Published in final edited form as:
Pest Manag Sci. 2010 April ; 66(4): 345–348. doi:10.1002/ps.1911.

Rapid vacuolar sequestration: the horseweed glyphosate resistance mechanism

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Abstract

BACKGROUND—Glyphosate-resistant (GR) weed species are now found with increasing frequency and threaten the critically importantGR weed management system.

RESULTS—The reported ^{31}P NMR experiments on glyphosate-sensitive (S) and glyphosate-resistant (R) horseweed, *Conyza canadensis* (L.) Cronq., show significantly more accumulation of glyphosate within the R biotype vacuole.

CONCLUSIONS—Selective sequestration of glyphosate into the vacuole confers the observed horseweed resistance to glyphosate. This observation represents the first clear evidence for the glyphosate resistance mechanism in *C. canadensis.*

Keywords

glyphosate; glyphosate-resistant; *in vivo*^{31}P NMR; *Conyza canadensis*

1 INTRODUCTION

The evolution of glyphosate-resistant (GR) weed variants has been significantly slower than the evolution of resistance to most other commonly used herbicides,[1] but their numbers are increasing and represent a threat to the worldwide usage of GR crops.[2] Understanding the mechanism of glyphosate resistance in weeds is urgently required to ensure the continued use and further development of this immensely important GR technology.[3 – 5] The ^{31}P NMR studies reported in the present work support a plausible explanation for the resistance mechanism in GR horseweed, *Conyza canadensis* (L.) Cronq., found on five continents.[6] Advantage was taken of the pH-dependent ^{31}P NMR chemical shift of glyphosate to identify and quantify its presence in cytoplasm versus vacuole compartments in leaf tissue.[7 – 10] The results unambiguously show differences between R and S plant tissue in (i) the occupancy of two pools of glyphosate *in vivo* – cytoplasm and vacuole, (ii) the concentrations of glyphosate available for translocation and (iii) the kinetics of transport between glyphosate pools. These results provide a conceptual framework by which to gain a better understanding of existing published work[11 – 15] related to GR horseweed, and form the basis for identifying the accumulation of glyphosate in the cell vacuole as the resistance mechanism.

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2 METHODS

The homozygous (selfed four generations) R and S plant lines of horseweed used for this study were grown side by side from seed in Readi-Earth® (Osmocote 14-14-14) soil and controlled climate conditions in a greenhouse (27/21 °C day/night temperatures with supplemental lighting). To mimic glyphosate application conditions in the field, R and S horseweed plants were sprayed with glyphosate 540 g AE L^{-1} SL (WeatherMAX®, Monsanto) at 3.3 kg AE ha^{-1} (4× field use rate) and tissue was later harvested for *in vivo* ^{31}P NMR studies. Young, growing leaves in the apical meristem (sink tissue) were protected from direct exposure to the herbicide with aluminum foil. Leaf tissues were harvested at various times after spraying, washed repeatedly with deionized water prior to vacuum infiltration with the perfusion buffer, placed in a 10 mm NMR tube and fitted with a perfusion system for NMR studies at 11.74 T (Varian Inova-500).[10] Relative glyphosate partitioning (cytoplasm and vacuole) was established from signal magnitudes using Bayesian-probability-theory-based signal analysis algorithms and associated software.[16] For pulse chase studies, untreated mature-source leaf tissue was harvested from plants and handled as noted above. The tissue was perfused with a buffered 10 mM glyphosate media for 10 h and then replaced with a glyphosate-free perfusion solution, and data were collected in the same fashion for the next 14 h.

3 RESULTS AND DICUSSION

Panels A and B in Fig. 1 show treated mature-leaf ^{31}P NMR spectra, with insets of the glyphosate region, from R and S plants respectively, harvested sequentially at 4, 24 and 44 h after treatment with glyphosate. Signals from high-energy phosphates (ATP, UDPG) confirm tissue viability.[17,18] Two chemical-shift resolved resonances were observed for glyphosate inside the plant tissue and are indicative of its presence in two different pH environments.[19] [The glyphosate ^{31}P chemical shift versus pH relationship is shown in Fig. 1D, and the signals for inorganic phosphate (Pi) corroborate the two pH environments.] The narrower signal at 8.04 ppm for glyphosate was observed several hours after spraying in both R and S tissue and was assigned to glyphosate in the cell cytoplasm (CG, pH ~7.0).[9] The broader signal at 8.58 ppm appeared later in the time course and was assigned to glyphosate in the cell vacuole (VG, pH ~5.5). The amplitude of the vacuole signal increased at the expense of the cytoplasm signal with time, suggesting that the two compartments were directly related. Notably, at 24 h after spraying, treated mature leaves from R plants showed significant (greater than 85%) glyphosate fractional occupancy of the vacuole, whereas the corresponding occupancy of the vacuole S tissue was much less, at approximately 15% occupancy.[16] (The vacuole pH of 5.5, inferred from the ^{31}P NMR chemical shifts of both glyphosate and Pi, was the same for both R and S variants.) It is emphasized that the rate of vacuole accumulation of glyphosate was far more rapid in R tissue than in S tissue, even though the net uptake of glyphosate into the cell was the same.[11] Indeed, vacuole sequestration of glyphosate was evident shortly (within hours) after spraying in mature R leaf tissue and only clearly obvious in treated mature S leaves 24 h after spraying. This strikingly rapid rate of vacuole sequestration explains prior reports of decreased translocation of glyphosate in R compared with S horseweed.[11,13]

