

## Possible Glyphosate Tolerance Mechanism in Pitted Morningglory (*Ipomoea lacunosa* L.)

Daniela N. Ribeiro,<sup>†,||</sup> Vijay K. Nandula,<sup>\*,‡</sup> Franck E. Dayan,<sup>§</sup> Agnes M. Rimando,<sup>§</sup> Stephen O. Duke,<sup>§</sup> Krishna N. Reddy,<sup>‡</sup> and David R. Shaw<sup>†</sup>

<sup>†</sup>Department of Plant and Soil Sciences, Mississippi State University, P.O. Box 9555, Mississippi State, Mississippi 39762, United States

<sup>‡</sup>Crop Production Systems Research Unit, USDA-ARS, P.O. Box 350, Stoneville, Mississippi 38776, United States

<sup>§</sup>Natural Products Utilization Research Unit, USDA-ARS, P.O. Box 1848, University, Mississippi 38677, United States

### S Supporting Information

**ABSTRACT:** Natural tolerance of *Ipomoea lacunosa* to glyphosate has made it problematic in the southeastern U.S. since the adoption of glyphosate-resistant crops. Experiments were conducted to determine (i) the variability in tolerance to glyphosate among accessions, (ii) if there is any correlation between metabolism of glyphosate to aminomethylphosphonic acid (AMPA) or sarcosine and the level of tolerance, and (iii) the involvement of differential translocation in tolerance to glyphosate. Fourteen *I. lacunosa* accessions had  $GR_{50}$  values ranging from 58 to 151 grams of acid equivalent per hectare (ae/ha) glyphosate, a 2.6-fold variability in tolerance to glyphosate. There was no evidence of the most tolerant (MT) accession metabolizing glyphosate to AMPA more rapidly than the least tolerant (LT) accession. Metabolism to sarcosine was not found. <sup>14</sup>C-glyphosate absorption was similar in the two accessions. LT accession translocated more <sup>14</sup>C-glyphosate than MT accession at 24 and 48 h after treatment. Differential translocation partly explains glyphosate tolerance in MT accession.

**KEYWORDS:** absorption, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), glyphosate, herbicide resistance, *Ipomoea lacunosa*, metabolism, pitted morningglory, translocation

### INTRODUCTION

The nonselective herbicide glyphosate was first introduced in 1971 and commercialized in 1974.<sup>1</sup> Since then, it has been extensively used in agriculture worldwide to become the most important herbicide ever developed.<sup>1,2</sup> First used as a noncrop, preplant, or orchard and vine crop herbicide, it is now also used in no-tillage systems and in glyphosate-resistant (GR) crops.<sup>1</sup>

The adoption of GR crops has been remarkable, resulting in heavy reliance on glyphosate for weed management.<sup>1</sup> This has caused great selection pressure on the weed communities, resulting in the evolution of glyphosate resistance in 31 weed species.<sup>3</sup> Several weed species have varying degrees of natural tolerance to glyphosate. These species include *Chenopodium album* L. (common lambsquarters), *Ambrosia trifida* L. (giant ragweed, currently considered to have evolved resistance),<sup>3</sup> *Abutilon theophrasti* Medik (velvetleaf), *Dicliptera chinensis* L. Juss. (jussieu), *Oenothera biennis* L. (common evening-primrose), *Pastinaca sativa* L. (wild parsnip), *Phytolacca americana* L. (common pokeweed), *Equisetum arvense* L. (field horsetail), *Commelina* spp., and *Ipomoea* spp.<sup>4</sup>

Among *Ipomoea* spp., *I. lacunosa* L. (pitted morningglory), one of the most common and troublesome weed species in southern U.S. row crops,<sup>5</sup> has considerable genetic variability in its response to glyphosate at typical GR crop application rates.<sup>6,7</sup> Differences in levels of tolerance to glyphosate in *I. lacunosa* have been attributed to limited absorption,<sup>8</sup> but no evidence of differential absorption and translocation or reduced spray coverage was found in other reports on this species.<sup>6,9</sup> Whether glyphosate tolerance in *I. lacunosa* is due to natural

variation or to evolution of low levels of resistance has not been established.

Research by Baucom and Mauricio<sup>10–12</sup> on *I. purpurea* has suggested that less glyphosate-sensitive biotypes of this species are due to selection with glyphosate, as resistant populations could be selected for artificially.<sup>13</sup> Their definitions of tolerance and resistance differ from those of the Weed Science Society of America (which defines tolerance as natural, nonselected herbicide insensitivity and resistance as evolved herbicide insensitivity).<sup>14</sup> No population of any *Ipomoea* species has thus far been recognized as having evolved resistance.<sup>3</sup> *I. purpurea* has been reported to metabolize glyphosate to aminomethylphosphonic acid (AMPA) (Figure 1) to a limited extent,<sup>15</sup> so all or part of its tolerance and/or resistance could be due to enhanced metabolic degradation of glyphosate.

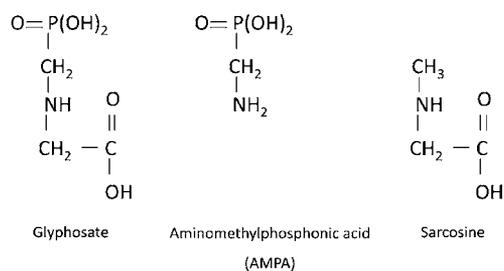
The most frequently detected degradation product of glyphosate is AMPA, with detection of sarcosine (Figure 1) in only a few species.<sup>16</sup> Evidence for either of these two degradation pathways is lacking for many species.<sup>16</sup> AMPA is produced by oxidative cleavage of the C–N bond of glyphosate by a glyphosate oxidoreductase (GOX) enzyme.<sup>16</sup> AMPA is much less phytotoxic than glyphosate.<sup>16</sup> GR canola (*Brassica napus* L.) is at least in part resistant due to a GOX transgene derived from the microbe *Ochrobactrum anthropic*.<sup>1,17</sup> There is

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**Figure 1.** Chemical structure of glyphosate and its metabolites aminomethylphosphonic acid and sarcosine.

no established proof that metabolic degradation plays a role in either glyphosate tolerance or resistance in nontransgenic plants.

