Accumulation of Shikimic Acid: A Technique for Screening Glyphosate Efficacy

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Biochemical changes in leaves of 3-week-old oil seed rape (*Brassica napus* L. cv. Iris) plants were determined shortly after treatment with different doses of glyphosate. Secondary effects of *N*-(phosphonomethyl)glycine (glyphosate) on activity of the enzyme phenylalanine ammonia-lyase was not related to dose of glyphosate by any known model within the first 48 h after spraying. Accumulation of shikimic acid was related to the dose of glyphosate by a nonlinear logistic dose−response model—as early as 5 h after spraying. Within the same period shikimic acid accumulation made it possible to distinguish between a formulation of the pure isopropylamine salt of glyphosate and formulations containing different surfactants. ED_{50} estimates based on accumulation of shikimic acid gave a good indication of the relative strength of the evaluated glyphosate formulations. Ranking of ED_{50} based on accumulation of shikimic acid was the same as achieved by visual assessment of plant death 14 days after spraying.

Keywords: *Glyphosate; shikimic acid; efficacy; dose-response*

A foliar-applied herbicide such as glyphosate must pass through several barriers in order to reach its site of action. Many investigators have looked at epicuticular wax as the barrier for glyphosate uptake, but during recent years attention has been focused on the plasma membrane as the main barrier (Denis and Delrot, 1993; Riechers et al., 1994; Røyneberg et al., 1992). Attempts to increase uptake through these barriers have led to many different formulations of glyphosate and adjuvants, which have been tested and are commercially available.

Testing the rate of uptake of these formulations has included [^{14}C]glyphosate and the use of isolated cuticles, cell suspension cultures (Burton and Nelson, 1988; Holländer-Czytko and Amrhein, 1983), isolated protoplasts (Watson et al., 1980), and artificial membranes (Miller and St. John, 1974) depending on the aim of the study. However, testing the efficacy has always been based on time-consuming bioassays including visual assessment of plant death or measurement of biomass, e.g., fresh weight, 10−14 days after spraying. Because visual symptoms develop slowly after glyphosate treatment, investigators have attempted to develop fast and reliable ways to evaluate glyphosate efficacy by using early physiological plant responses. Changes in leaf temperature, caused by stomatal closure upon glyphosate treatment, have to some degree differentiated glyphosate dose and formulations (Lourtie et al., 1995). Likewise, photosynthetic parameters including fluorescence have been tested and related to the dose of glyphosate (Madsen et al., 1995). To the best of our knowledge, work of this kind has never resulted in any automated, reliable, and fast method for evaluation of plant response upon glyphosate treatment.

At the biochemical level the primary mode of glyphosate action is inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) (Rubin et al., 1982; Steinrücken and Amrhein, 1980). This results in blockage of the shikimate pathway causing a reduction in aromatic amino acid synthesis, reduced protein synthesis, reduced growth, and premature cellular death (Duke, 1988). Duke et al. (1980), Duke and Hoagland (1985), and Hoagland (1990) have reported that the pool of aromatic amino acids is further reduced by increased activity of phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5).

Blockage of the shikimate pathway results in accumulation of high levels of shikimic acid (Amrhein et al., 1980; Berlin and White, 1981; Lydon and Duke, 1988). Accumulation is exacerbated by loss of feedback control, allowing an unregulated flow of carbon to be diverted into intermediates prior to the blockage point of the shikimate pathway (Jensen, 1985). One would have expected accumulation of the EPSP synthase substrate—shikimic acid 3-phosphate, but this is not the case (Holländer-Czytko and Amrhein 1983; Mollenhouer et al., 1987), probably due to a phosphatase that hydrolyzes the ester and releases shikimic acid. The phosphatase may be localized either in the tonoplast or within the vacuole (Holländer-Czytko and Amrhein, 1983).

Becerril et al. (1989) found accumulation of shikimic acid in leaves of velvetleaf (*Abutilon theophrasti*) 6 days after treatment with glyphosate. The shikimic acid levels were significantly reduced 12 and 18 days after treatment (Becerril et al., 1989). Wan Kim and Amrhein (1995) found glyphosate induced accumulation of shikimic acid within 24 h after treatment of tomato

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plants. Fast accumulation is in agreement with Stasiak et al. (1992).

Thus, glyphosate's action on the development of phytotoxic symptoms and final plant death is complicated due to unregulated flow of carbon into the shikimate pathway, depletion of aromatic amino acids, and greatly disrupted secondary metabolism. The slow development of symptoms in whole plants precludes rapid screening for new formulation and adjuvant leads using traditional methods.

The aim of this study was to detect biochemical changes shortly after herbicide treatment that could be used as sensitive parameters in screening assays. A further requirement is that results of the assays should correlate with the effect at the whole plant level. Development of such a screening assay must be able to identify parameters that show reproducible dose−response effects. This study included glyphosate action on PAL activity and shikimic acid accumulation.

MATERIALS AND METHODS

Chemicals. Glyphosate formulations were Monsanto products (Roundup Bio and Roundup Ultra) and a Cheminova product (Glyfos) all containing 360 g of a.i./L. The pure isopropylamine salt of glyphosate (IPA) was used for comparison. All other chemicals were Sigma products unless otherwise mentioned.

Plant Material. Seeds of oil seed rape (*Brassica napus* L. cv. Iris) were soaked overnight in aerated tap water, before being planted in a sand peat mixture containing (in mg of nutrient/100 g of sand peat mixture) 35−50 NO_3-N, 40−55 P, 35−50 K, 15−25 Mg, Ca > 175 plus micronutrients. Plants were grown in a greenhouse at 22 ± 2 °C under a 16-h photoperiod provided by natural light and supplemented with high-pressure sodium lamps ensuring proton flux densities greater than 400 μmol m^{-2} s^{-1}. Unless otherwise stated, the third leaf of 3-week-old plants was used in the experiments.

