Glyphosate Transport and Early Effects on Shikimate Metabolism and Its Compartmentation in Sink Leaves of Tomato and Spinach Plants

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Glyphosate (N[phosphonomethyl]glycine) applied to an assimilate-exporting leaf of tomato or spinach plants is translocated *via* the phloem to the young growing areas of the shoot apex and root where it causes the rapid accumulation of shikimate and shikimate 3-phosphate to up to 16% of the dry weight or these tissues. Using the technique of non-aqueous fractionation of spinach leaves it was shown that the herbicidal target of glyphosate, the shikimate pathway enzyme 5-enolpyruvlyshikimate 3-phosphate synthase, is located within the chloroplast and that, in the presence of glyphosate, shikimate 3-phosphate accumulates in the same organelle, while shikimate was preferentially localized in the vacuole. The major part of [^{14}C]glyphosate imported into the leaf *via* the phloem was detected in the cytosolic fraction of the mesophyll cells.

Introduction

Glyphosate (N[phosphonomethyl]glycine) is a widely used potent nonselective postemergence herbicide which effectively controls a large number of annual and perennial weeds [1, 2]. When applied to the foliage, it is rapidly absorbed and transported to the meristematic and young growing tissues of both the shoot and the root following the source-sink gradient within the plant [3–5]. Its biochemical mode of action has been shown to be the inhibition of the shikimate pathway enzyme 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase (EC 2.5.1.19) resulting in a block in the synthesis of the aromatic amino acids and other aromatic compounds in plants [6, 7]. While attention has focussed on the molecular biology and biochemistry of EPSP-synthase and its interaction with glyphosate [7], few studies have been concerned with i) how glyphosate reaches its target at the subcellular level, and ii) what are the early biochemical consequences of the interaction of glyphosate with its target both at the subcellular, cellular and whole plant level. We have previously reported on glyphosate toxicity in the shoot apical region of the tomato plant and shown that plastid swelling is the initial ultrastructural feature following *in vivo* inhibition of EPSP-synthase [8]. Glyphosate's early effect on plastid ultrastructure is particulary intriguing because EPSP synthase has been shown to be a plastidial enzyme [9, 10] which is synthesized on cytoplasmic ribosomes as a higher molecular weight precursor [11] and which is then transported through the plastid envelope and proteolytically processed inside the organelle to yield mature EPSP synthase [12]. In extension of previously published work [8, 13–15] we wish to report here on biochemical alterations observed in young growing leaves of tomato and spinach plants during the import of glyphosate from an assimilate-exporting leaf.

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Materials and Methods

Tomato plants (*Lycopersicon esculentum* Mil var. "Moneymaker") were grown as previously described [8]. Spinach plants (*Spinacia oleracea* L. var. "Matador") were grown for 6 weeks in a greenhouse at 15 ± 3 °C under natural light conditions with supplementary fluorescent light (10 kLux) 8 h per day. The source of glyphosate,

its mode of application to the plants, as well as the extraction and determination of glyphosate, shikimate and shikimate 3-phosphate (S3P) are also found in ref. [8]. Cotransport of [^{14}C]glyphosate and [^{3}H]sucrose (6,6′(*n*)-^{3}H-sucrose, Amersham-Buchler, Braunschweig, F.R.G.) was investigated by depositing a 20 µl drop containing 200 nmol [^{14}C]glyphosate (8.7 kBq) and 580 nmol [^{3}H]sucrose (16.3 kBq) in 5 mM potassium phosphate buffer, pH 5.5, and 0.1% Tween 80 onto one of the middle lobes of the third leaf of a six week old tomato plant. The plants were returned to the growth room [8] and shoot apices and roots were excised at short intervals and their ^{3}H- and ^{14}C-activities determined after combustion in a Packard Tri-Carb Sample Oxidizer B 306. The fractionation of freeze-stopped spinach leaves in non-aqueous media, the assay of marker enzyme activities and of metabolites for the chloroplast stroma, cytosol and vacuolar compartments, as well as the evaluation of the subcellular distribution of metabolites followed closely the procedures given by Gerhardt and Heldt [16, 17]. EPSP synthase activity was determined as in ref. [18].