Enhanced metabolic degradation of herbicides is a common mechanism of resistance to many herbicide classes.<sup>18</sup> Because several plant species, including all *Ipomoea* species thus far studied, can metabolize glyphosate, we hypothesized that enhanced metabolism of glyphosate would be a likely response to strong selection with glyphosate (resistance) and could also explain biotype differences in susceptibility to glyphosate that are not due to selection with glyphosate (tolerance). The objectives of this research were to investigate (i) the variability in tolerance to glyphosate among *I. lacunosa* accessions, (ii) if there is any correlation between metabolism of glyphosate to AMPA or sarcosine and the level of tolerance, and (iii) the role of differential translocation as a potential mechanism of tolerance to glyphosate.

## MATERIALS AND METHODS

**Plant Material and General Experimental Conditions.** During 2004 through 2006, seeds from *I. lacunosa* were randomly collected at multiple locations across the U.S. that had or had not been exposed to glyphosate for several years in GR crop management systems.<sup>19</sup> *I. lacunosa* seeds were stored at 10 °C until used. Germination of seed, transplanting of seedlings, growth of plants, and all experiments were conducted under greenhouse growing conditions (30/22 °C day/night, 12 h photoperiod under natural sunlight) unless otherwise described. Seeds were planted at 1 cm depth in 50 × 20 × 6 cm plastic trays with drain holes containing a commercial Metro-Mix 360 potting mix (Sun Gro Horticulture, Bellevue, WA). Two weeks after emergence, seedlings were transplanted into 6 × 6 × 6 cm pots containing the same soil mix. Plants were watered as needed. Plants were fertilized once by subirrigating the pots with a Miracle-Gro nutrient solution (The Scotts Company, Marysville, OH) containing 200 mg/L each of N, P<sub>2</sub>O<sub>5</sub>, and K<sub>2</sub>O at 4 weeks after transplanting. All herbicide treatments were applied with an air-pressurized indoor spray chamber equipped with an 8002E flat-fan nozzle (Spraying Systems Company, Wheaton, IL) delivering 140 L/ha at 280 kPa and were made on plants of each accession at the four- to five-leaf stage (beginning to vine).

**Glyphosate Dose Response in *I. lacunosa* Accessions.** Fourteen *I. lacunosa* accessions were selected strictly on the basis of their response to preliminary screening, being the seven most tolerant (MS-WAS-8, MS-VJ, MS-SCO-1, MS-PAN-1, MS-YAZ-2, MS-COA-1, and MS-ITA-1) and the seven least tolerant (MS-LEE-2, MS-WAS-2, MS-MAR-1, MS-99, MS-90, MS-QUI-1, and MS-YAZ-1) accessions, among 76 initial accessions. Plants of each of the 14 accessions were treated with the Roundup WeatherMAX formulation of glyphosate (Monsanto Company, St. Louis, MO) at 0, 105, 210, 420, 840, and 1680 g of ae (acid equivalent)/ha. Shoot fresh weights were recorded at 2 weeks after treatment (WAT) only, as the separation of vines between control plants becomes problematic at later growth stages. Shoot fresh weight was expressed as a percent of nontreated plants. There were four replications per treatment, one plant per replication,

in a completely randomized design, and the experiment was conducted twice.

**Glyphosate Metabolism Studies in Intact Plants.** The most tolerant (MT) (MS-WAS-8) and least tolerant (LT) (MS-YAZ-1) accessions were selected on the basis of the dose response assay described above and were analyzed for metabolism of glyphosate to AMPA and sarcosine as well as shikimate, the precursor of aromatic amino acids. Two experiments were conducted. In the first, five plants were treated with glyphosate at 420 g of ae/ha (0.5 × field rate) and harvested at 2 WAT, and the experiment was conducted twice. In the second, three plant replications were treated at their respective GR<sub>50</sub> doses (MT, 151 g of ae/ha; LT, 58 g of ae/ha) and at 1, 3, and 6 days after treatment (DAT) plants were harvested. The GR<sub>50</sub> rate for each plant accession was selected so that the two accessions would have the same level of phytotoxicity interfering with metabolism of glyphosate. There was one plant per replication in a completely randomized design, and the experiment was repeated over time. At harvesting, plants from both experiments were excised at the soil surface, washed with running water, rinsed with distilled water to remove glyphosate remaining on the leaf surface, and blotted dry with paper towels. Each sample consisted of all leaves from each single plant (replicate) and leaves were predried in a greenhouse and then oven-dried at 80 °C for 2 weeks, ground with a mortar and pestle, and analyzed for glyphosate, AMPA, shikimate, and sarcosine.

Extraction and derivation were performed for glyphosate and AMPA analysis according to previously reported procedures,<sup>20,21</sup> with modifications. Ground tissue (0.25 g) was extracted with 8 mL of water in a 20 mL scintillation vial, shaken, placed in a sonicating bath for 20 min, and then centrifuged at 5000g, 20 °C, for 20 min. The supernatant was collected, the remaining tissue sample pellet was extracted a second time by adding 4 mL of water, and procedures were performed as in the first extraction. The volume of the combined supernatant was measured and syringe-filtered (0.45 μm) into a new 20 mL plastic vial. Then, 45 μL of 12.1 N HCl was added to the supernatant and shaken. Four milliliters was transferred to a 20 mL scintillation vial with a Teflon-lined cap, shaken with 2 mL of CH<sub>2</sub>Cl<sub>2</sub>, and centrifuged at 300g, 25 °C, for 10 min. A portion (1.8 mL) of the top water layer was taken, and 200 μL of acidic modifier [(16 g of KH<sub>2</sub>PO<sub>4</sub>:160 mL of H<sub>2</sub>O):13.4 mL of 12.1 N HCl] was added and vortexed. One milliliter was loaded to a cation exchange (CAX) resin column, 0.8 × 4 cm AG 50W-X8 Resin 200–400 mesh, H<sup>+</sup> (Bio-Rad Laboratories, Hercules, CA), previously equilibrated with two 5 mL portions of water. The sample was eluted until the column bed was reached. Seven hundred microliters of CAX mobile phase (160 mL of H<sub>2</sub>O:40 mL of MeOH:2.7 mL of HCl) was added, eluted, and discarded. Twelve milliliters of CAX mobile phase was again added to the column to elute the analytes. The eluate was collected in a 20 mL vial and evaporated to dryness using a Savant speed vac. A 1.5 mL volume of CAX mobile phase was added to the dried sample, and then the vial was placed in a sonicating bath for 30 min. A 20 μL aliquot was syringe-filtered (0.2 μm) and added to 640 μL of a solution of 2,2,3,3,4,4,4-heptafluoro-1-butanol and trifluoroacetic anhydride (1:2, v/v) in a chilled 4 mL vial with a Teflon-lined lid in a duplicate extraction experiment. The mixture was equilibrated at room temperature for 10 min. The vial was transferred to a heating block at 90 °C for 1 h and then allowed to cool to room temperature. The solvent was evaporated under a stream of nitrogen at 50 °C, and the residue was dissolved in 80 μL of ethyl acetate containing 0.2% citral.