Glyphosate Application. Plants were sprayed in a spray cabin using a Hardi 4680-21E flat fan nozzle applying water at a rate of 210 L ha^{-1}. A complete randomized design with three replicates was used in all experiments. Plants were always treated 3 h after light onset.

Shikimic Acid Analyses. Leaves was harvested into a zipbag and stored on ice for up to 10 min before it was weighed and further stored at 4 °C for a maximum of 30 min before extraction. Leaves were homogenized in liquid nitrogen and extracted with H_2SO_4 (4 mL of 0.01 M g^{-1} of fresh weight (FW)) for 60 min on a shaker. $NaHCO_3$ (1 mL of 0.4 M) was added, and the solution was centrifuged at 25000*g* for 20 min at 4 °C. The supernatant was filtered and stored at −30 °C. HPLC analysis was carried out according to the methods of Lydon and Duke (1988).

PAL Extraction and Assay. The third leaf was ground in liquid nitrogen. The powder was carefully transferred to 12-mL centrifuge tubes containing 1 g of cooled polyvinyl−polypyrrolidone (insoluble) and 1 g of cooled Amberlite XAD-2 (Lancaster). Cold Tris-HCl buffer (8 mL, 50 mM Tris, 1 mM dithiothreitol, pH 7.5) was added. The mixture was mixed thoroughly with a glass spatula and centrifuged at 25000*g* for 30 min at 4 °C. The supernatant was filtered through a Whatman No. 1 paper. The filtrate was centrifuged once more at 25000*g* for 10 min at 4 °C (Lin and Northcote, 1990). The activity was determined shortly after extraction, but the supernatant could be stored for several months at −30 °C.

The assay for PAL activity was a modified procedure by Lin and Northcote (1990); 0.04 M L-phenylalanine, 50 mM borate buffer, pH 8.8, containing 0.2 mL of enzyme extract in a final volume of 1.5 mL was incubated at 37 °C for 1 h, and the reaction was stopped by addition of HCl (1.5 mL of 2 M). Absorbance at 290 nm was read using an enzyme-free reaction mixture as reference. As blank was used a reaction mixture where L-phenylalanine was substituted by D-phenylalanine. The activity is expressed as nmol of cinnamic acid produced per mg of protein/min.

Protein Determination. Protein concentration was determined by the method of Bradford, using bovine serum albumin (fraction V) as standard (Bradford, 1976).

Visual Measurement. Visual measurements of herbicide effect were determined 14 days after spraying using a scale from 0 to 100% plant death, zero effect meaning no difference between treated plant and control and 100% meaning death of plants.

Statistical Analysis. A completely randomized design was used for all studies. Each experiment had at least three replicates. The data from the PAL assay was described by a nonparametric method because we could not find a model that properly described the relationship for the dose response. The systematic part of the plant response (shikimic acid accumulation or percent of plant death) (U) on herbicide concentration (z) was assumed to follow the logistic model (Streibig, 1988):

$$U_{ij} = D + \frac{C_i - D}{1 + \exp[b_i(\log(z_{ij}) - \log(\mathrm{ED}_{50(i)}))]} \qquad (1)$$

where U_{ij} denotes the response at the jth concentration of the ith herbicide preparation. For negative b_i; C_i denotes the upper limit at infinite concentration of herbicide i and D the lower asymptote at zero concentration (for shikimic acid in oil seed rape this was not significantly different from zero). $ED_{50(i)}$ denotes the concentration required of herbicide i to achieve shikimic acid accumulation or visual scoring halfway between D and C, and b_i is the proportional slope of the curve around $ED_{50(i)}$.

Within an experiment the nonlinear regression models were fitted simultaneously to individual data points for the four herbicides applied. To stabilize the variance, a transform-both-sides method (Carroll and Rupert, 1988) was used. The regressions were assessed by test for lack-of-fit and by graphical analysis of the distribution of residuals (Streibig et al., 1993).

For individually fitted curves in Table 1 and Figure 2 we deemed ED_{50} estimates significantly different if either of the estimates was outside the confidence limits of the other ED_{50} estimate. In all other experiments (Tables 2−5) we fitted the curves with different or similar ED_{50} values and used tests for lack of fit ($P \leq 0.05$) in a consecutive way to determine which ED_{50} estimates were significantly different (Streibig et al., 1993).

RESULTS AND DISCUSSION

PAL. PAL activity was assayed 24 and 48 h after spraying 3-week-old oil seed rape plants with glyphosate in doses from 25 to 800 g of a.i. ha^{-1}. The activity of PAL tended to decrease with increasing dose of glyphosate (Figure 1). It was not possible to find a satisfactory dose−response curve, but the nonparametric regression clearly illustrated the depletion of PAL activity. Earlier results have been diverging. Ishikura and Takeshima (1984) and Ishikura et al. (1986) showed marked inhibition of PAL activity by glyphosate in *Perilla* cell suspension culture. Induction of PAL activity, as one of the early biochemical effects of glyphosate, has been published (Duke et al., 1979; Duke and Hoagland, 1978), but Holländer and Amrhein (1980) have suggested that increased PAL activity should be considered a secondary logical consequence of reduced synthesis of certain phenolic compounds that, in addition to others, regulate PAL activity (Engelsma, 1979), an effect that may easier be seen in cell suspension culture.

