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GLYPHOSATE EFFECTS ON SHIKIMATE PATHWAY PRODUCTS IN LEAVES AND FLOWERS OF VELVETLEAF

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Key Word Index—*Abutilon theophrasti*; Malvaceae; benzoic acids; cinnamic acids; glyphosate; herbicide; *N*-(phosphonomethyl)glycine; shikimate pathway.

Abstract—The effects of sublethal treatments of the herbicide glyphosate [*N*-(phosphonomethyl)glycine] on the accumulation of shikimate, hydroxybenzoic acids, and hydroxycinnamic acids in *Abutilon theophrasti* (velvetleaf) were measured. In newly developing tissues (both leaf and flower), the concentrations of shikimate and protocatechuate were greatly increased by the herbicide, whereas hydroxybenzaldehyde and *p*-coumarate levels were decreased. During recovery from glyphosate treatment there was a rapid loss of protocatechuate and the levels of other phenolic acids rapidly returned to normal. These data indicate that phenolic acid pools in higher plants are regulated and that simple phenolic acids do not accumulate as end products.

INTRODUCTION

The herbicide glyphosate acts by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19), thus blocking the shikimate pathway [1, 2]. This blockage causes a reduction in synthesis of aromatic amino acids, cinnamate, and all cinnamate-derived phenolic compounds [2, 3]. Pools of aromatic amino acids are apparently further reduced by increased phenylalanine ammonia-lyase (EC 4.3.1.5) activity in glyphosate-treated tissues [3, 4]. Blockage of the shikimate pathway also results in the accumulation of high levels of shikimate [2, 5, 6]. This accumulation is exacerbated by the loss of feedback control of the pathway and unregulated carbon flow into this pathway [7, 8]. Certain benzoic acids that are derived primarily from shikimate rather than cinnamate also accumulate in glyphosate-treated tissues [2, 3, 5, 9, 10]. Thus, the mechanism of development of phytotoxic symptoms is complicated, due to depletion of aromatic amino acids, unregulated drain of carbon into the shikimate pathway, and greatly disrupted secondary metabolism.

Glyphosate-treated yellow nutsedge (*Cyperus esculentus* L.) leaves accumulated high levels of gentisic acid and, to a lesser extent, salicylic and vanillic acids 14 days after treatment [9]. However, in other studies in which leaf tissues of several species were harvested six days after treatment or earlier, the major hydroxybenzoic acids found to accumulate were protocatechuic and gallic acids [5, 10]. These results suggest that gallate and protocatechuate, both relatively simple hydroxybenzoic acids, accumulate early after glyphosate treatment, and that more complex derivatives such as gentisate, vanillate, and salicylate accumulate later. One objective of this study was to observe over an extended time this hypothetical shift in hydroxybenzoic acid content in tissues exposed to sublethal levels of glyphosate.

As discussed before [6, 11], the long term effects of sublethal glyphosate exposure on phenolic compound metabolism could have significant ecological and physiological implications. Thus, another objective was to determine the magnitude of effect of a sublethal glyphosate treatment on phenolic compound metabolism after an extended period.

We chose velvetleaf (*Abutilon theophrasti* Medic) for this study for several reasons. It is a major weed problem and any long term effect on this species could be important in many agricultural ecosystems. Furthermore, we have previously demonstrated that it accumulates high levels of both gallate and protocatechuate in response to lethal glyphosate levels [6]. Finally, in previous studies [6], we found that there is a high level of uniformity in response to glyphosate among individual plants of this species.

RESULTS

The effects of glyphosate on the accumulation of cinnamate-derived phenolic acids and benzoic acids in mature, contact-treated leaves (data not shown) were much less than those on young, non-contact-treated, developing leaves and flowers. No shikimate accumulation could be detected in the mature, treated leaf. This is to be expected because glyphosate is readily translocated to metabolic sinks, where is generally has its greatest effects [2, 12]. Therefore, we report only the data from non-contact treated, developing leaves and flowers. The 5 mM glyphosate treatment caused some observable phytoxicity (mild chlorosis), however, plant growth was

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not significantly altered. The 1 mM glyphosate treatment caused no observable symptoms.