For the analysis of shikimate and sarcosine, a 1 g powdered sample was placed in a 20 mL scintillation vial and extracted with 15 mL of water, shaken, placed in a sonicating bath for 20 min, and then centrifuged at 5000g, 20 °C, for 20 min. A 4 mL aliquot of supernatant was removed to a new 20 mL vial. The tissue sample pellet was extracted a second time by adding 5 mL of water, and procedures were performed as in the first extraction. A 2 mL aliquot of supernatant was removed and combined with the previous 4 mL aliquot, totaling 6 mL of supernatant. Then, 30 μL of HCl was added to supernatant and shaken. One half of the total supernatant was transferred to a tared vial, frozen, and lyophilized. The dry product was recovered, and 5 mg of lyophilized extract was transferred to gas chromatography (GC)

vial, in a duplicate extraction experiment, to be treated with 50  $\mu\text{L}$  of *N,O*-bis(trimethylsilyl)trifluoroacetamide and *N,N*-dimethylformamide (1:1, v/v) and vortexed. The vial was transferred to a heating block at 70 °C for 30 min, allowed to cool to room temperature, and centrifuged at 300g, 25 °C, for 10 min. Then, 25  $\mu\text{L}$  of clear liquid was transferred to a GC vial and analyzed by GC–mass spectrometry (MS).

Analysis of glyphosate and AMPA was performed by GC–MS on a 6890 GC gas chromatograph (Agilent, Santa Clara, CA) coupled to a JEOL GCMateII mass spectrometer (JEOL USA, Peabody, MA), using a 30 m length  $\times$  0.25 mm i.d., 0.25  $\mu\text{m}$  film, DB-5 capillary column (Agilent Technologies, Foster City, CA). The GC temperature program: initialized at 80 °C, held for 2.5 min, raised to 160 °C at 30 °C/min rate, raised to 270 °C at 40 °C/min rate, raised to 300 °C at 35 °C/min rate, and kept at this temperature for 1.5 min. The carrier gas was ultrahigh purity helium at a 1 mL/min flow rate. The injection port was kept at 250 °C, and the GC–MS interface and the ionization chamber were kept at 230 °C. The volume of injection was 1  $\mu\text{L}$  (splitless injection). The mass spectrum was acquired in the positive, low resolution, selected ion monitoring mode, with electron impact of 70 eV. AMPA was monitored using  $m/z$  571, 502, 446, and 372 ( $t_{\text{R}}$  5.97 min); glyphosate was monitored using  $m/z$  611, 584, 486, and 460 ( $t_{\text{R}}$  6.77 min). Glyphosate and AMPA in the samples were quantitated from a calibration curve of the respective standards: glyphosate, purity 99.5% (Chem Service, West Chester, PA), and AMPA, purity 99% (Sigma-Aldrich, Saint Louis, MO). Analysis was performed in duplicate. The LOD and LOQ for glyphosate were 19.9 and 160 pg on column (1  $\mu\text{L}$  injection), respectively. The LOD and LOQ for AMPA were 4.16 and 12.61 pg on column (1  $\mu\text{L}$  injection), respectively.

Analysis of sarcosine and shikimate was performed by GC–MS using the same conditions as in the analysis of glyphosate and AMPA, except the GC temperature program: initialized at 120 °C, held for 2 min and raised to 300 °C at 17 °C/min rate, and then held at this temperature for 0.5 min. Sarcosine was monitored using  $m/z$  233, 218, 190, and 160 ( $t_{\text{R}}$  5.58 min); shikimate was monitored at  $m/z$  462, 447, 372, and 255 ( $t_{\text{R}}$  8.67 min). Sarcosine and shikimic acid in the samples were quantitated from a calibration curve of the respective standards: shikimic acid, purity 99% (Sigma-Aldrich), and sarcosine (Sigma-Aldrich). Analysis was performed in duplicate. Sarcosine was not detected in any of the samples. The LOD and LOQ for shikimate were 929 and 2815 pg on column (1  $\mu\text{L}$  injection), respectively.

**Glyphosate Metabolism Studies in Leaf Discs.** In a study to eliminate uptake and translocation effects, 4 mm diameter leaf discs were cut with a cork borer from uniform leaves of 18-day-old, greenhouse-grown-MT and LT plants. Three hundred discs were floated on 20 mL of aqueous solutions of technical grade glyphosate for 1 day in 10 cm diameter polystyrene Petri dishes. Treatments were quadruplicated. After incubation, samples were taken for analysis of shikimate, AMPA, and glyphosate content. The 300 discs from each dish were washed in a total of 1 L of Millipore filtered water and then frozen at –80 °C in 50 mL centrifuge tubes until analysis by the methods above. Relative shikimate content was also determined by the method of Shaner et al.<sup>22</sup>

**Glyphosate Absorption and Translocation.** MT and LT *I. lacunosa* plants were treated with glyphosate as described before at a rate of 840 g of ae/ha, except that the second youngest fully expanded leaf was covered with a water-resistant paper sleeve. This sleeve was removed immediately after herbicide treatment for subsequent (within 30 min of overspray) application of solutions containing <sup>14</sup>C-glyphosate, <sup>14</sup>C-methyl labeled with 2.0 GBq/mmol specific activity, 99.5% radiochemical purity in an aqueous stock solution of 7.4 MBq/mL (American Radiolabeled Chemicals, Inc., St. Louis, MO). A solution containing glyphosate at a final concentration equivalent to 0.84 kg/ha in 140 L was prepared using <sup>14</sup>C-glyphosate, a commercial formulation of glyphosate, and distilled water. A 10  $\mu\text{L}$  volume of the treatment solution, containing 5 kBq of <sup>14</sup>C-glyphosate, was applied to the adaxial surface of the not oversprayed second true leaf of 15 cm tall plants in the form of 25 droplets with a microapplicator. Plants were harvested at 1, 4, 24, 48, 96, and 168 h after <sup>14</sup>C-glyphosate treatment