Accumulation of Shikimic Acid. The logistic dose−response model (eq 1) described data well, and the parameters and the appropriate corresponding 95% confidence intervals are shown in Tables 1−4 and

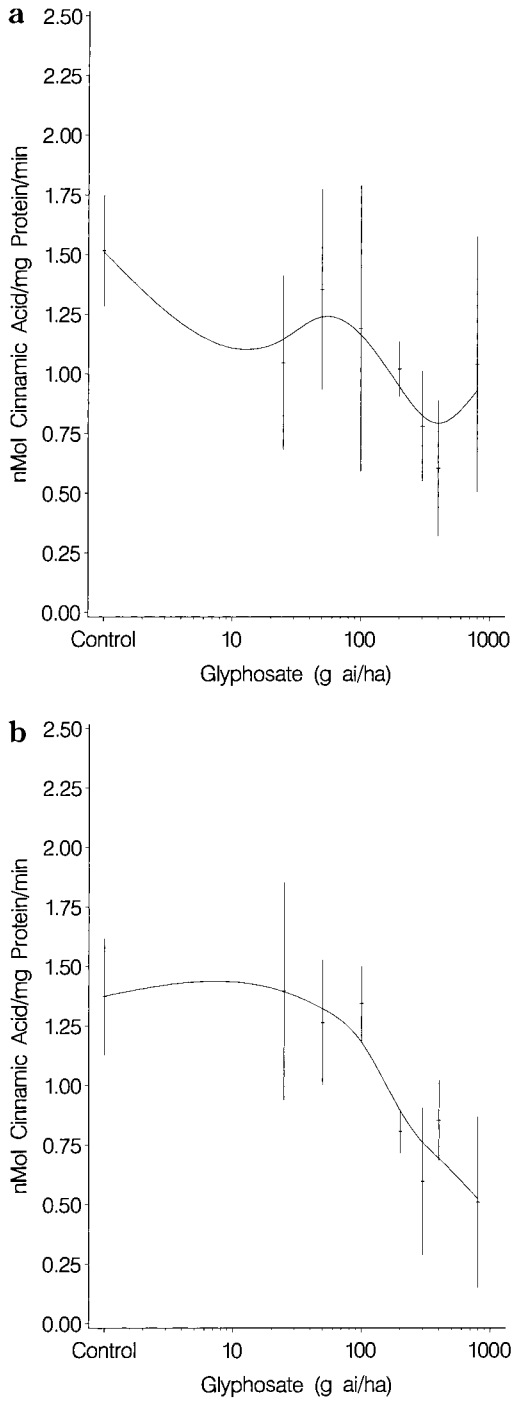

Figure 1. PAL activity in the third leaf of 3-week-old oil seed rape 24 h (a) and 48 h (b) after glyphosate spraying. The size of standard deviations is shown as a vertical bar. A nonparametric regression was fitted to the data. The glyphosate source was Glyfos.

Figures 2−7. Apart from the assay in Table 1 the lower limits of the curves, *D*, were not significantly different from zero and could be omitted from the model. Between 6 and 48 h posttreatment regression slopes increased and corresponding ED_{50} estimates decreased (Table 1, Figures 2 and 3).

Figure 2 illustrates accumulation of shikimic acid in leaves of 3-week-old oil seed rape 6, 24, and 48 h after spraying with glyphosate. According to Table 1 and Figure 2 changes in the shikimic acid content could be related to the dose of glyphosate as early as 6 h after spraying.

Table 1. Regression Parameters from Regression of Shikimic Acid Content in the Third Leaf of 3-Week-Old Oil Seed Rape on Dose of Glyphosate 48, 24, and 6 h after Spraying[a]

hours after spraying	D, μg of shikimic acid g^{-1} of FW	C, μg of shikimic acid g^{-1} of FW	*b*	ED_{50}, g of a.i. ha^{-1}
48	24.0	3539	−6.59	77.8 (65.7−89.9)
24	19.8	2097	−4.37	57.3 (45.3−69.2)
6	36.5	938	−4.47	52.9 (44.6−61.2)

[a] 95% Confidence intervals in parentheses. Glyphosate source: Glyfos.

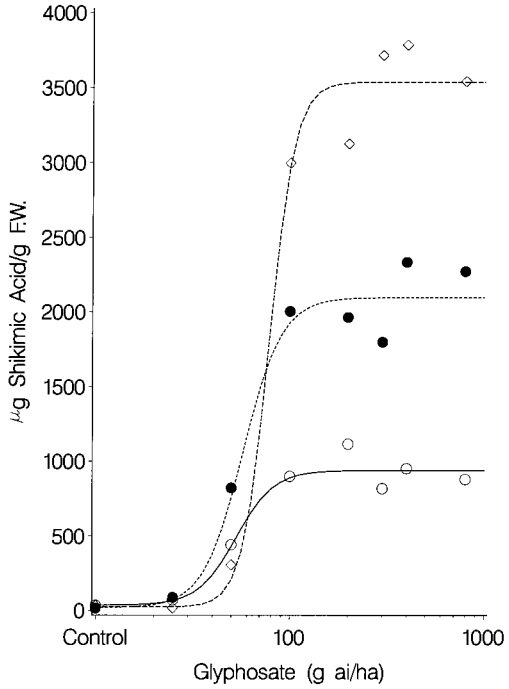

Figure 2. Accumulation of shikimic acid in the third leaf of 3-week-old oil seed rape 48 h (◇), 24 h (○), and 6 h (○) after spraying with glyphosate. Glyphosate source was Glyfos. Dots are mean values of three replicates.