Caffeate, *p*-coumarate, and ferulate were the only cinnamate-derived phenolic acids that were detectable in the extracts by our methods. In addition to the cinnamate-derived acids found in the treated leaves, *p*-hydroxybenzaldehyde was detected in the young, non-treated developing leaves. The tissue concentrations of *p*-hydroxybenzaldehyde were reduced *ca* 50% by both 1 and 5 mM glyphosate treatments after six days, but there was no effect at 12 or 18 days (Fig. 1a). Caffeate concentrations were not affected at any sampling time at either glyphosate concentration (Fig. 1b). The highest concentration of glyphosate reduced *p*-coumarate concentrations at all sampling times, however, the degree of effect decreased with time (Fig. 1c). On a dry weight basis, the concentrations of hydroxybenzaldehyde and coumarate in control tissues decreased during the course of the experiment. The observed reductions were somewhat less on a per leaf basis (data not shown) due to growth during the experiment. However, expressing the data on a per leaf basis would be difficult to interpret because leaves at several states of development were pooled at each sampling time. Ferulate concentrations were unaffected by glyphosate treatments in these tissues after six days, however, there was an increase in concentration at 12 and 18 days with the 5 mM treatment (Fig. 1d).

Vanillin and syringylaldehyde were detectable in leaves at low levels after six days of glyphosate exposure. The vanillin contents of the control, 1 mM glyphosate and 5 mM glyphosate treatments were 0.111 ± 0.005, 0.112 ± 0.001 and 0.085 ± 0.003 μmol/g dry wt, respectively. The syringylaldehyde contents of the same treatments were 0.261 ± 0.008, 0.266 ± 0.020 and 0.129 ± 0.004 μmol/g dry wt, respectively. Cinnamate was not detectable in any samples.

Gallate concentrations were increased *ca* two-fold over control levels by the 5 mM glyphosate treatment at all sampling times in developing leaves (Fig. 2a). The 1 mM glyphosate treatment caused only a barely detectable increase at 18 days in this tissue. There was no effect of the 1 mM glyphosate treatment on protocatechuate (Fig. 2b). However, protocatechuate levels were *ca* 10-fold higher than those of the control in the 5 mM treatment at six days. The effect was greatly attenuated at 12 and 18 days, but was still highly significant.

Shikimate was not detected in the leaves of non-treated control or 1 mM glyphosate treatments (Fig. 2c). However, a high level of shikimate was detected in the 5 mM glyphosate treatments at six days after treatment. The shikimate levels in this treatment were much lower after 12 and 18 days, however, shikimate was still readily detectable. In the untreated leaves, the pattern of shikimate changes was virtually identical to that of protocatechuate (Fig. 2b).

Both control and glyphosate-treated plants flowered during the course of the experiment. Flowers were harvested 18 days after treatment and analysed for shikimate, benzoates, and cinnamate-derived phenolic acids. The concentrations of caffeic, *p*-coumaric, and ferulic acid were decreased by 75, 56 and 39%, respectively by the 5 mM glyphosate treatment (Fig. 3a). The levels of extractable *p*-coumarate in the flowers were more than five-fold higher than those of caffeic or ferulic acid in