Glyphosate is translocated via the phloem, following along a sucrose gradient from mature source leaves to rapidly growing sink tissues.[20] To investigate glyphosate delivered by the phloem, ^{31}P NMR studies were carried out on sink tissues protected during the sprayed application of glyphosate. Figure 1E shows representative ^{31}P NMR spectra from unexpanded sink leaves harvested 24 h after treatment of mature-source leaves from R and S plants. Shikimate-3-phosphate (S3P) is observed to accumulate at a much higher concentration in sink leaves compared with source leaves (Fig. 1E).[9] There are two reasons

that could explain the enhanced sensitivity (evidenced by S3P build-up) of sink compared with source tissue to glyphosate. One is that there are higher levels of EPSPS enzyme in the sink tissue.[21] A second is that young, rapidly growing sink tissue commonly has smaller vacuoles and thus cannot shield the chloroplast effectively from glyphosate. From prior-sprayed ^{14}C-glyphosate delivery experiments, it was shown that within 24 h about 35% of the glyphosate entering the leaf is translocated from the source tissue to sink tissues in S horseweed.[11] The amount translocated in R horseweed is about half this amount. Note that, while only the cytoplasmic glyphosate signal (8.04 ppm) was observed in S sink leaves, signals from glyphosate in both compartments, cytosol and vacuole, were observed in R sink leaves (Fig. 1E inset). As with source leaves, R sink-leaf vacuoles accumulate the majority of the observed glyphosate. Thus, preferential vacuole sequestration in R variants not only makes less glyphosate available for translocation from source to sink tissues, but within sink tissue this sequestration also reduces the cytosolic glyphosate pool available to enter the chloroplast and inhibit EPSPS, as is requisite for herbicide action. The observed sink-leaf vacuole sequestration of phloem-translocated glyphosate is fully consistent with the prior observation that glyphosate in sink tissue is less toxic in R versus S plants.[11] From the present experiments, the unique ability of R tissue cells to sequester glyphosate into the vacuole (not the apoplast as hypothesized [15]) appears to be plant wide [14] and affords progressive removal of glyphosate to the cell vacuole at all points in the R plant. The rapidly growing sink tissue is ultimately protected by its own vacuolar glyphosate sequestration, in addition to that of the source leaves.[15,22]

Given that vacuolar sequestration of glyphosate in R horseweed occurs rapidly and can be easily quantified by *in vivo* ^{31}P NMR, 'pulse chase' studies were carried out to investigate the dynamics of this process.[23] Data from pulse chase experiments are summarized in Fig. 2. Here, ^{31}P NMR measurements were averaged over successive 2 h time blocks following exposure of source-leaf tissue to flowing media containing glyphosate (pulse period 10 h). During the 14 h chase period, the perfusate was switched to a glyphosate-free solution. In the absence of the dominant, interfering signal from glyphosate in the perfusate, this allowed quantification of vacuole ingress as well as the mass balance of the herbicide in both R (Fig. 2A) and S (Fig. 2B) tissues. Similar to the glyphosate spray application studies, the ^{31}P resonance from glyphosate in the cytosol is observed in both R and S, but only in the R variant is the vacuole signal observed. Glyphosate migrates substantially (65–85%) to the vacuole R tissue over the 24 h monitoring period, yet is absent from the vacuole in S tissue over the same period. The bar graphs show glyphosate partitioned between vacuole and cytoplasm at selected times during the chase period from trials with both R (Fig. 2C) and S (Fig. 2D) leaf tissue. Note that the R tissue loses little glyphosate during the chase phase to the circulating perfusate, while S tissue loses a significant amount (~35%). This implies that glyphosate entry and exit from the cytoplasm (apoplast and symplast) is diffusion controlled and reversible.[12] However, once glyphosate crosses the tonoplast, it is effectively trapped. This suggests that there may be a tonoplast transporter for glyphosate.[24,25]

The following view of horseweed resistance to glyphosate emerges from the data presented herein. Glyphosate enters the cytoplasm of both R and S plant variants at the same rate. Within hours, however, glyphosate begins to occupy the vacuole in the R but not the S biotype. The identical pH values of R and S vacuoles speak against the possibility of a pH-driven process. This, coupled with the preferential movement of glyphosate from the cytosol to the vacuole in R tissue but not in S, suggests the presence of a transporter for glyphosate either specific to R or at a substantially greater concentration in R than in S tissue. Glyphosate in the cytoplasmic pool is available for translocation to sink tissues. However, glyphosate sequestered within the vacuole is effectively removed from the phloem-accessible pool of glyphosate and explains the reduced translocation previously observed.[11, 13] Sensitive plants succumb to the lethal effects of glyphosate largely because there is a

greater reservoir of glyphosate in the cytoplasm of source and sink tissues. The resistance mechanism for R plants reflects an inherent ability[14] to sequester glyphosate in the vacuole, where, presumably, it stays indefinitely or is released slowly at a sublethal rate.