The upper level of accumulated shikimic acid 48 h after spraying (3000−3500 μg of shikimic acid g^{-1} of fresh weight or 480−560 ng of shikimic acid mg^{-1} of dry weight) is in agreement with the level observed by Wan Kim and Amrhein (1995) 2−5 days after application of 200 nmol of glyphosate to leaves of 6-week-old tomato plants. Lydon and Duke (1988) found 100 and 200 ng of shikimic acid mg^{-1} of dw in leaves of soybean (*Glycine max*) and velvetleaf (*A. theophrásti*), respectively. Their extraction procedure included 1 h of boiling in 1 M HCl. Our experiments showed at least 50% reduction in recovery when boiled in either 1 M HCl or 0.1 M H_2SO_4.

Accumulation of shikimic acid clearly depended on the dose of glyphosate applied. This was earlier indicated by Lydon and Duke (1988) 3 and 6 days after application of a 50-μL drop containing 1, 5, or 10 mM glyphosate in 0.1% Triton X-100. Accumulation of shikimic acid 6 h after glyphosate treatment was earlier found by Schulz et al. (1990) in tomato leaves treated with 200 nmol of glyphosate.

The dose−response experiment in Figure 2 showed that accumulation of shikimic acid could not be elevated by adding glyphosate in excess of 200 g of a.i. ha^{-1}. It could be postulated that Michaelis−Menten saturation

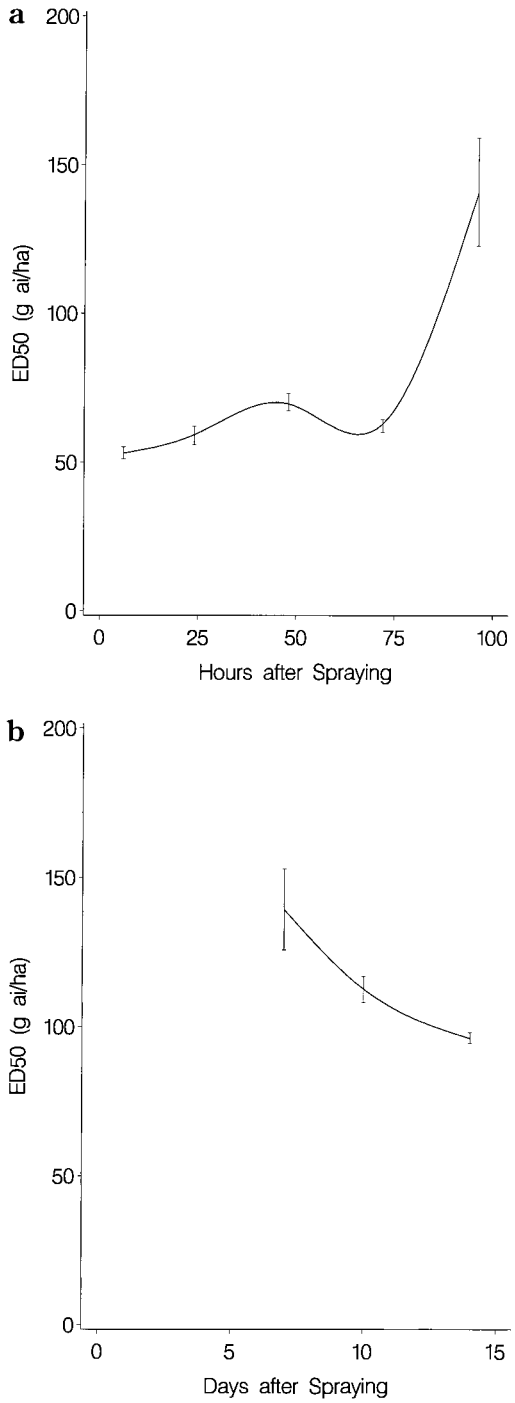

Figure 3. Time course for ED_{50} values for shikimic acid accumulation in the third leaf 5−96 h after spraying 3-week-old oil seed rape plants with glyphosate (a) and time course for ED_{50} values based on visual assessment of plant death 7−14 days after spraying 3-week-old oil seed rape with glyphosate (b). Glyphosate source was Glyfos.

of EPSP synthase is achieved at 100 g of a.i. ha^{-1}. Other factors could obviously contribute to these findings too: e.g., higher concentration of glyphosate or surfactant can rupture the cell or cell membrane and thus prevent shikimic acid accumulation. Similarly at low dose rates insufficient surfactant concentration may leave glyphosate deposited on the leaf surface, in the waxy cuticle or in the apoplast, depending on where the uptake barrier is located.

Increasing ED_{50} values throughout the time course (Table 1 and Figure 3a) could be a consequence of the above or metabolism of glyphosate in the plant. Unfortunately, only little literature on glyphosate metabolism in plants exists (Coupland, 1985; Franz et al., 1997); Duke (1988) pointed out that this is largely due to a general lack of metabolic degradation of glyphosate in higher plants.

Table 2. Regression Parameters of the Effect of Four Different Glyphosate Formulations on Shikimic Acid Accumulation in the Third Leaf of 3-Week-Old Oil Seed Rape 5 h after Spraying[a]

glyphosate source	ED_{50}, g of a.i. ha^{-1}
IPA	820 (323−1317)
Roundup Bio	131 (64−198)
Roundup Ultra	182 (90−273)
Glyfos	124 (61−187)

[a] 95% confidence intervals in parentheses. The four curves all had the same upper limit ($C = 1493$, $s = 208$) and slope ($b = -1.56$, $s = 0.22$).