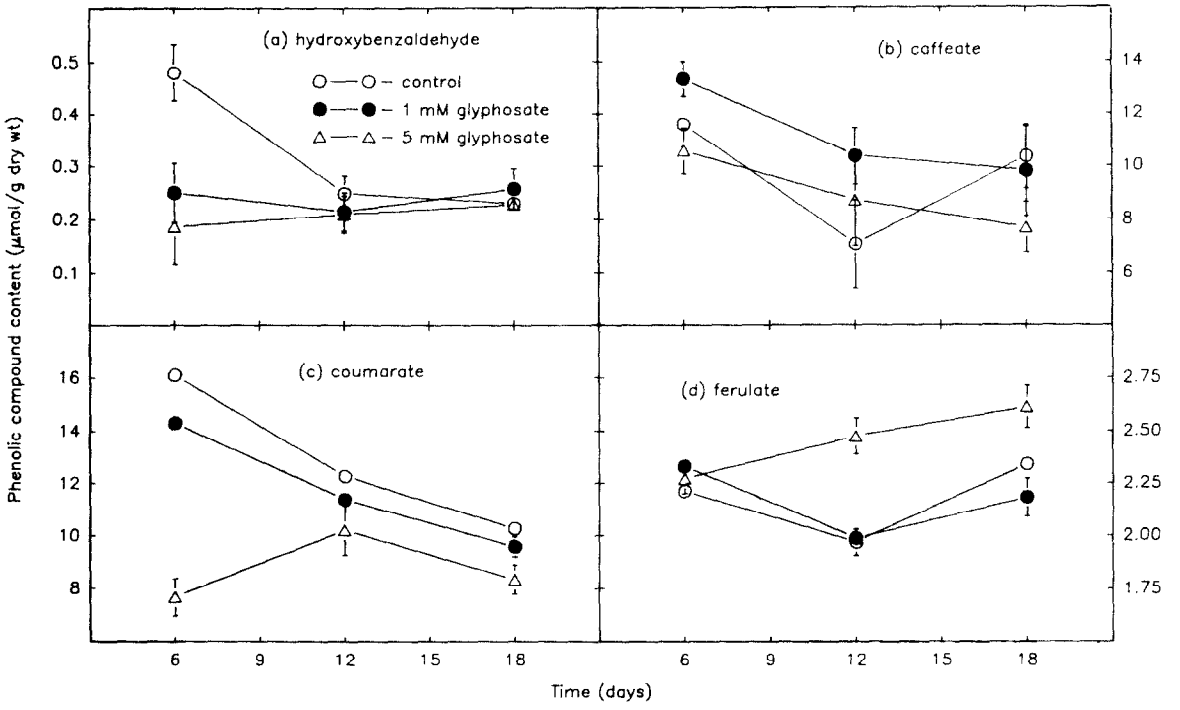

Fig. 1. Effects of glyphosate on *p*-hydroxybenzaldehyde and cinnamate-derived phenolic acid content in young, untreated leaves of plants to which 0.1 ml (1 or 5 mM) glyphosate solution was applied to lower, fully mature leaves at 6, 12 or 18 days before samples were taken. Control plants received no glyphosate. Error bars represent the s.e.s of the means. Where errors bars are not shown, they are obscured by the data symbols.

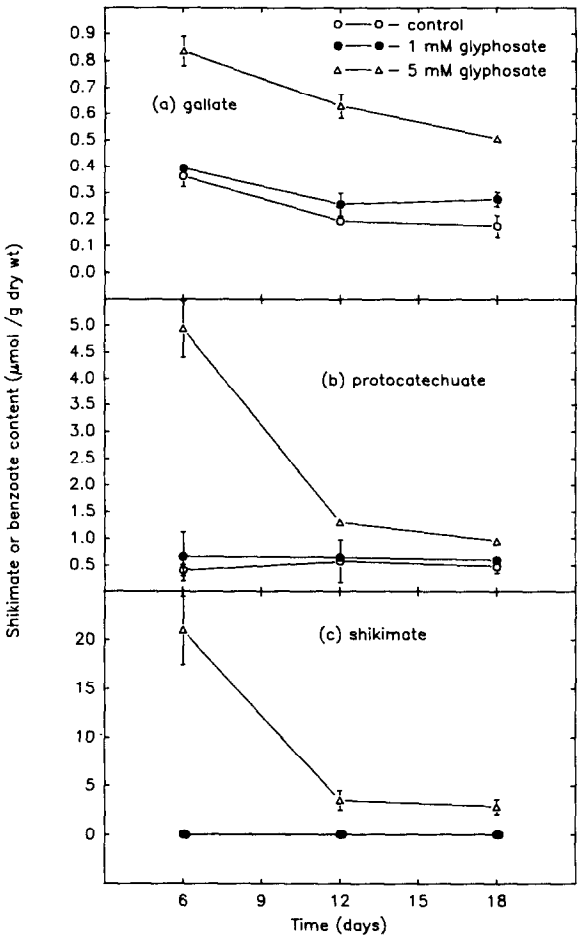

Fig. 2. Effects of glyphosate on benzoic and shikimic acid contents in young, untreated leaves of plants to which 0.1 ml (1 to 5 mM) glyphosate solution was applied to lower, fully mature leaves at 6, 12 or 18 days before samples were taken. Control plants received no glyphosate. Error bars represent the s.e.s of the means. Where error bars are not shown, they are obscured by data symbols.

