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Uptake and Distribution of *N*-Phosphonomethylglycine in Sugar Beet Plants[1]

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ABSTRACT

Glyphosate (*N*-phosphonomethylglycine) was readily transported in sugar beet plants (*Beta vulgaris* L., Klein E type, monogerm). Concentrations in sink leaves reached 2.5 to 13.7 micromolar in 10 hours from a 15 millimolar solution supplied to one mature leaf. Distribution of glyphosate followed that of [^{3}H]sucrose used as a marker for materials transported by phloem, indicating that this is the primary means for distribution of glyphosate. Possible mechanisms of entry into the sieve tubes were evaluated using isolated leaf discs. Concentration dependence of uptake and kinetics of exodiffusion from tissue indicate a passive, nonfacilitated mechanism. Uptake was not affected by pH, eliminating the passive, weak acid mechanism. Permeability of the plasmalemma to glyphosate was calculated as 1.7×10^{-10} meters per second. This characteristic would allow slow entry and exit from the phloem, and together with other physiological parameters of the plant, is postulated to allow accumulation and transport in the phloem.

Glyphosate,[2] a nonselective post-emergence herbicide, has been shown to be phloem-mobile in several species. This mode of distribution has been concluded from experiments in which labeled glyphosate followed a typical source-sink relationship when applied to leaves (6, 7, 13, 15, 16). Phloem-mobility of glyphosate in sugar beet plants was also found in this study. Transport of any xenobiotic substance in the phloem must be preceded by its entry into the symplast. The mechanism of entry into the symplast will be a major factor in determining both its phloem mobility and distribution throughout the plant. The purpose of this study was to investigate possible mechanisms of cellular uptake and phloem transport of glyphosate. Results with isolated leaf tissue support a passive, nonfacilitated uptake of glyphosate into cells. An intermediate permeability mechanism, described by Tyree *et al.* (14), is suggested for phloem transport of glyphosate based on permeability calculations of plant cell membranes to glyphosate.

MATERIALS AND METHODS

Plant Material. Sugar beet plants (*Beta vulgaris* L., Klein E type monogerm) were grown 6 to 8 weeks in a mixture of equal amounts of sand and Jiffy-Mix. Plants were watered twice daily with nutrient solution as described by Snyder and Carlson (11) with the following modifications. KH_2PO_4 at 1 mM was omitted, the concentration of KNO_3 was increased to 3.5 mM, and the concentration of H_3BO_3 was increased to 20.6 μM. Plants were grown in a growth chamber with a 14-h light period at 24 C and a 10-h dark period at 17 C. Photon flux density at leaf blade level was 370 μE m^{-2} s^{-1}.

Distribution of Glyphosate. One mature leaf, about 0.7 dm^2 in size, was supplied with [^{3}H]sucrose and [^{14}C]glyphosate in separate locations on the leaf. Light was provided by two metal-halide lamps at photon flux density of 350 μE m^{-2} s^{-1} at the level of the lamina. Ten discrete areas of the leaf were abraded with alumina to facilitate entry of sucrose. Sucrose concentration was 10 mM in 5 mM KH_2PO_4 buffer at pH 6.5. A total of 31 μCi [^{3}H]sucrose, 3.86 μCi μmol^{-1} (Amersham, Arlington Heights, IL) was added. Mylar discs, 1 cm in diameter, were placed over the sucrose. Areas under the Mylar discs were kept wet by adding H_2O during the experiment. Glyphosate was added as a solution containing the surfactant MON 0818 (Monsanto Agricultural Products) at 1.5% w/v and 1.1% w/v monoisopropylamine. Final glyphosate concentration was 15 mM. A total of 4 μCi [^{14}C]methylglyphosate, 0.5 μCi μmol^{-1} (Monsanto Agricultural Products), was added to unabraded areas of the leaf in 25-μl drops with a microsyringe and not covered with Mylar discs. After 6 h, the leaves, crown, beet, taproot, and fibrous roots were separated and frozen. Plant parts were homogenized in deionized H_2O, 1 ml H_2O/0.2 g fresh weight. The homogenate was centrifuged at 17,300*g* for 5 min and the pellet was extracted twice with 0.5 N NH_4OH, 1 ml NH_4OH solution/0.4 g fresh weight. Aliquots of the combined supernatant solutions were assayed for both ^{3}H- and ^{14}C-labeled materials using dual-isotope scintillation spectrometry. Concentrations were calculated using 1 g fresh weight equivalent to 1 ml.