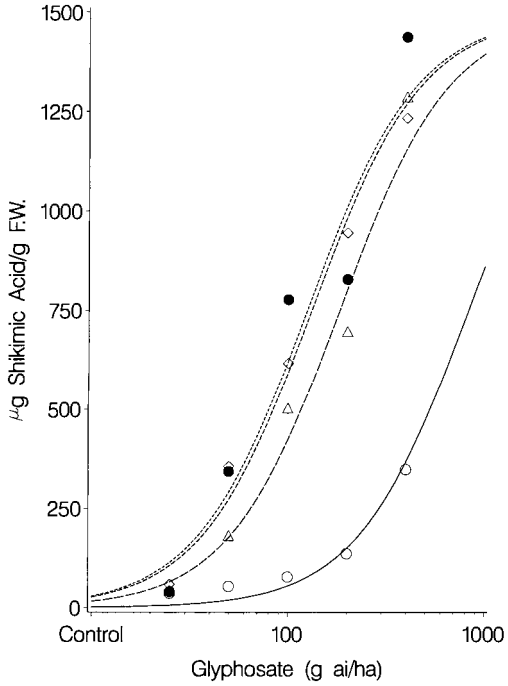

Figure 4. Dose−response curves of (from the top of the graph) Glyfos (●), Roundup Bio (◇), Roundup Ultra (△), and the pure IPA (○) (isopropylamine salt of *N*-(phosphonomethyl)glycine) based on the accumulation of shikimic acid g^{-1} of FW in the third leaf of 3-week-old oil seed rape plants 5 h after spraying. Dots are mean values of three replicates.

Reduction in shikimic acid levels, in sublethal glyphosate treatments, was earlier reported by Wan Kim and Amrhein (1995) starting 4 days after application of sublethal glyphosate levels (200 nmol) to leaves of tomato plants. Mollenhauer et al. (1987) suggested that plant survival at sublethal concentrations is a consequence of protection of the apical meristem through rapid cell division, which progressively dilutes cellular glyphosate. No further reports on the physiological mean of the disappearance of accumulated shikimic acid exist, but a theoretical explanation could be enhanced synthesis of EPSP synthase—like in glyphosate-tolerant plant species or rapid turnover of this enzyme.

Effect of Glyphosate Formulations 5 h after Treatment. In view of the results above, it seems reasonable to evaluate different glyphosate formulations by measuring the shikimic acid content 5 h after treatment with glyphosate. This was done in a dose−

Table 3. Regression Parameters of the Effect of Four Different Glyphosate Formulations on Shikimic Acid Accumulation in the Third Leaf of 3-Week-Old Oil Seed Rape 48 h after Spraying[a]

glyphosate source	*b*	ED_{50}, g of a.i. ha^{-1}
IPA	−1.87	710 (408−1011)
Roundup Bio	−5.11	84 (73−96)
Roundup Ultra	−2.23	164 (128−202)
Glyfos	−3.30	69 (57−82)

[a] 95% Confidence intervals in parentheses. The upper limit (C = 1294 μg of shikimic acid g^{-1} of fresh weight, s = 64) was the same for all curves.

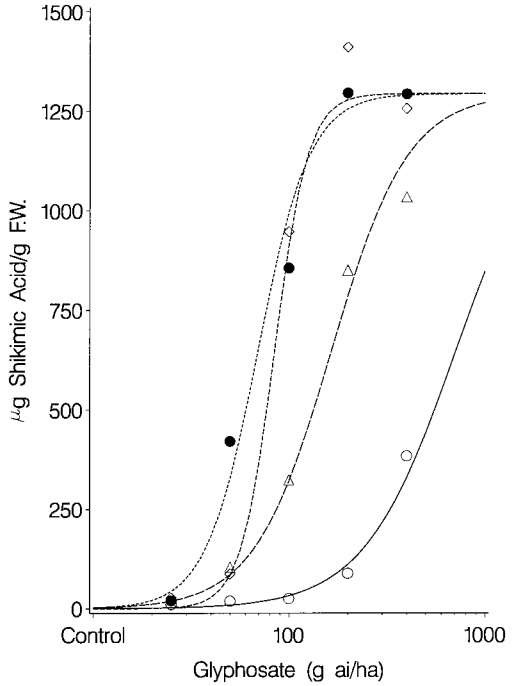

Figure 5. Dose−response curves of (from the top of the graph) Glyfos (●), Roundup Bio (◇), Roundup Ultra (△), and the pure IPA (○) (isopropylamine salt of *N*-(phosphonomethyl)glycine) based on the accumulation of shikimic acid g^{-1} of FW in the third leaf of 3-week-old oil seed rape plants 48 h after spraying. Dots are mean values of three replicates.

response experiment with four different glyphosate products (Figure 4 and Table 2). In this case the curves were assumed to have the same upper limit at high doses of glyphosate. The results clearly demonstrated that the formulated products (Roundup Bio, Roundup Ultra, and Glyfos) had a markedly better effect than the pure active component (IPA). The ED_{50} values of Glyfos and Roundup Bio were significantly lower than ED_{50} for Roundup Ultra, while there was no significant difference between Roundup Bio and Glyfos.