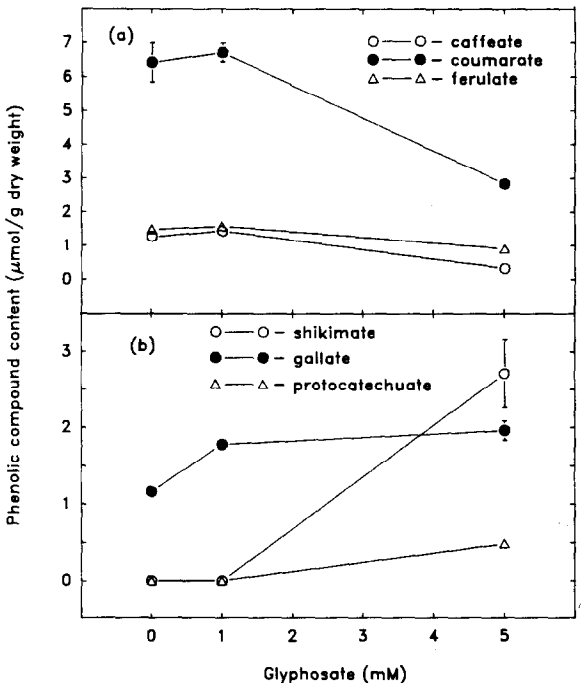

Fig. 3. Effects of glyphosate on phenolic compound contents of flowers from plants to which 0.1 ml (1 or 5 mM) glyphosate solution was applied to lower, fully mature leaves 18 days before samples were taken. Control plants received no glyphosate. Error bars represent the s.e.s of the means. Where error bars are not shown, they are obscured by data symbols.

control tissues. No shikimate or protocatechuate was detectable in the control or 1 mM glyphosate treatment flowers. However, both compounds were readily detectable in the 5 mM treatment (Fig. 3b). Gallate was detected in all treatments, but its level was increased by about 50% by both glyphosate treatments. There were no significant differences between the concentrations of vanillic and syringic acids in any of the treatments (data not shown). The level of vanillic and syringic acids in the flowers was 725 ± 18 and 273 ± 12 nmol/g dry wt, respectively.

DISCUSSION

In an earlier study, we examined the effects of a very phytotoxic and eventually lethal dose of glyphosate (10 mM) on shikimate and hydroxybenzoic acids, but not on cinnamic acids, in young velvetleaf plants [6]. Sampling was done only at six days after glyphosate treatment in the previous study. The 5 mM treatment in the present study caused only 10% as much shikimate to accumulate in non-treated leaves as the 10 mM treatment of the previous study six days after treatment. In the previous study, 10 mM glyphosate caused 5- and 60-fold increases in gallate and protocatechuate, respectively, whereas, in the present study, the increases were 2.3- and 12-fold, respectively. These results are consistent, in that protocatechuate was influenced much more than was gallate by both treatments.

Glyphosate is not metabolized by higher plants and does not degrade by physical mechanisms in growing plants [2]. Thus, recovery from glyphosate exposure is probably due to exudation of the compound from roots [e.g. 13], sequestration of this water-soluble ion in the vacuole, and dilution of the compound by plant growth. Recovery of these plants from glyphosate after day six of treatment was reflected by the decrease of shikimate and protocatechuate in the non-treated leaves. At days 12 and 18, the effects of 5 mM glyphosate on gallate and protocatechuate were similar in magnitude. Therefore, the high levels of protocatechuate that were observed at day six were not later reflected in gallate levels, even though protocatechuate is a precursor of gallate. Furthermore, there were no effects of glyphosate on any other, more complex benzoates such as vanillic acid, the methylated form of protocatechuic acid. These data suggest that gallate levels are more strongly regulated by the plant than are those of shikimate and protocatechuate (its immediate precursors) and that benzoates found later in the benzoic acid pathway are very strongly regulated as well.