Transport of Glyphosate. A total of 17.8 μCi [^{14}C]glyphosate, 0.7 μCi μmol^{-1}, in solution as described for distribution of glyphosate was uniformly added to the upper surface of one mature leaf. Accumulation in this mature leaf was monitored by clamping a 1.6- to 2-mg cm^{-2} end-window GM tube (TGM Corp., model N 1003) beneath one corner of the leaf. Arrival of [^{14}C]glyphosate in a young sink leaf importing material from the leaf supplied with [^{14}C]glyphosate was monitored by clamping a GM tube over the upper surface of the leaf. Concentrations of glyphosate in the leaves after 10 h were determined as described for distribution of glyphosate.

Concentration Series. Leaf discs (0.38 cm^2) were punched from a mature leaf after the lower epidermis had been removed. Four discs were floated, lower surface down, on 2 ml [^{14}C]glyphosate solution in 5 mM KH_2PO_4 buffer at pH 6.5. Each solution of different concentration contained 0.5 μCi/ml [^{14}C]glyphosate. After 3 h uptake, discs were floated over 5 mM KH_2PO_4 for 20 min to remove free space glyphosate. Discs were immediately frozen over Dry Ice, vacuum dried at −70 C, and pressed at a pressure of 36,000 p.s.i. Flattened discs were glued to planchets and counted with Nuclear-Chicago ultrathin-end-window, gas-flow GM detector.

pH Dependence. Leaf discs were prepared as for the concentration series except the epidermis was not peeled. [^{14}C]Glyphosate, 0.06 μCi μmol^{-1}, was 17.6 mM in 5 mM KH_2PO_4 buffer at pH 6.5.

[1] Supported by a grant from Monsanto Agricultural Products Co.
[2] Abbreviation: glyphosate, *N*-phosphonomethylglycine.

The pH was adjusted with monoisopropylamine. Uptake time was 3 h. Radioactivity was determined as for concentration series. Significance was evaluated using Newman-Keuls multiple comparison test. Autoradiographs were made on Kodak Industrex type-M film.

Compartmental Analysis. Leaf discs were prepared as for pH dependence experiments. Three leaf discs were floated on 1 ml 17.6 mM [^{14}C]glyphosate, 0.11 μCi μmol^{-1}, in 5 mM KH_2PO_4 buffer at pH 6.5. After 3 h uptake, each set of three discs was transferred to 300 μl buffer every 10 min for 2.5 h. After exodiffusion, discs were counted as for concentration series. Radioactivity released in each transfer was determined by liquid scintillation spectrometry. The amount of glyphosate in a leaf disc at the beginning of each 10-min interval was calculated by summing the amounts exodiffused during that time interval, all subsequent intervals, and the amount of glyphosate remaining in the disc at the end of exodiffusion.

RESULTS

Distribution of Glyphosate. The distribution of glyphosate throughout the plant was followed using [^{14}C]glyphosate. [^{3}H]-Sucrose was used as a marker for materials recently translocated in the phloem. Tissue concentrations of glyphosate and [^{3}H]sucrose after 6 h for each plant part are expressed relative to concentrations in an actively growing sink leaf importing material from the leaf supplied with the labeled materials. Glyphosate and [^{3}H]sucrose had the same relative concentrations for most plant parts (Fig. 1). There were proportionally lower concentrations of [^{3}H]sucrose in the two youngest leaves. This could have resulted from a higher rate of sucrose metabolism in these young leaves. Only trace amounts of labeled material were found in two of the older leaves as we expected. The slightly higher levels found in one older leaf were probably from re-circulation of both labeled species in the xylem. The crown and beet were extracted in two halves cut perpendicular to the axis of the labeled leaf. For both plant parts, the half corresponding to the leaf supplied with labeled material had significantly higher concentrations than the other half.