Effect of Glyphosate Formulations 48 h after Treatment. Over time, shikimic acid accumulation caused by herbicide formulation components, may be reduced or eliminated due to (1) different mechanism of surfactant action, (2) general agreement that under optimal spray conditions the surfactant is of minor importance, and (3) our previous findings concerning raising ED_{50} values over time (Figure 3). Indeed, this was not the case. The relative content of shikimic acid observed in plants treated with the formulated products remains higher than in plants treated with IPA 48 h after treatment (Figure 5); 48 h after treatment (Table 3) we had significantly different ED_{50} values for all glyphosate preparations: the Roundup Ultra ED_{50} remained significantly higher than the ED_{50} of Glyfos

Table 4. Regression Parameters from Investigations of Four Different Glyphosate Formulation Effects on Shikimic Acid Accumulation in Oil Seed Rape with Two Mature Leaves 5 h after Spraying[a]

glyphosate source	ED_{50}, g of a.i. ha^{-1}
IPA	590 (372−809)
Roundup Bio	105 (70−140)
Roundup Ultra	155 (104−207)
Glyfos	95 (64−128)

[a] Both leaves were harvested and pooled. 95% Confidence intervals in parentheses. The four curves all had the same upper limit (C = 793 μg of shikimic acid g^{-1} of fresh weight, s = 67) and slope (b = −1.56, s = 0.17).

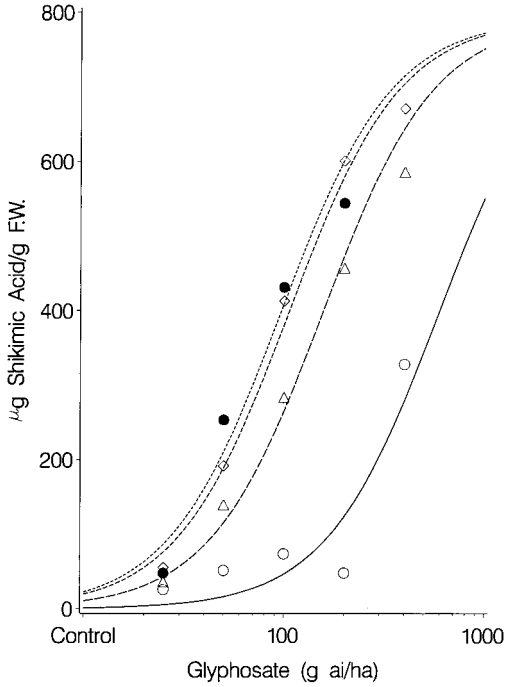

Figure 6. Dose−response curves of (from the top of the graph) Glyfos (●), Roundup Bio (◇), Roundup Ultra (△), and the pure IPA (○) (isopropylamine salt of *N*-(phosphonomethyl)glycine) based on the accumulation of shikimic acid g^{-1} of FW in leaves of 18-day-old oil seed rape plants with only two mature leaves 5 h after spraying. Dots are mean values of three replicates.

and Roundup Bio as it did after 5 h (Table 2). Also, the ED_{50} of Roundup Bio was significantly higher than was the ED_{50} of Glyfos.

The upper limits of shikimic acid accumulation 48 h after spraying were generally lower than expected, compared to results in Table 1. This may reflect different growth conditions in the greenhouse over the summer. This implies that results from different experiments are not comparable in terms of actual measurement, but the relative ranking of formulations stayed the same.

Attempts To Optimize the Assay. Efforts were made to reduce the variation of response curves within dose to significantly distinguish the effect between Glyfos and Roundup Bio 5 h after treatment. Optimizing spray conditions (e.g., changing spray volume, droplet size, or stabilizing leaf angle), to try reducing variation originating from different retention and absorption, did not pay off. Changing sampling procedure, by including only leaf plate by cutting away the midvein of the leaf, changing the development stage of the plants to the stage with only two mature leaves, and pooling these leaves before extraction had, however, some effect

Table 5. Regression Parameters from Visual Assessment of Plant Death Caused by Four Different Glyphosate Formulations 14 Days after Spraying 3-Week-Old Oil Seed Rape Plants[a]

glyphosate source	*b*	ED_{50}, g of a.i. ha^{-1}
IPA	−1.59	374 (290−458)
Roundup Bio	−3.58	118 (104−132)
Roundup Ultra	−2.52	190 (164−216)
Glyfos	−4.32	96 (87−104)

[a] 95% Confidence intervals in parentheses. The four curves all had the same upper limit (C = 92% plant death, s = 3).

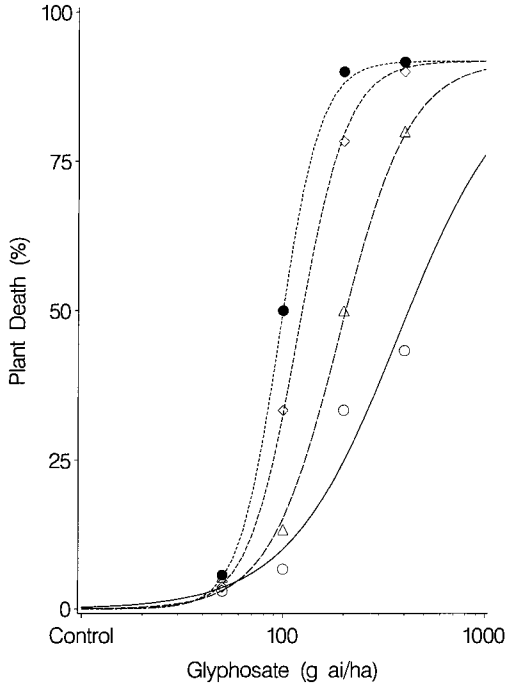

Figure 7. Dose−response curves of (from the top of the graph) Glyfos (●), Roundup Bio (◇), Roundup Ultra (△), and the pure IPA (○) (isopropylamine salt of *N*-(phosphonomethyl)glycine) based on visual assessment of plant death 14 days after spraying 3-week-old oil seed rape plants. Dots are mean values of three replicates.

