitudinally during three phases of altered perfusion conditions. NMR monitoring during each phase consisted of three 1-h data collection periods. In the first phase (control), the leaves were perfused with glyphosate-free buffer. In the second phase (alkalinization and ammonia infusion), leaves were perfused with an aqueous solution of 10 mM N-Tris (hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), 20 mM ammonium sulfate, and 50 mM Suc with pH adjusted to 9. In the third phase (recovery), the leaf tissue was perfused with glyphosate-free buffer. These experiments were performed in triplicate.

**Glyphosate Delivery: Effect of Vanadate**

Freshly harvested GR horseweed leaves were immersed in glyphosate-free buffer with pH adjusted to 6.5, either with or without 20 mM orthovanadate, and incubated (20°C and 100% oxygen) in the dark for 6 h. Following this incubation phase, the leaf tissue was washed quickly with vanadate-free buffer, vacuum infiltrated with vanadate-free buffer containing 10 mM glyphosate, placed within the NMR spectrometer, and perfused with vanadate-free buffer containing 10 mM glyphosate for 14 h (pulse phase), followed by a 4-h wash phase and a 6-h chase phase, and both phases used glyphosate-free buffer. ³¹P-NMR data were acquired during the 6-h chase phase. This protocol was repeated three times.

**Time Course of Glyphosate Uptake: GR versus GS Horseweed**

Leaf tissue from GR and GS horseweed was harvested, vacuum infiltrated with glyphosate-free buffer, and exposed directly within the ³¹P-NMR perfusion apparatus to a 12-h continuous perfusion with buffer containing 10 mM glyphosate. ³¹P-NMR data were acquired during this 12-h period.

The glyphosate ³¹P-resonance chemical shift is pH dependent. Glyphosate present in the buffer (pH 5) has a chemical shift that is very similar to that for vacuole-sequestered glyphosate (vacuole pH approximately 5.5) but dissimilar to that for cytosolic glyphosate (cytosol pH approximately 7; Ge et al., 2010, 2011, 2012). Thus, this protocol results in a buffer-born glyphosate ³¹P-NMR signal that obscures the vacuole-sequestered glyphosate signal, and only cytosolic glyphosate can be quantified.

**Dependence of Glyphosate Uptake Rate on Buffer Glyphosate Concentration with GR Horseweed**

Leaf tissue from GR horseweed was harvested, vacuum infiltrated with glyphosate-free buffer, and exposed directly within the ³¹P-NMR perfusion apparatus to a 10-h continuous perfusion (pulse phase) with buffer containing one of seven different, fixed glyphosate concentrations, which varied from 2 to 20 mM. The pulse phase was followed by a 2-h wash phase and 12-h chase phase, and both phases used glyphosate-free buffer. ³¹P-NMR data were acquired throughout the protocol.

**AMPA, NMG, and Glyphosate: Compartmentalization and Competition**

Leaf tissue from GR horseweed was harvested, vacuum infiltrated with glyphosate-free buffer, and exposed directly within the ³¹P-NMR perfusion apparatus to a 10-h continuous perfusion (pulse phase) with buffer containing (1) 10 mM AMPA, (2) 10 mM NMG, (3) both 10 mM glyphosate and 10 mM AMPA, or (4) both 10 mM glyphosate and 10 mM NMG. The pulse phase was followed by a 2-h wash phase and 12-h chase phase, and both phases used buffer free of glyphosate, AMPA, and NMG. ³¹P-NMR data were acquired throughout the chase phase. (Glyphosate uptake and compartmentalization data obtained when glyphosate is delivered alone were reported previously [Ge et al., 2010], and this protocol was not repeated here.)

**Supplemental Data**

**Supplemental Figure S1.** ³¹P-NMR spectral observables for GS and GR horseweed.

**Supplemental Figure S2.** ³¹P-NMR spectra of GR horseweed treated with vanadate.

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**LITERATURE CITED**


Kulichikhin KY, Aitio O, Chirkova TV, Fagerstedt KV (2007) Effect of oxygen concentration on 


Lolium rigidum


therapeutic targets in autoimmunity and cancer. J Leukoc Biol 86: 1075–1087


CORRECTIONS

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Figure 7 showed incorrect chemical structures of glyphosate (PMG) and N-methyl glyphosate (NMG). Both structures should have included an additional –CH$_2$- group between the nitrogen and the carbonyl group. The revised Figure 7 included below shows the corrected chemical structures.

![Chemical structures](image)

**Figure 7.** Chemical structures.