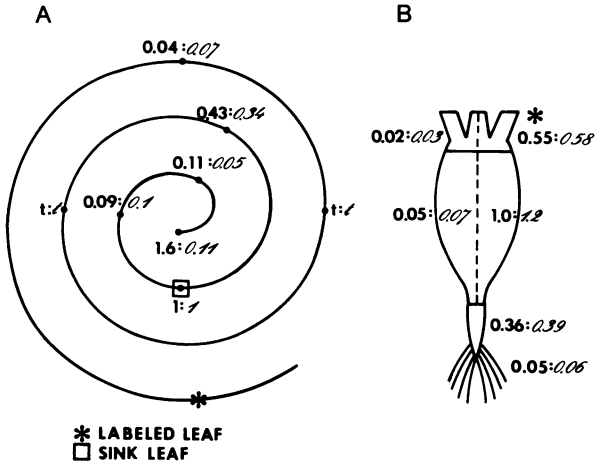

FIG. 1. Relative distribution of glyphosate and [^{3}H]sucrose in sugar beet plant. Data are from one of three experiments. Marked leaf (*) was supplied with [^{3}H]sucrose and [^{14}C]glyphosate at separate locations on the leaf as described. A: diagram of leaves showing phyllotaxy. Next to each leaf are concentrations of glyphosate, in bold numerals, and [^{3}H]sucrose, in italics, relative to the concentrations of each in a young, importing leaf (⊡). Glyphosate concentration in this leaf was 74 nmol/g fresh weight and [^{3}H]sucrose concentration was 240 nmol/g fresh weight. Trace amounts are indicated by t. B: diagram of crown, beet, taproot, and fibrous roots showing relative concentrations as in A. Dashed lines show positions of cuts separating plant parts. Lower petiole of leaf supplied with [^{14}C]-glyphosate and [^{3}H]sucrose is marked (*).

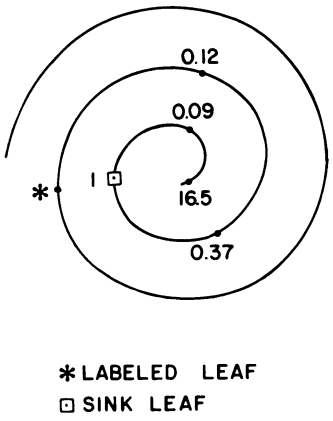

FIG. 2. Relative distribution of glyphosate in sugar beet plant supplied with only [^{14}C]glyphosate on marked leaf (*) as described. Spiral represents leaf phyllotaxy. Concentrations of glyphosate are relative to concentration of 13.7 nmol/g fresh weight glyphosate for young importing leaf (⊡). Data are for one of three experiments.

A potential problem with this method is that addition of exogenous, 10 mM sucrose to the leaf might increase export from the leaf and might change the distribution of glyphosate. This was checked by comparing the distribution of glyphosate in plants supplied with [^{3}H]sucrose and [^{14}C]glyphosate to that of plants supplied only with [^{14}C]glyphosate. There was a 10-fold greater concentration of glyphosate in the actively growing sink leaf of the three plants supplied with both labeled materials. However, the distribution pattern of glyphosate under the two sets of conditions was the same (Figs. 1A and 2).

Transport of Glyphosate. A single mature leaf of an intact plant was supplied with 15 mM [^{14}C]glyphosate plus surfactant. Labeled glyphosate reached the monitored sink leaf within 130 min (Fig. 3). After 10 h, glyphosate concentration in this sink leaf was 2.5, 7.6, and 13.7 μM in three experiments. Rate of arrival at the sink leaf began to increase steadily after 6 h. This can be accounted for by a steady rate of increase in uptake into the leaf supplied with [^{14}C]glyphosate. The nonlinear uptake into the source leaf was most likely due to drying out of the added glyphosate solution on the surface of the leaf resulting in an increased concentration gradient across the leaf.

Concentration and pH Dependence of Glyphosate Uptake. Uptake of glyphosate into leaf discs was directly related to glyphosate concentration up to 10 mM (Fig. 4). Autoradiographs of leaf discs (Fig. 5) show no phloem loading of [^{14}C]glyphosate above that in the surrounding mesophyll. The pH of the uptake solution had no effect on uptake of [^{14}C]glyphosate into leaf discs (data not shown).