(HAT) for absorption measurements carried out as described previously.<sup>6</sup>

MT and LT plants were processed for phosphorimaging prior to translocation analysis. The treated leaves from the plants were removed at respective harvest times to wash off unabsorbed radioactivity and set aside. The remaining above ground part of the plant was excised from the roots and mounted on a 27  $\times$  21.25 cm piece of plain white paper. Shoot parts were evenly spread and kept in place with thin strips of clear office tape. Care was taken to avoid contact of the washed treated leaf with other parts of the plant. Roots were gently rinsed with water to remove soil, blotted dry with paper towels, and mounted on a separate sheet as with the shoot. The mounted plant parts were pressed between one or more layers of newspaper, and bookended with two hard cardboard sections. The assembled plant press was held together with large binder clips and stored at –20 °C for later drying. The plant samples were dried in a gravity convection oven set at 60 °C for 24 h. Phosphorimaging was used to develop an image of the plant samples. After the dried sample was cooled to room temperature, the plant was placed in a 20  $\times$  40 cm exposure cassette (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and brought into contact with a BAS IPSR 2025 E storage phosphor screen (GE Healthcare Bio-Sciences Corp.) under diffused lighting. The apparatus was placed in a dark cabinet for 24 h. A Typhoon FLA 7000 phosphorimager (GE Healthcare) was used to detect distribution of <sup>14</sup>C-glyphosate and develop an image. Phosphorimaged plants were carefully removed from the mounting paper and divided into treated leaf, shoot above treated leaf (SATL), shoot below treated leaf (SBTL), and roots, and translocation was measured according to previously described procedures.<sup>6</sup> There were five replications per treatment (harvest) time per plant type (MT/LT).

**Statistical Analysis.** All data were analyzed by ANOVA via the PROC GLM statement using SAS software version 9.3 (SAS Institute, Cary, NC) to determine the main effects and interactions of the factors at  $P < 0.05$ . No significant experimental effect was observed in repeated experiments; therefore, data from experiments were pooled. Treatment means in selected experiments were separated using Fisher's Protected LSD test at  $P < 0.05$ .

Glyphosate dose response data were subjected to nonlinear regression analysis, and a sigmoidal log–logistic curve<sup>23</sup> of the form

$$y = L + \frac{U - L}{\left[ 1 + \left( \frac{D}{GR_{50}} \right)^s \right]} \quad (1)$$

where  $y$  represents shoot fresh weight reduction as compared to that of nontreated plants in percentage at herbicide rate  $D$ ,  $L$  is the mean response at very high herbicide rate (lower limit),  $U$  is the mean response when the herbicide rate is zero (upper limit),  $s$  is the slope of the line at  $GR_{50}$ , and  $GR_{50}$  is the glyphosate rate required for 50% growth reduction of *I. lacunosa* plants, was fit to the data. The estimate of the four regression parameters was obtained using Sigma Plot version 11 (Systat Software, San Jose, CA) and tested for significance using the  $t$ -test method ( $P < 0.05$ ).  $GR_{50}$  values of tolerant accessions were compared to that of the least tolerant accession according to the Student  $t$ -test at  $P < 0.05$  with the open-source R software version 2.15.2 (R Foundation for Statistical Computing) using the drc package and drm function, and the comparisons were given by means of the selectivity index (SI) function.

In the glyphosate metabolism study, data variance was visually inspected by plotting residuals to confirm the homogeneity of variance prior to statistical analysis using SAS. The Shapiro–Wilk test was applied to verify if the data among accessions were normally distributed, and Hartley's  $F_{\text{max}}$  test was applied to verify if different populations have a similar variance using SAS. Means separation was performed using Fisher's Protected LSD test at  $P < 0.05$ .

## RESULTS AND DISCUSSION

**Glyphosate Dose Response in *I. lacunosa* Accessions.** The F-test in the ANOVA was significant ( $P < 0.0035$ ) for the pairing of dependent variable (fresh weight reduction) with

**Table 1. Effect of Glyphosate and  $GR_{50}$  Rate on Shikimate, Glyphosate, and Aminoethylphosphonic Acid (AMPA) Concentrations in the Most Tolerant (MT) and Least Tolerant (LT) *I. lacunosa* Accessions**

accession <sup>a</sup>	glyphosate rate, g of ae/ha	DAT	shikimate (SE), <sup>b,c</sup> $\mu\text{g/g}$ of tissue	glyphosate (SE), <sup>c</sup> $\mu\text{g/g}$ of tissue
MT	420	14	683.94 (554.37) b	$3.57 \times 10^{-5}$ ( $2.02 \times 10^{-5}$ ) b
LT			9868.30 (10 793.31) a	$9.78 \times 10^{-5}$ ( $3.63 \times 10^{-5}$ ) a
MT	151 <sup>d</sup>	1	190.85 (16.87) A	14.87 (2.66) B
		3	148.16 (40.42) AB	11.55 (3.01) BC
		6	97.44 (22.28) B	22.66 (7.48) A
LT	58 <sup>d</sup>	1	135.94 (34.93) AB	5.40 (2.62) C
		3	133.35 (27.20) AB	5.19 (1.61) C
		6	118.39 (40.23) B	4.77 (3.78) C

accession <sup>a</sup>	glyphosate rate, g of ae/ha	DAT	glyphosate/AMPA ratio (SE) <sup>c</sup>
MT	420	14	$1.28 \times 10^{-5}$ ( $0.46 \times 10^{-5}$ ) b
LT			$3.76 \times 10^{-5}$ ( $1.11 \times 10^{-5}$ ) a
MT	151 <sup>d</sup>	1	23.55 (2.69) A
		3	20.35 (6.02) AB
		6	26.26 (9.06) A
LT	58 <sup>d</sup>	1	17.69 (5.61) AB
		3	10.82 (5.03) BC
		6	6.91 (5.01) C