(Table 4 and Figure 6). Now the ED_{50} estimates of glyphosate preparations were significantly different and with the very same ranking of compounds as in Table 2.

Thus, changing the sampling procedure paid off by giving significantly different separation of ED_{50} estimates and an additional quality check on data in that we could now assume similar curves for the upper limit, C, and the slope, b, were the same for all curves. As pointed out elsewhere (Streibig et al., 1993) the assumption of similar curves (also called parallel) is a necessary but not a sufficient condition for assuming the same mode of action of the compounds.

Visual Assessment. To validate the glyphosate screening technique, we must ensure that the ranking of ED_{50} values with shikimic acid response is the same as achieved by visual assessment after 14 days. As seen in Table 5 and Figure 6 the ranking does not change which supports the hypothesis that shikimic acid is a viable alternative to the slower visual assessment method. Not only is time after spraying reduced to 5 h, but secondary effects are eliminated, especially attacks or invasion of fungi or other pathogens which may weaken plants and make them more susceptible. These effects may be disguised by the herbicide effect.

CONCLUDING REMARKS

PAL activity was reduced 24 and 48 h after spraying different doses of glyphosate, and the effect of doses was significant, but results could not be explained by either a linear or a nonlinear logistic model (eq 1). Therefore, this secondary effect of glyphosate cannot easily be used for evaluation of different formulations. Accumulation of shikimic acid, on the other hand, was related to the dose of glyphosate by a nonlinear logistic dose−response model (eq 1) as early as 5 h after spraying. This relationship was utilized for evaluation of different glyphosate formulations and gave an extremely good indication of the relative strength of the evaluated products.

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LITERATURE CITED

Amrhein, N.; Deus, B.; Gehrke, P.; Steinrücken, H. C. The site of the inhibition of the shikimate pathway by glyphosate. *Plant Physiol.* **1980**, *66*, 830−834.

Becerril, J. M.; Duke, S. O.; Lydon, J. Glyphosate effects on shikimate pathway products in leaves and flowers of velvetleaf. *Phytochemistry* **1989**, *28*, 695−699.

Berlin, J.; White, L. Effects of glyphosate on shikimic acid accumulation in tobacco cell cultures with low and high yields of cinnamoyl putrescines. *Z. Naturforsch. C Biosci.* **1981**, *36*, 210−215.

Bradford, M. M. A rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Burton, J. D.; Nelson, B. E. Glyphosate uptake by suspension-cultured potato (*Solanum tuberosum and S. brevidens*) cells. *Weed Sci.* **1988**, *36*, 146−153.

Carroll, R. J.; Ruppert, D. *Transformation and Weighting in Regression*; Chapman and Hall: New York, 1988; p 249.

Coupland, D. Metabolism of glyphosate in plants. In *The Herbicide Glyphosate*; Grossbard, E., Atkinson, D., Eds.; Butterworth: London, 1985; pp 25−34.

Denis, M. L.; Delrot, S. Carrier-mediated uptake of glyphosate in broad bean (*Vicia faba*) via a phosphate transporter. *Physiol. Plant.* **1993**, *87*, 569−575.

Duke, S. O. Glyphosate. In *Herbicides: Chemistry, Degradation and Mode of Action*; Kearney, P. C., Kaufman, D. D., Eds.; Dekker: New York, 1988; pp 1−70.

Duke, S. O.; Hoagland, R. E. Effects of glyphosate on the metabolism of phenolic compounds I. Induction of phenylalanine ammonia-lyase in dark-grown maize roots. *Plant Sci. Lett.* **1978**, *11*, 185−190.

Duke, S. O.; Hoagland, R. E. Effects of glyphosate on metabolism of phenolic compounds. In *The Herbicide Glyphosate*; Grossbard, E., Atkinson, D., Eds.; Butterworth: London, 1985; pp 75−91.

Duke, S. O.; Hoagland, R. E.; Elmore, C. D. Effects of glyphosate on metabolism of phenolic compounds. IV. Phenylalanine ammonia-lyase activity, free amino acids, and soluble hydroxyphenolic compounds in axes of light-grown soybeans. *Physiol. Plant.* **1979**, *46*, 307−317.

Duke, S. O.; Hoagland, R. E.; Elmore, C. D. Effects of glyphosate on metabolism of phenolic compounds. V. L-α-Aminooxy-β-phenylpropionic acid and glyphosate effects on

phenylalanine ammonia-lyase in soybean seedlings. *Plant Physiol*. **1980**, *65*, 17−21.

Engelsma, G. Inhibition of phenylalanine ammonia-lyase by cinnamic acid derivatives. In *Regulation of Secondary Product and Plant Hormone Metabolism*; Luckner, M., Schreiber, K., Eds.; 55 Symposium S8, 12th FEBS Meeting Dresden; Pergamon Press: Oxford, 1979; pp 163−172.

Franz, J. E.; Mao, M. K.; Sikorski, J. A. *Glyphosate a Unique Global Herbicide*; American Chemical Society: Washington, DC, 1997; p 653.

Hoagland, R. E. Interaction of indoleacetic acid and glyphosate on phenolic metabolism in soybeans. *Pestic. Biochem. Physiol*. **1990**, *36*, 68−75.

Hoagland, R. E.; Duke, S. O.; Elmore, D. C. Effects of glyphosate on metabolism of phenolic compounds. III. Phenylalanine ammonia-lyase activity, free amino acids, soluble proteins and hydroxyphenolic compounds in axes of dark-grown soybeans. *Physiol. Plant*. **1979**, *46*, 357−366.