Exodiffusion of Glyphosate and Calculation of Permeability. Glyphosate was loaded into leaf discs for 3 h. The leaf discs were then exodiffused by transfer to fresh buffer at 10-min intervals. Assuming a passive uptake and efflux of glyphosate from cells of leaf tissue, the amount leaking out should be directly related to the amount of glyphosate in the tissue. It follows that $\ln N = \ln N_0 + Kt$, where N is amount of material in tissue at time t, N_0 is amount present at time zero, and K is rate constant equal to ln 2/half-time. The data for glyphosate exodiffusion were plotted in this form (Fig. 6). There were two phases of efflux from leaf tissue, each consistent with the passive permeation model. The second phase probably represents efflux from the cytoplasm and vacuoles. It has a half-time of 389 min with a range of 128 min averaged over nine experiments. The first phase is most likely exit from the free space. Half-time of exit from the first compartment was calculated by subtracting extrapolated amounts of glyphosate exiting from the cytoplasm during the first 20 min efflux. The

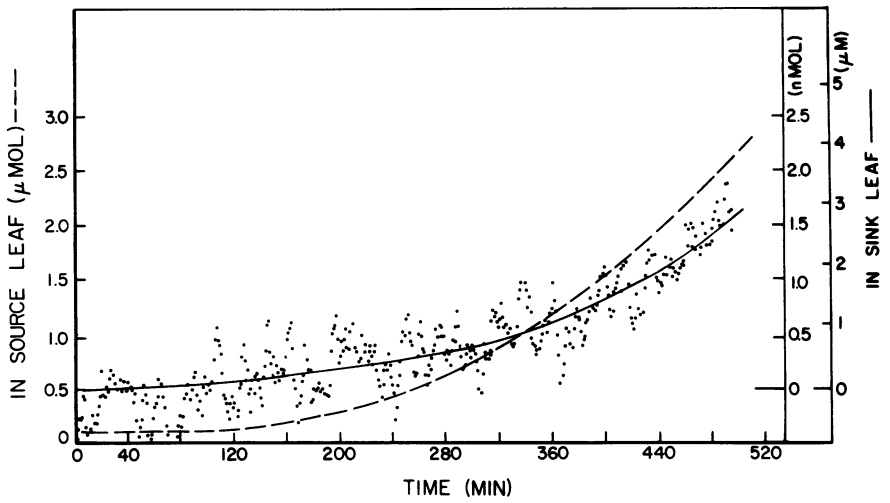

FIG. 3. Arrival of [^{14}C]glyphosate in sink leaf and accumulation in source leaf each monitored with a GM tube. [^{14}C]Glyphosate at 15 mM, plus surfactant was added to upper surface of source leaf. Low initial values for source leaf represent the very small proportion of β^- particles which were able to penetrate the lamina of the mature leaf. Individual data points are not shown for source leaf accumulation. Figure is representative of three experiments.

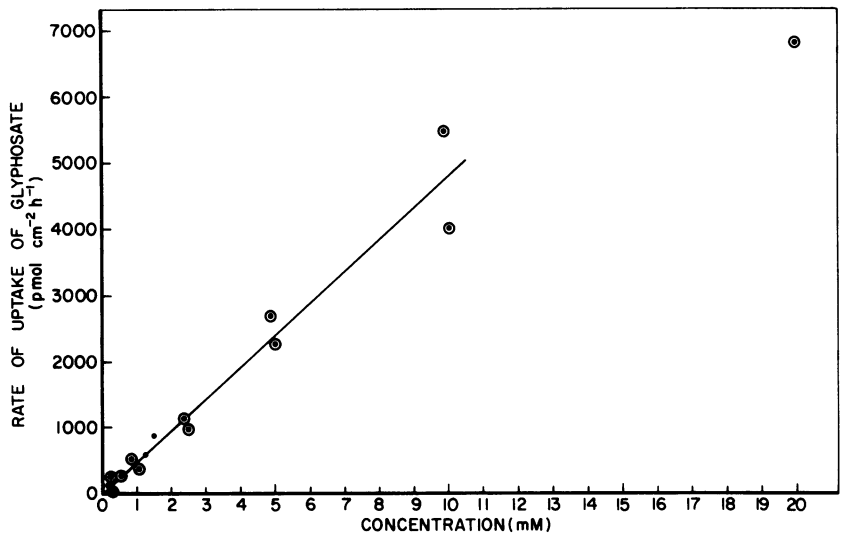

FIG. 4. Concentration dependence of uptake of glyphosate into leaf discs from [^{14}C]glyphosate in 5 mM KH_2PO_4 (pH 6.5). Each point is average of two experiments, each with three or four leaf discs. Both coordinates of noncircled points were multiplied by 10 to allow plotting.