<sup>a</sup>The most tolerant (MT) was MS-WAS-8 and least tolerant (LT) was MS-YAZ-1. <sup>b</sup>SE represents the standard error of the mean where  $n = 10$  (pooled data from two experiments of glyphosate at 420 g of ae/ha) and  $n = 6$  (pooled data from two experiments of glyphosate at  $GR_{50}$  rate). <sup>c</sup>Values followed by the same letter (lowercase when MT and LT were treated with glyphosate at 420 g ae/ha, uppercase when MT and LT were treated with glyphosate at 151 and 58 g ae/ha, respectively) within each column are not significantly different at the 5% level as determined by the LSD test. <sup>d</sup> $GR_{50}$  indicates the glyphosate dose required to cause a 50% reduction in plant growth, 151 g of ae/ha for the MT accession, and 58 g of ae/ha for the LT accession.

main effect terms (accessions and glyphosate dose) and interaction terms, indicating that the response to glyphosate varied among the 14 *I. lacunosa* accessions (data not shown). The accessions with less previous exposure to glyphosate in GR crops were the ones with numerically smaller  $GR_{50}$  values (MS-99 106, MS-90 91, MS-QUI-1 59, and MS-YAZ-1 58 g/ha glyphosate) (data not shown). Thus, the higher  $GR_{50}$  values of some accessions could be due to selection with glyphosate. If so, these more “tolerant” accessions may actually be categorized as resistant (genetically changed by selection with glyphosate). *I. lacunosa* went from the fifth to the second most common weed in Mississippi soybean six years after the introduction of GR soybean.<sup>24</sup> Therefore, it appears that the emergence of *I. lacunosa* as a major weed problem has coincided with the widespread adoption of GR crops. The tolerance/resistance of *I. lacunosa* may be a consequence of a mutation(s) arising after the introduction of the herbicide, or the resistance gene(s) may predate the widespread use of the herbicide with selection from preexisting genetic variation in the population.<sup>11</sup> Also, localized adaptations have probably resulted in the evolution of several variably glyphosate tolerant *I. lacunosa* ecotypes in North America, which could, in turn, be unrelated to exposure of the population to glyphosate.

Intersimple sequence repeat (ISSR) markers were used to study intraspecific population structure in *I. lacunosa*.<sup>25</sup> Subpopulation differentiations were detected in those accessions from proximal locations and clustered together populations with high similarity of agricultural environments, like Arkansas and Mississippi. However, intraspecific accessions almost always clustered together among various *Ipomoea* species.<sup>26</sup> Moreover, it is possible that the colonization of *I. lacunosa* in the southern U.S. started with only one genotype and evolved with time due to localized adaptations and hybridization with compatible species. Consequently, the morphological and genetic variance of *I. lacunosa* in the

southern U.S. may impact the efficacy of weed management strategies.

The MS-YAZ-1 accession had the lowest  $GR_{50}$  value of 58 g/ha glyphosate and MS-WAS-8 accession had the highest  $GR_{50}$  value of 151 kg/ha glyphosate, resulting in a R/S (proportion of  $GR_{50}$  values of most and least tolerant accessions) ratio of 2.6. The 2.6-fold R/S ratio of glyphosate tolerance is more than that reported for other *I. lacunosa* accessions from the southern U.S., which was 1.9-fold less when their most tolerant and least tolerant populations were compared.<sup>19</sup> In weed species that have evolved glyphosate resistance, the resistance mechanisms thus far elucidated are target-site-based and non-target-site-based.<sup>27,28</sup> The following processes have been reported to provide different levels of resistance to glyphosate: reduced glyphosate absorption ( $\sim 3$ -fold),<sup>29,30</sup> impaired glyphosate translocation ( $\sim 3$ - to 13-fold),<sup>31–34</sup> sequestration to the vacuole (2.9–5.6-fold),<sup>35</sup> EPSPS mutations ( $\sim 2$ - to 15-fold),<sup>36,37</sup> and overproduction of target enzyme (6–8-fold)<sup>38</sup> in weedy species.

Because legume species have been reported to metabolize glyphosate,<sup>7,39</sup> different sensitivities to glyphosate in certain populations of *I. lacunosa* may be due to differences in the levels of degradation of glyphosate to the much less phytotoxic metabolite of glyphosate, AMPA, or even sarcosine. To test this hypothesis, glyphosate, shikimate, AMPA, and sarcosine levels were measured in glyphosate-treated leaves of the MT and LT accessions.

**Glyphosate Metabolism Studies.** Two experiments were performed. In the first, the LT and MT accessions were each treated with 420 g/ha glyphosate (half the recommended field rate), after which shikimate, glyphosate, and AMPA levels in the entire plant shoot were determined at 14 DAT (Table 1). In the second experiment, the LT and MT biootypes were treated with  $GR_{50}$  doses (58 and 151 g/ha, respectively), after which shikimate, glyphosate, and AMPA shoot contents were

measured at 1, 3, and 6 DAT (Table 1). Sarcosine was not detected in either accession in either experiment.

In the first experiment, shikimate levels were much more elevated (ca. 15-fold more) in the LT than the MT accession. There were no differences in AMPA levels between the two accessions, but glyphosate levels were more than twice as high in the LT than the MT accession. Such an experiment provides only pool sizes at one time point, without any information on flux of compounds through those pools. The higher glyphosate pool in the LT could be due to a slower rate of glyphosate metabolism in the LT than the MT accession. An alternative explanation could be the reduced metabolism of glyphosate in the LT accession caused by metabolic dysfunction, due to the fact that this accession was more severely affected by the herbicide. Also, reduced growth during the 14 DAT would mean that the glyphosate would be more concentrated in the LT accession.

The second experiment in which the two accessions should have been equally affected by the glyphosate treatment was designed to better assess whether glyphosate metabolism differs in the two accessions, as the higher rates used in the first experiment may have disrupted metabolic processes. By blocking EPSPS, glyphosate causes many-fold increases in shikimate levels in non-GR plants and, consequently, elevated shikimate levels are used as an early and highly sensitive indicator of glyphosate effects on glyphosate-sensitive plant tissue.<sup>40,41</sup> The nonsignificantly different levels of shikimate between the two accessions at 1, 3, and 6 DAT indicated that the  $GR_{50}$  doses for the two accessions had similar physiological effects (Table 1). The only significant difference between the two accessions was a higher shikimate level at 1 DAT of the MT accession than at 6 DAT in the LT accession. Shikimate levels decreased from 1 to 6 DAT for both accessions, but the only statistically significant change with time for an accession was a reduction for the MT accession between 1 and 6 DAT (Table 1).