Holländer, H.; Amrhein, N. The site of the inhibition of the shikimate pathway by glyphosate. I. Inhibition by glyphosate of phenylpropanoid synthesis in buckwheat (*Fagopyrum esculentum* Moench). *Plant Physiol*. **1980**, *66*, 823−829.

Holländer-Czytko, H.; Amrhein, N. Subcellular compartmentation of shikimic acid and phenylalanine in buckwheat cell suspension cultures grown in the presence of shikimate pathway inhibitors. *Plant Sci. Lett*. **1983**, *29*, 89−96.

Ishikura, N.; Takeshima, Y. Effects of glyphosate on caffeic acid metabolism in *Perilla* cell suspensions cultures. *Plant Cell Physiol*. **1984**, *25*, 185−189.

Ishikura, N.; Teramoto, S.; Takeshima, Y.; Mitsui, S. Effects of glyphosate on the shikimate pathway and regulation of phenylalanine ammonia-lyase in *Cryptomeria* and *Perilla* cell suspension cultures. *Plant Cell Physiol*. **1986**, *27*, 677−684.

Jensen, R. A. The shikimate arogenate pathway: Link between carbohydrate metabolism and secondary metabolism. *Physiol. Plant*. **1985**, *66*, 164−168.

Lin, Q.; Northcote, D. H. Expression of phenylalanine ammonia-lyase gene during tracheary-elements differentiation from cultured mesophyll cells of *Zinnia elegans* L. *Planta* **1990**, *182*, 591−598.

Lourtie, E.; Bonnet, M.; Bosschaert, L. New glyphosate screening technique by infrared thermometry. Fourth International Symposium on Adjuvant Agrochemistry, Melbourne, Australia, 1995; pp 297−302.

Lydon, J.; Duke, S. O. Glyphosate induction of elevated levels of hydroxybenzoic acids in higher plants. *J. Agric. Food Chem*. **1988**, *36*, 813−818.

Madsen, K. H.; Heitholt, J. J.; Duke, S. O.; Smeda, R. J.; Streibig, J. C. Photosynthetic parameters in glyphosate-treated sugarbeet (*Beta vulgaris* L.). *Weed Res*. **1995**, *35*, 81−88.

Miller, G. M.; St. John, J. B. Membrane-surfactant interactions in lipid micelles labeled with 1-anilino-8-naphthalenesulfonate. *Plant Physiol*. **1974**, *54*, 527−531.

Mollenhauer, C.; Smart, C. C.; Amrhein, N. Glyphosate toxicity in the shoot apical region of the tomato plant. I. Plastid swelling is the initial ultrastructural feature following *in vivo* inhibition of 5-enolpyruvylshikimic acid 3-phosphate synthase. *Pestic. Biochem. Physiol*. **1987**, *29*, 55−65.

Nilsson, G. Effects of glyphosate on the amino acid content in spring wheat plants. *Swedish J. Agric. Res*. **1977**, *7*, 153−157.

Riechers, D. E.; Wax, L. M.; Liebl, R. A.; Bush, D. R. Surfactant-increased glyphosate uptake into plasma membrane vesicles isolated from common lambsquarters leaves. *Plant Physiol*. **1994**, *105*, 1419−1425.

Rubin, J. L.; Gaines, C. G.; Jensen, R. A. Enzymological basis for the herbicidal action of glyphosate. *Plant Physiol*. **1982**, *70*, 833−839.

Røyneberg, T.; Balke, N. E.; Lund-Høie, K. Effects of adjuvants and temperature on glyphosate absorption by cultured cells of velvetleaf (*Abutilon theophrasti* Medic.). *Weed Res*. **1992**, *32*, 419−428.

Schulz, A.; Munder, T.; Holländer-Czytko, H.; Amrhein, N. Glyphosate transport and early effects on shikimate metabolism and its compartmentation in sink leaves of tomato and spinach plants. *Z. Naturforsch*. **1990**, *45*, 529−534.

Singh, B. K.; Shaner, D. L. Changes in free amino acid pools can predict the mode of action of herbicides. *Pestic. Sci*. **1995**, *43*, 221−225.

Smith, E. A.; Oehme, F. W. The biological activity of glyphosate to plants and animals: A literature Review. *Vet. Hum. Toxicol*. **1992**, *34*, 531−543.

Stasiak, M. A.; Hofstra, G.; Fletcher, R. A. Physiological changes in birch seedlings by sublethal applications of glyphosate. *Can. J. For. Res*. **1992**, *22*, 812−817.

Steinrücken, H. C.; Amrhein, N. The herbicide glyphosate is a potent inhibitor of 5-enolpyrovyl-shikimic acid 3-phosphate synthase. *Biochem. Biophys. Res. Commun*. **1980**, *94*, 1207−1212.

Streibig, J. C. Herbicide bioassay. *Weed Res*. **1988**, *28*, 479−484.

Streibig, J. C.; Rudemo, M.; Jensen, J. E. Dose−response curves and statistical models. In *Herbicide Bioassays*; Streibig, J. C., Kudsk, P., Eds.; CRC Press: Boca Raton, FL, 1993; pp 29−55.

Wan Kim, T.; Amrhein, N. Glyphosate toxicity: Long-term analysis of shikimic acid accumulation and chlorophyll degradation in tomato plants. *Kor. J. Weed Sci*. **1995**, *15*, 141−147.

Watson, M. C.; Bartels, P. G.; Hamilton, K. C. Action of selected herbicides and Tween 20 on oat (*Avena sativa*) membranes. *Weed Sci*. **1980**, *28*, 122−127.

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