Results and Discussion

The first visible symptom observed after application of 200 nmol of glyphosate to the third oldest leaf of 6- to 8 week old tomato plants is a chlorosis of the apical shoot region [8]. It is this region, as well as the root system of the plants, into which glyphosate is translocated and in which shikimate accumulates [8, 14]. Even 5 days after application of [^{14}C]glyphosate, the radioactivity resided exclusively, with the exception of the leaf area to which it had been applied, in these two parts of the plant as is evident from the radioautogram shown in Fig. 1. At this time, the entire apical region is already necrotic. The distribution of label from glyphosate (which is not metabolized by tomato plants) is indicative of phloem transport of the herbicide which follows the source to sink gradient in plants. This conclusion is supported by experiments in which phloem transport, but not translocation in the xylem, was incapacitated by heat girdling of the stem (Fig. 2): depending on the position of the heat girdle relative to the site of application of the herbicide, glyphosate translocation to either the shoot or the root was severely blocked. The residual fraction of glyphosate transported upwards through a heat-inactivated region of the stem probably reflects xylem transport as a very minor component of total glyphosate transport. It is apparent from Fig. 3 that the distribution of glyphosate in the heat-girdled plants is reflected by the levels of shikimic acid in the tissues. Likewise, it had previously been shown that the pattern of shikimic acid (and S3P) distribution in

Fig. 1. Autoradiogram of 8 week old tomato plant 5 days after application of 200 nmol [^{14}C]glyphosate (17.8 kBq) to a lobe of the third oldest leaf (arrow).

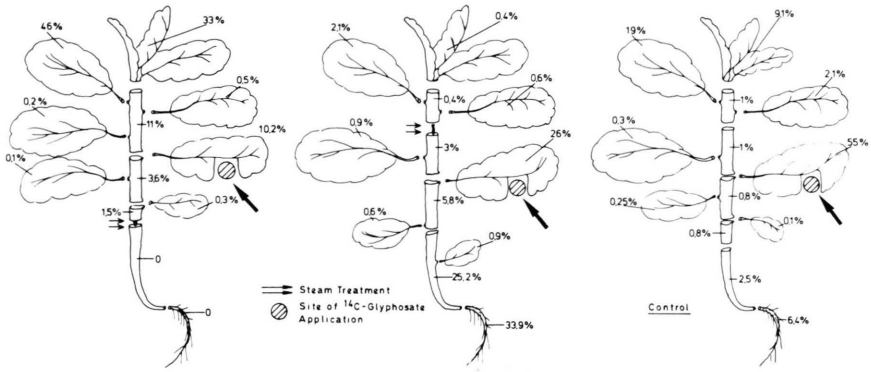

Fig. 2. Effect of heat girdling on glyphosate translocation. Distribution of radioactivity in 6 week old tomato plants was determined 12 days after application of 200 nmol [^{14}C]glyphosate (4.5 kBq) to the third oldest leaf. Numbers represent percentage of total radioactivity taken up by and distributed within the plants. Radioactivity remaining at the site of application (large arrows) was not considered. Small double arrows indicate areas where heat girdling by a 1 min treatment with vapor from boiling water was performed.

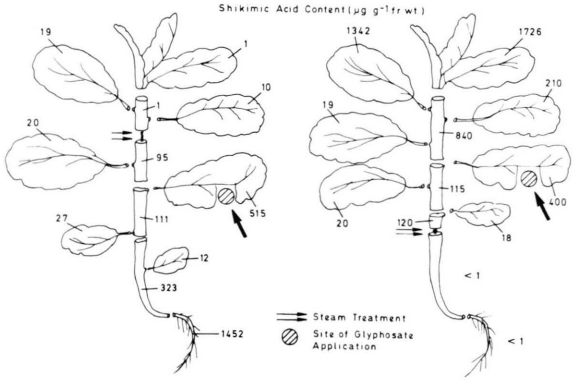

Fig. 3. Effect of heat girdling on shikimate accumulation in glyphosate-treated plants. Conditions were as in Fig. 2 except that non-radioactive glyphosate was used. Values indicate shikimate content in µg per g fresh weight.

apical leaves matched precisely with the prevalent glyphosate concentration [8].

Co-transport of sucrose (as a marker of phloem transport) and of glyphosate is clearly indicated by an experiment in which the import of the two compounds, labelled with different isotopes, into the shoot apex and root was followed (Fig. 4). Both glyphosate and sucrose are rapidly imported into these sink tissues, with a constant ratio of the respective radioisotopes. The increase in the ^{3}H/^{14}C-ratio from 2 (in the applied droplet) to appr. 8 (in the sink tissues) reflects preferential uptake of sucrose into the phloem, but the constant ^{3}H/^{14}C-ratio over the transport period confirms that both compounds are co-transported once they have entered the phloem. Essentially similar results have previously been obtained for sugar beet [4] and other plants [2], and the same results were found with cyanide poisoning of phloem transport (A. Schulz and N. Amrhein, unpublished). Analysis of the time course of the changes of shikimate concentration in the sink tissues, and calculation of glyphosate tissue concentrations clearly reveal that less than 1 µM glyphosate in the target tissue is sufficient to initiate the accumulation of shikimic acid (Fig. 4).