AMPA levels were the same in both accessions at all times after treatment (Table 1). Although AMPA levels trended upward from 1 to 6 DAT for both accessions, the increase was not statistically significant. However, glyphosate concentrations were higher at 1 and 6 DAT (ca. 3-fold and 5-fold, respectively) in the MT than the LT accession. Under different growth conditions, Reddy et al.<sup>21</sup> found that AMPA levels in treated soybean leaves were highest 1 DAT and decreased over a period of 22 days.

If there were enhanced metabolism of glyphosate in the MT accession, one would not expect the higher levels of glyphosate than LT observed above. If the uptake and translocation as a percentage of glyphosate applied were the same in the two accessions, one would expect about 2.6-fold more glyphosate in the MT than the LT accession when each is treated with their respective  $GR_{50}$  dose. This is approaching the 1 DAT result for both glyphosate and AMPA (2.75-fold and 2.13-fold more in the MT than the LT) (Table 1). The nonsignificant differences in the glyphosate to AMPA ratio between the two accessions at 1 and 3 DAT suggest similar levels of metabolism as a percent of glyphosate taken up. However, the 6 DAT glyphosate to AMPA ratio was significantly higher in the MT than the LT (ca. 3.7 fold), indicating that metabolism could be higher as a percent of glyphosate taken up in the LT accession (Table 1). Again, these data are only a snapshot in time of pool sizes that do not take into account flux through the pools due to the translocation of glyphosate and AMPA to roots and metabolic

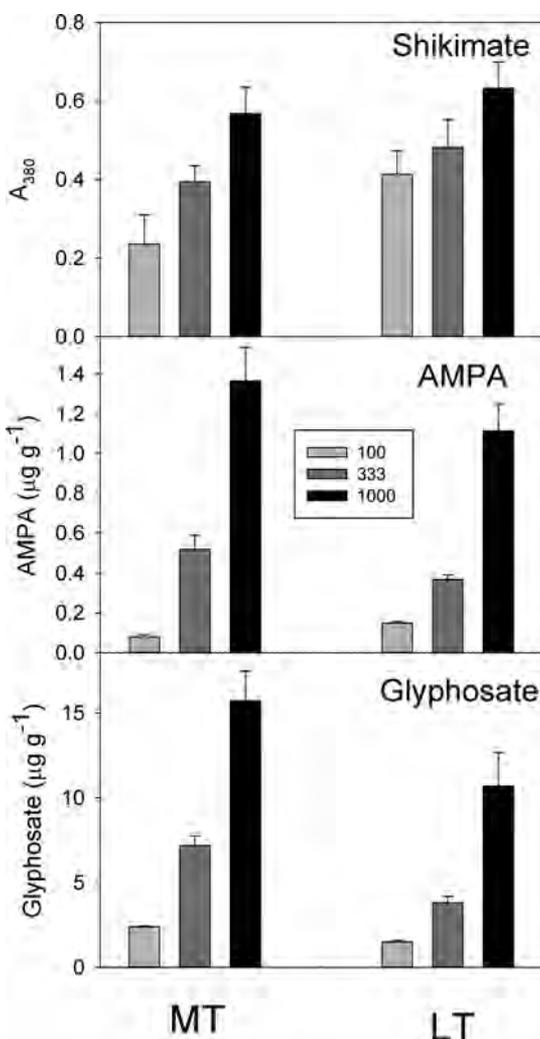
degradation of AMPA. Nevertheless, these data do not support the theory that metabolism of glyphosate explains the relative sensitivities to glyphosate in the two *I. lacunosa* accessions tested.

There has been no conclusive evidence of metabolic degradation as an important mechanism of evolved resistance to glyphosate.<sup>8</sup> The fact that *I. lacunosa* accessions with variable levels of tolerance accumulated the same amount of AMPA does not support the view that enhanced metabolism of glyphosate is involved in the tolerance of the MT accession. Gene mutation or amplification of plant genes for GOX-like enzyme activity or horizontal transfer of microbial genes for glyphosate-degrading enzymes could result in GR weeds.<sup>16</sup> This mechanism of tolerance could be combined with another mechanism such as differential absorption or translocation of glyphosate. This is supported by our results of increased glyphosate concentration from 1 to 6 DAT in the MT, suggesting that glyphosate may be slowly taken up in this accession. This hypothesis of single or multiple mechanisms of glyphosate tolerance was investigated using studies of absorption and translocation of glyphosate in the *I. lacunosa* accessions.

Metabolism in cut leaf discs was determined to avoid effects of potential differences in uptake and translocation between the two accessions. Glyphosate content was slightly higher in the MT than the LT accession at each glyphosate treatment concentration (Figure 2). AMPA levels rose with glyphosate dose for both accessions (Figure 2), but the percentage of AMPA as a fraction of glyphosate as glyphosate dose increased was 3.5, 7.2, and 8.7% for the MT accession vs 13, 9.7, and 10.4% for the LT accession. These data do not support the view that differences in glyphosate metabolism have anything to do with the differences in tolerance to glyphosate between these two accessions. Shikimate levels after 24 h of incubation in 0.1, 0.33, or 1 mM glyphosate were the same or slightly higher in the LT accession at the three concentrations (Figure 2). The differences in shikimate levels between MT and LT plants were not as great as in whole plants (Table 1). These results provide further support for the view that metabolism is not involved in the difference between the LT and MT accessions.

**<sup>14</sup>C-Glyphosate Absorption and Translocation.** The absorption pattern of <sup>14</sup>C-glyphosate in the MT and LT accessions of *I. lacunosa* was similar throughout the time course of 168 HAT (Figure 3A). Glyphosate absorption amounts reported here (21–25%, 42–43%, and 45–46% of applied at 24, 48, and 96 HAT, respectively) are similar to previously reported estimates (23% and 43% of applied at 24 and 96 HAT, respectively),<sup>6</sup> but lower than levels (57–63%, 52–58%, and 53–59% of applied at 26, 50, and 74 HAT, respectively) reported for three different formulations of glyphosate in *I. lacunosa*.<sup>42</sup> In *I. cordatotriloba* Dennstedt (sharpshod morning-glory), glyphosate absorption was much higher (75% of absorbed at 72 HAT)<sup>43</sup> than for MT and LT accessions of *I. lacunosa* reported here.