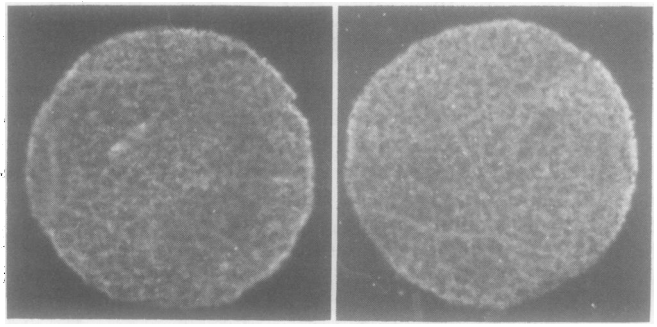

FIG. 5. Autoradiographs of leaf discs after 3 h uptake from 2 mM [^{14}C]glyphosate in 5 mM KH_2PO_4 (pH 6.5).

half-time was 6.7 min with a range of 0.3 min.

Using the half-time of exit for the cell contents inside the plasmalemma, the permeability of the plasmalemma to glyphosate can be calculated using the following equation (14),

$$P^* = \frac{V_1}{A}\frac{V_2}{V_1 + V_2}\frac{\ln 2}{t_{1/2}}$$

where V_1 = volume of cytoplasm, V_2 = volume of external solution, and A = surface area of membrane. Since, in this case, V_2 is much larger than V_1, the second factor reduces to one. V_1 and A can be calculated assuming mesophyll cells are 35-μm diameter spheres and cells are tightly packed in seven cell layers in leaf tissue. Substituting, $V_1 = 12.7 \times 10^{-9}$ m^3 and $A = 22.1 \times 10^{-4}$ m^2, $P^* = 1.7 \times 10^{-10}$ m s^{-1} with a range of 0.83×10^{-10} m s^{-1}. This calculation was confirmed using influx of glyphosate in pH dependence experiments at pH 6.7 and the following equation (8),

$$P = \frac{V}{At}\ln\frac{C^o - C^i(o)}{C^o - C^i(t)}$$

where V and A have the same values as above, t is time, C^o and C^i are concentrations outside and inside tissue at initial time (0) and final time (t). Permeability calculated with this method was 8.5×10^{-11} m s^{-1}, within the range of permeability calculated above.

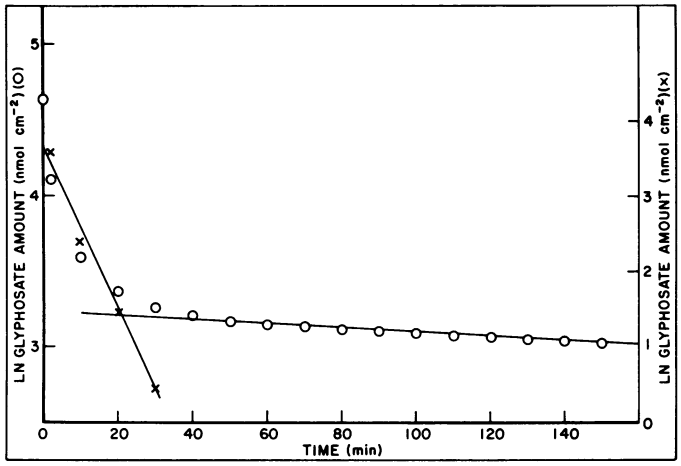

FIG. 6. Exodiffusion of [^{14}C]glyphosate from leaf discs. Uptake time was 3 h from 17.6 mM [^{14}C]glyphosate in 5 mM KH_2PO_4 (pH 6.5). Discs were then transferred to fresh buffer every 10 min for 2.5 h. Amount of glyphosate remaining in discs was calculated as described. Data points (×) for the first phase of efflux were calculated by subtracting extrapolated amounts due to second phase from observed points (○) during that time. Data from 1 of 9 experiments.

DISCUSSION

Glyphosate was readily transported throughout the plant within the first several h when applied to leaves (Fig. 3). Concentrations in an actively growing sink leaf ranged between 2.5 to 13.7 μM in 10 h from a 15 mM solution added to one mature leaf. This transport was shown to occur in the phloem by double-labeling experiments using [^{3}H]sucrose as a marker for material recently translocated in the phloem. The ratio of glyphosate to [^{3}H]sucrose was similar thoughout the plant indicating a common transport mode (Fig. 1).