Under the conditions of our experimental setup, shikimic acid began to accumulate in the shoot apex 4 h after application of glyphosate to the third-oldest leaf of a 6 week old tomato plant, while in the root shikimate began to accumulate after 12 h (Fig. 4 and 5). Shikimate levels then increased linearly for extended periods of up to 5 days and, in the shoot apex, shikimic acid finally constituted almost 16% of the dry weight of the tissue after 5 days (Fig. 5). Such figures seem to support the notion that loss of feedback control of the shikimate pathway and unregulated carbon flow into this pathway are involved in the action of glyphosate [19, 20]. Induction of the first enzyme of the shikimate pathway, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase, recently shown

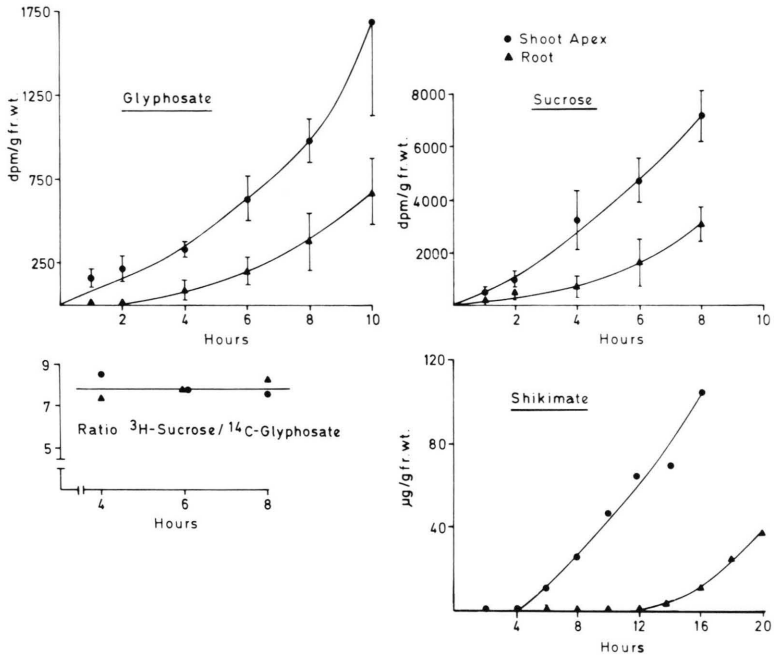

Fig. 4. Contransport of [^{3}H]sucrose and [^{14}C]glyphosate. For experimental conditions see Materials and Methods. Shikimic acid was analyzed in a second batch of plants treated with unlabelled glyphosate only.

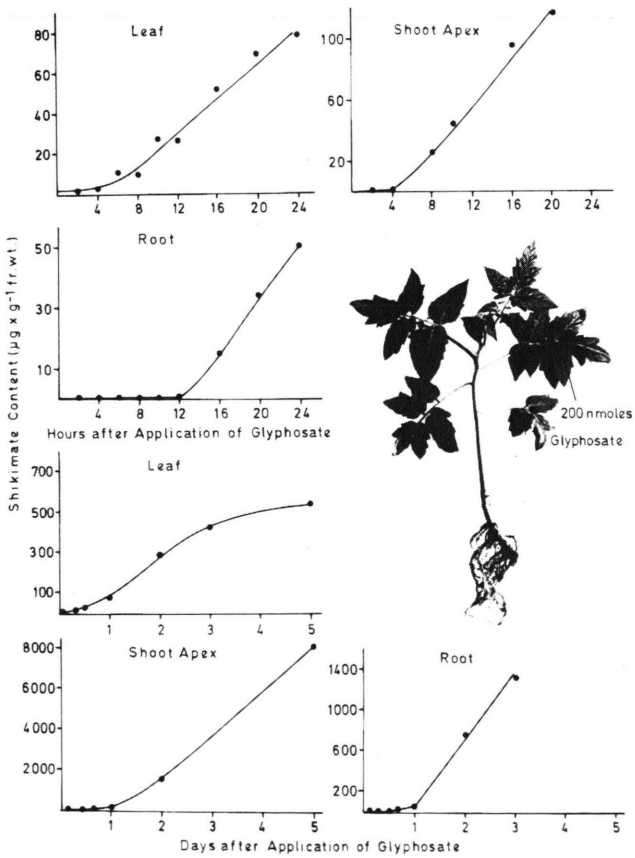

Fig. 5. Time course of shikimate accumulation in the root, shoot apex and glyphosate-treated leaf of a 6 week old tomato plant. A 20 µl drop containing 200 nmol glyphosate was applied to the indicated lobe. Note that the time scale in the upper three graphs represents hours, while in the lower three graphs it represents days.

to be caused by glyphosate in cultured potato cells [21], may contribute to the excessive flow of carbon into the pathway in the presence of glyphosate.