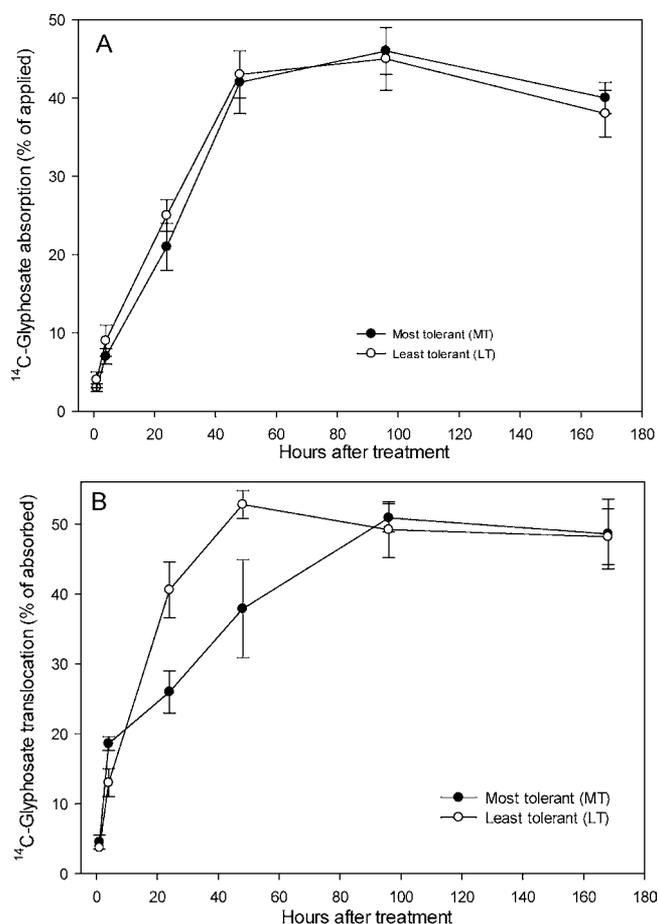
The amount of <sup>14</sup>C-glyphosate that translocated out of the treated leaves of the MT and LT plants was similar up to 4 HAT (Figure 3B). Thereafter, the LT accession translocated more (41 and 53% of absorbed at 24 and 48 HAT, respectively) <sup>14</sup>C-glyphosate than the MT accession (26 and 38% of absorbed at 24 and 48 HAT, respectively) out of the treated leaf, with translocated amounts leveling off to similar levels in both populations by 96 HAT (49 and 51% of absorbed in LT and MT, respectively) and 168 HAT (48 and 49% of absorbed



**Figure 2.** Shikimate, glyphosate, and AMPA levels in leaf discs of MT and LT *I. lacunosa* floating on 0.100, 0.333, and 1.000 mM glyphosate for 24 h.

in LT and MT, respectively). The translocation levels of  $^{14}\text{C}$ -glyphosate out of the treated leaf reported here (26–41%, 38–53%, and 49–51% of absorbed at 24, 48, and 96 HAT, respectively) are greater than those reported previously in *I. lacunosa* (5 and 23% of absorbed at 24 and 96 HAT, respectively)<sup>6</sup> and *I. cordatotriloba* (13% of absorbed at 72 HAT),<sup>43</sup> with the same formulation of the potassium salt of glyphosate used in all cases.

Distribution of absorbed  $^{14}\text{C}$ -glyphosate in the MT and LT accessions is summarized in Table 2. The quantity of  $^{14}\text{C}$ -glyphosate that remained in the treated leaf was higher in the LT (87% of absorbed) than in the MT plants (81% of absorbed) at 4 HAT. Thereafter, the MT accession retained more glyphosate in the treated leaf (74 and 62% of absorbed at 24 and 48 HAT, respectively) than the LT accession (59 and 47% of absorbed at 24 and 48 HAT, respectively). A similar trend was reflected in the SATL where the level of glyphosate in the MT plants was higher (15% vs 5% of absorbed at 4 HAT) and lower (14% vs 20% and 19% vs 25% of absorbed at 24 and 48 HAT, respectively) than the LT plants. The MT accession retained more  $^{14}\text{C}$ -glyphosate in SBTL (12% of absorbed) than the LT accession (8% of absorbed) at 48 HAT. However, the LT accession roots contained more glyphosate



**Figure 3.** Absorption (A) and translocation (B) of  $^{14}\text{C}$ -glyphosate in most (MT, closed circles) and least (LT, open circles) tolerant *Ipomoea lacunosa* accessions. Vertical bars represent standard error of mean ( $n = 5$ ).

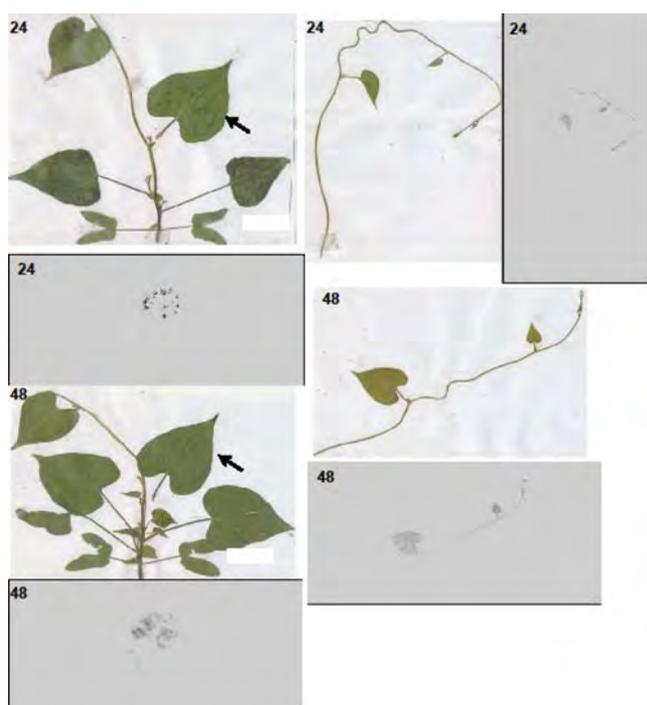
(20% of absorbed) than those of the MT accession (7% of absorbed) at 48 HAT. Distribution of  $^{14}\text{C}$ -glyphosate in the treated leaf and other parts of the plant was generally lower and higher, respectively, across the LT and MT accessions compared to previous reports in *I. lacunosa*<sup>6</sup> and *I. cordatotriloba*<sup>43</sup> within 24–96 HAT, which is an extension of translocation observations.