Work is in progress to: (i) establish the dose dependence of glyphosate pool-partitioning in horseweed, (ii) search for a glyphosate transporter, (iii) establish the extent to which this mechanism of glyphosate resistance is relevant for other plant species and (iv) determine the substrate diversity for the transporter that selectively sequesters xenobiotics into the R horseweed vacuole. The present observation of glyphosate preferentially loading in the R vacuole is similar to reports of ABC transporter systems for detoxification. Yuan *et al*.[25] have demonstrated that a normal ABC transporter in *Arabidopsis* can be used to pump a xenobiotic into the vacuole and shield the cell from the normally toxic effects of the antibiotic, thereby creating a selection mechanism for cells overexpressing the transporter as they are resistant to higher doses of the phytotoxin. Horseweed might be using a similar strategy, whereby a tonoplast transporter can sequester glyphosate and shield the chloroplast EPSPS from glyphosate. The functional ability to move glyphosate into the tonoplast exists in sensitive horseweed but is dramatically improved in resistant horseweed, suggesting overexpression or upregulation of the putative transporter.

Acknowledgments

Funding for this research was provided by Monsanto Company.

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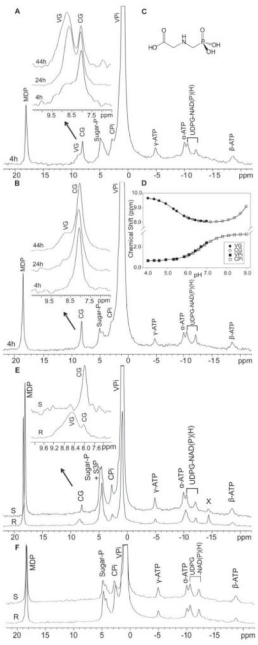

Figure 1.

In vivo ^{31}P NMR spectra of R and S horseweed leaves treated with glyphosate at 3.3 kg AE ha^{-1}. **A**, ^{31}P NMR spectra of R horseweed source leaves obtained at 4, 24 and 44 h after spraying. **B**, ^{31}P NMR spectra of S horseweed source leaves obtained at the same times after spraying. **C**, Glyphosate chemical structure. **D**, Glyphosate and Pi ^{31}P chemical shift dependence as a function of pH determined from standard solutions that mimic environments of the vacuole and cytoplasm: vacuolar glyphosate (●), cytoplasmic glyphosate (○), vacuolar Pi (■), cytoplasmic Pi (□). **E**, ^{31}P NMR spectra acquired from R and S horseweed sink leaves 24 h after glyphosate spraying of source leaves. S3P, shikimate-3-phosphate; X indicates an unknown esterified phosphoryl metabolite. **F**, ^{31}P NMR spectra of R and S horseweed leaves without glyphosate treatment. *Note*: 0.00 ppm assigned to 85% phosphoric acid (external) in separate experiments. Abbreviations: MDP, methylene diphosphonate, an external concentration reference; VG, vacuolar glyphosate; CG, cytoplasmic glyphosate; VPi, vacuolar phosphate; CPi, cytoplasmic phosphate; Pi, inorganic phosphate; UDPG, uridine 5′-diphosphoglucose; *α*-, *β*-, *γ*-ATP refer to corresponding ATP phosphate groups; Sugar-P, sugar phosphates.

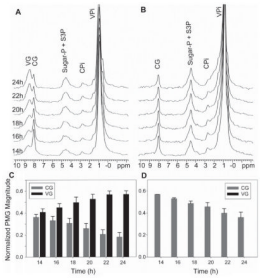

Figure 2.

In vivo ^{31}P NMR time course data for R and S horseweed source leaves collected during the chase phase after a 10 h pulse period with 10 mM glyphosate. **A**, ^{31}P NMR stacked plot of data obtained during the chase phase with R horseweed mature leaves. **B**, ^{31}P NMR stacked plot of data obtained during the chase phase with S horseweed mature leaves. Each plot represents a 2 h data collection. Note that, at all time periods, glyphosate occupies the cell vacuole (broad signal at 8.68 ± 0.10 ppm) appreciably in R tissue, whereas little or none is observed in S tissue. **C**, Total observable glyphosate (PMG, phosphonomethylglycine) during the 'chase' period for R horseweed plant tissue (n = 3). For R tissue, glyphosate progressively leaves the cytoplasm (gray) and occupies the vacuole (black) during this period. The Y-axis units are normalized to the external reference MDP signal magnitude. **D**, Total observable glyphosate during the 'chase' period for S horseweed plant tissue (n = 3). Error bars indicate SEM.