The correlation between the import of glyphosate into its target tissues with the initial biochemical changes occurring in these tissues prompted us to study these changes at the subcellular level. It is generally accepted that the plastid is the major site of aromatic amino acid biosynthesis in the plant cell [9], even though a separate pathway in the cytosol with its own complement of enzymes has been postulated [19]. We had previously found that shikimate accumulating in buckwheat cells cultured in the presence of glyphosate was preferentially localized in the vacuole [22]. In these experiments vacuoles were isolated from protoplasts in a time-consuming procedure. Metabolite redistribution during the isolation procedure could therefore not be excluded. In the present study we used the fractionation of freeze-stopped leaf tissue in nonaqueous media developed by Gerhardt and Heldt [16] for spinach leaves in order to avoid such possible complications. In preliminary experiments (results not shown) glyphosate was found to be translocated and to cause shikimate accumulation and chloroplast swelling in spinach in a manner fully comparable with its effects in tomato plants. The subcellular compartmentation of EPSP synthase, of shikimate and its 3-phosphate, as well as of glyphosate in a mesophyll cell of a spinach sink leaf three days after application of glyphosate to a source leaf is shown in Table I. In control leaves, the levels of shikimate and S3P were too low for quantitative analysis, and EPSP synthase activity was found exclusively in the stroma fraction. The fraction of EPSP synthase comigrating with the cytosolic markers in gradients prepared from leaf material exposed to glyphosate for three days may either be the result of the release of EPSP synthase from ruptured chloroplasts into the cytosol [8] or may represent the activity of the EPSP synthase precursor protein. This alternative is not unplausible because i) it has been shown that the *Petunia* EPSP-synthase precursor protein is enzymatically active [12] and ii) that glyphosate inhibits the uptake of the precursor into the chloroplast to some extent [23]. Even though the fractionation of freeze-stopped material in non-aqueous media permitted only a relatively inaccurate estimation of the distribution of the metabolites and of glyphosate in the subcellular fractions of the mesophyll cells, it is evident from Table I that the substrate of EPSP synthase, S3P, accumulates predominantly, if not exclusively, in the chloroplast, whereas shikimate accumulates in the vacuole, in agreement with the previous finding for cultured cells [22]. As to be expected on the basis of its highly acidic nature, glyphosate imported into the mesophyll *via* the phloem is found in a high concentration in the cytosolic fraction. Taking into consideration that glyphosate inhibits EPSP synthase from higher plants with a $K_i <$ 1 μM [7], the concentration of glyphosate found in

Table I. Subcellular distribution of EPSP synthase, shikimate and its 3-phosphate, as well as glyphosate in spinach mesophyll cells.

Compound	Content in % of total (concentration) Stroma	Cytosol	Vacuole
EPSP-synthase[a]	88 ± 4	12 ± 4	0
Shikimate 3-phosphate[b]	82 ± 23 (12 ± 9 mM)	–*	–*
Shikimate[b]	–*	–*	87 ± 15 (3.5 ± 1.3 mM)
Glyphosate[c]	5 ± 2 (57 ± 20 μM)	74 ± 2 (1.1 ± 0.2 mM)	21 ± 4 (43 ± 17 μM)

* Values too low and too variable for calculation.
[a] $n = 12$; [b] $n = 6$; [c] $n = 5$; values are given ± SD.

A 20 μl droplet containing 200 nmol glyphosate (31 kBq when radiolabelled glyphosate was used) was applied to an older leaf of a six week old spinach plant. After 2 days, the youngest leaves were excised and freeze-stopped in liquid nitrogen. The ground and lyophilized material was subjected to fractionation by density gradient centrifugation in non-aqueous media and further processed as described by Gerhardt and Heldt [16].

the stroma would suffice to inhibit the enzyme; however, this calculation cannot be convincing without knowledge of the concentration of phosphoenolpyruvate (the EPSP synthase substrate competing with glyphosate for the binding site on the enzyme) in the stroma, and more data on glyphosate concentrations in the subcellular fractions of the mesophyll cells at earlier stages of its import into the leaf are clearly also required. While our data appear to support the view that glyphosate exerts its effect on the shikimate pathway by inhibiting the EPSP synthase which is exclusively found in the chloroplast, an additional effect on the uptake of the precursor of the enzyme into this organelle [23], as well as the possibility of the interference of glyphosate with an extra-plastidial EPSP synthase [19] cannot be excluded with certainty.

In conclusion, our work has confirmed that glyphosate is translocated almost entirely *via* the phloem and that it causes rapid inhibition of its target enzyme, EPSP synthase, in assimilate importing tissues as evident from the accumulation of shikimate and its phosphate ester. To explain the lethal action of glyphosate at the whole plant level one should, however, concentrate not only on changes occurring in the shikimate pathway. As Geiger and his colleagues have clearly demonstrated, glyphosate rapidly affects the assimilation, as well as the allocation of carbon in sugar beet plants [24–26]. Future studies will have to show how inhibition of EPSP synthase brings about these and other pleiotropic effects of glyphosate reported in the literature [1, 2].

Acknowledgements

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