Phosphorimaging was used to generate autoradiographs of MT and LT plants treated with  $^{14}\text{C}$ -glyphosate to visualize  $^{14}\text{C}$ -glyphosate translocation patterns.  $^{14}\text{C}$ -glyphosate movement was discernible in the SATL of the LT accession (Figure 4) at 24 HAT, but not the MT accession (Figure 5). The intensity of the autoradiograph of SATL was greater in the LT accession (Figure 4) at 48 HAT compared to that of the MT accession (Figure 5), reflecting the translocation and distribution data at 48 HAT. A closer examination of the colored plant specimen of the LT accession at 168 HAT indicated extensive chlorosis compared to the case of the MT population (data not shown) at the same harvest time. Inhibition of the apical meristems in the LT population may have caused a feedback inhibition of glyphosate loading, thereby resulting in numerically reduced glyphosate levels in SATL of LT plants (22 and 23% of absorbed at 96 and 168 HAT, respectively) compared to the level of the MT plants (30 and 34% of absorbed at 96 and 168 HAT, respectively). The progressive increase in intensity of the root in LT (data not shown) could be partly due to a switch to

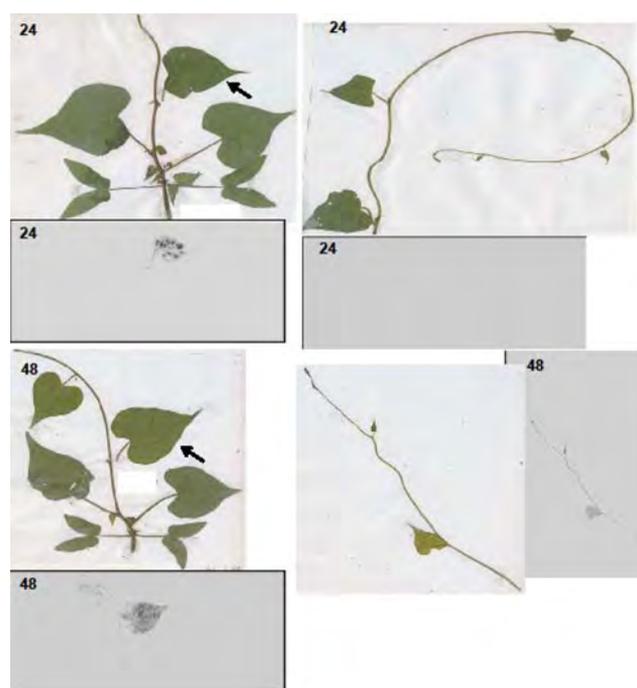
**Table 2.** Distribution of  $^{14}\text{C}$ -Glyphosate (% Absorbed) in Most Tolerant (MT) and Least Tolerant (LT) *Ipomoea lacunosa* Accessions<sup>a,b</sup>,

accession	hours after treatment											
	1	4	24	48	96	168	1	4	24	48	96	168
	TL						SATL					
MT	96	81	74	62	49	51	1	15	14	19	30	34
LT	96	87	59	47	51	55	2	5	20	25	22	23
LSD (0.05) <sup>c</sup>	ns	5	12	4	ns	ns	ns	4	4	3	ns	ns
	SBTL						Root					
MT	1	2	8	12	8	5	2	2	4	7	13	10
LT	1	4	7	8	6	6	1	4	14	20	21	16
LSD (0.05)	ns	ns	ns	4	ns	ns	ns	ns	ns	4	ns	ns

<sup>a</sup>Abbreviations: LT, least tolerant; MT, most tolerant; ns, not significant; SATL, shoot above treated leaf; SBTL, shoot below treated leaf; TL, treated leaf. <sup>b</sup>Distribution represents partitioning of absorbed  $^{14}\text{C}$ -glyphosate between the treated leaf, shoot above treated leaf, shoot below treated leaf, and root. <sup>c</sup>Number indicates significance at the 5% level of probability and ns indicates no significant difference between means within the same column and within the same plant part.



**Figure 4.** Above ground plant parts and corresponding phosphor-images of least tolerant (LT) *Ipomoea lacunosa* accession. Arrow indicates TL. A part of the SATL appears above the TL. Numbers indicate hours after treatment (HAT) with  $^{14}\text{C}$ -glyphosate.



**Figure 5.** Above ground plant parts and corresponding phosphor-images of most tolerant (MT) *Ipomoea lacunosa* accession. Arrow indicates treated leaf. A part of the SATL appears above the TL. Numbers indicate hours after treatment (HAT) with  $^{14}\text{C}$ -glyphosate.

basipetal translocation brought about by inhibition of aerial sinks by glyphosate.

In summary, glyphosate dose response studies were conducted on selected *I. lacunosa* accessions suspected to be tolerant to glyphosate. Results indicated variable tolerance with the most tolerant (MT) and least tolerant (LT) accessions exhibiting  $GR_{50}$  values of 151 and 58 g of ae/ha of glyphosate, with a resistance index of 2.6. Contrary to our hypothesis, metabolism of glyphosate to AMPA did not explain the tolerance mechanism. The absence of enhanced metabolic degradation of glyphosate as a resistance or tolerance mechanism of any case of resistance or tolerance thus far studied remains unresolved.  $^{14}\text{C}$ -glyphosate absorption patterns were similar in the MT and LT accessions, but the LT accession translocated more glyphosate at 24 and 48 HAT than

the MT accession. Differential glyphosate translocation may explain, at least in part, tolerance in the MT accession.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

A table summarizing regression parameters from glyphosate dose response curves of 14 *I. lacunosa* accessions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

### ✉ Corresponding Author

\*V.N. tel: 662-686-3760. Fax: 662-686-5422. Email: vijay.nandula@ars.usda.gov.

### 📍 Present Address

<sup>||</sup>Monsanto Co., 700 Chesterfield Pkwy West, Chesterfield, MO 63017, USA.

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## ABBREVIATIONS USED

AMPA, aminomethylphosphonic acid; DAT, days after treatment; GOX, glyphosate oxidoreductase; GR, glyphosate-resistant;  $GR_{50}$ , glyphosate rate required for 50% growth reduction of *I. lacunosa* plants; HAT, hours after treatment; LT, least tolerant; MT, most tolerant; SATL, shoot above treated leaf; SBTL, shoot below treated leaf; WAT, weeks after treatment

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