Any xenobiotic material transported in the phloem must first enter the symplast. Brecke and Duke (1) recently reported that glyphosate was readily absorbed into leaves of intact *Phaseolus vulgaris* L. plants, but on the order of 3,000 times less glyphosate was taken up by individual mesophyll cells isolated from a glyphosate-treated leaf, even after 24 h. This implies that glyphosate either never entered the mesophyll cells or was rapidly taken up into the veins. The latter possibility is in contrast to autoradiographs (Fig. 5) showing no phloem loading above that in the mesophyll. The possibility that glyphosate was not taken up by mesophyll cells is in contrast to our compartmental analysis showing glyphosate present within the plasmalemma. When leaf discs were loaded with glyphosate and then transferred to solution free of glyphosate, there was an initial rapid efflux (half-time 6.7 min), presumably exit from the free space, and a slower efflux (half-time 389 min), most likely from within the plasmalemma. A likely explanation for the lack of glyphosate in mesophyll cells in work of Brecke and Duke (1) is that glyphosate was lost during the cell isolation procedure.

A carrier-mediated mechanism for entry of a xenobiotic material seems unlikely because of the specificity of such processes (4). Results presented here support a passive, nonfacilitated uptake of glyphosate into the cells. Uptake into leaf discs increased linearly with increasing concentration as would be expected for simple diffusion not involving a saturable carrier. Glyphosate was not concentrated in cells of leaf discs. After 3 h uptake, glyphosate concentration within the cells was 3 mM, compared to 17.6 mM in the external solution. Efflux through the plasmalemma depended on the concentration of glyphosate in the leaf disc as would be expected for passive movement.

One passive mechanism for accumulation in the phloem is the weak acid mechanism (3). Glyphosate has four ionizable groups with pK values of less than 2, 2.6, 5.6, and 10.6 (12). Net charges on the molecule at pH values midway between adjacent pK points are 0, −1, and −2, respectively. Net charge on the molecule would increase as it moved from cell wall space at a slightly acidic pH into the sieve tubes, at pH about 8 for sugar beet (5). The weak acid mechanism predicts a higher permeability toward the less charged species. If this phenomenon were occurring, uptake would be expected to be greater from a solution lower in pH. This was shown for the diffusional uptake of IAA and 2,4-D, which increased with decreasing pH (10). However, pH of the external solution had no effect on uptake of glyphosate into leaf discs over the pH range 4.5 to 9.7. Therefore, it was concluded that the weak acid mechanism does not play an important role in phloem transport of glyphosate.

A second passive mechanism explaining accumulation and transport in the phloem is the intermediate permeability mechanism (9, 14). A substance capable of penetrating the sieve tubes, but to which membranes are not so permeable that the substance can easily leak out of the phloem and be carried away in the transpiration stream, will be distributed throughout the plant in the phloem. In the roots and other terminal sinks where transport slows to a halt, the chemical will slowly leak out of phloem and be re-circulated in xylem. Such a substance is classified as ambimobile (9). Tyree *et al.* (14) calculated an optimum permeability for a simplified model of a plant exhibiting this mechanism using an equation relating ambimobility to permeability. This relationship depended on several factors: linearized length of the plant (L), length of the phloem in the source leaf (l), radius of sieve tubes (r), and average daily translocation velocity (V). The radius of sieve tube alone, not including the contribution of companion cells, was used to calculate the flux into the phloem, which could affect the estimate of the optimum. Using the following values for a 6-week-old sugar beet (2), $L = 0.3$ m, $l = 0.05$ m, $r = 5.0$ μm, and $V = 1.0$ cm min^{-1} (1), the theoretical optimum permeability would be 9.6×10^{-10} m s^{-1}. The permeability calculated for membranes of sugar beet leaf cells toward glyphosate is 1.7×10^{-10} m s^{-1}, slightly lower than the theoretical optimum, but still within the range of permeabilities for which the measure of ambimobility is high (see Fig. 4, ref. 14). In contrast, an increase in permeabiity from the optimum results in a rapid decline in ambimobility. The assumption is made that permeability toward glyphosate of mesophyll cells, which constitute the bulk of the leaf, is of the same magnitude as that for sieve tubes. We concluded that an intermediate permeability mechanism can be used to explain phloem transport of glyphosate.

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