Because *p*-hydroxybenzaldehyde is a benzoic acid derivative [14], one might expect its levels to be elevated by glyphosate. However, *p*-hydroxybenzaldehyde is also known to be a hydrolysis product of dhurrin, the glucosylated form of *p*-hydroxy-(*S*)-mandelonitrile, a derivative of tyrosine [15]; and, to our knowledge, dhurrin has not been looked for in velvetleaf. If velvetleaf synthesizes dhurrin, our finding that glyphosate reduces levels of this compound is consistent with the other data on cinnamate-derived compounds. However, this does not explain the reductions in vanillin and syringylaldehyde, two other benzoic acid derivatives. All three of these benzoic acid derivatives are hydrolysis products of lignin [16]. Glyphosate has been reported to reduce lignification [10] due to inhibition of the synthesis of the cinnamate constituents of lignin. Our results are consistent with a reduction in lignin formation in velvetleaf by glyphosate. Thus, the large initial pools of shikimate and protocatechate are probably not eventually converted to lignin. Another possibility is that these complex benzoates are derivatives of cinnamate. In some species, benzoic acids have been found to originate from cinnamate by β-oxidation of the appropriate cinnamate-derived phenolic compound, as well as from shikimate [17].

The magnitude of effect of glyphosate on cinnamate-derived phenolic acids was generally less than that on benzoic acids. In the non-treated leaf, only reductions in *p*-coumarate could be detected at day six and little effect on this compound could be measured after this time. Increases in ferulate could be detected at days 12 and 18 in the 5 mM treatment. This could have been due to a decrease in lignin synthesis due to a factor other than substrate limitation. For example, peroxidase is required for lignin synthesis and a form of peroxidase (IAA oxidase) has been demonstrated to be inhibited by benzoates accumulated in extracts of plants that had been treated with glyphosate [18]. This increase might also be explained by Laanest's [19] recent finding that glyphosate strongly inhibits flavonoid synthesis in the presence of adequate levels of exogenously supplied aromatic amino acids. Thus, glyphosate may inhibit conversion of cinnamate-derived aromatic acids to more complex phenolic compounds.

In the flower, *p*-coumarate was the most strongly affected cinnamate-derived phenolic acid. However, significant reductions in all of the cinnamate-derived phenolic acids could be detected. This tissue is probably simpler, because accumulation patterns are not complicated by the synthesis of tannins or lignins. The loss of cinnamate-derived phenolic acids in the flower was approximately equal on a molar basis to the increase in shikimate and hydroxybenzoates measured. The flower may have been more strongly affected than the developing leaves because it never develops the capacity to export glyphosate. Because glyphosate is translocated with sucrose [2, 12], as leaves develop photosynthate exporting capacity, they probably export glyphosate that accumulated when they were metabolic sinks. Non-exporting organs such as flowers or fruits probably become terminal sites of accumulation of glyphosate.

One would not expect to find a stoichiometric relationship between reductions in cinnamate-derived phenolic acids and the increase in shikimate and hydroxybenzoates for two reasons. First, blockage of the shikimate pathway deregulates the modulation of carbon flow into the pathway [7]. Second, although our extractions were close to complete, we may not have measured the content of more complex phenolic compounds derived from hydroxybenzoates (e.g., certain tannins) and cinnamates (e.g., flavonoids). Nevertheless, the relative effects of glyphosate on the size of various phenolic pools is an indicator of the degree of regulation to which these pools are subject; and they demonstrate the relative efficiency of each of these pools as indicators of glyphosate effects at various times after a non-lethal dose of the herbicide.

In summary, our results demonstrate that sublethal glyphosate treatments do not have long-lasting effects on the levels of simple organic phenolic acids in higher plants. Soon after glyphosate treatment, effects on hydroxybenzoic acids are much more dramatic than effects on cinnamate-derived phenolic acids. However, even these effects are transitory and simple hydroxybenzoic acids are not terminal compounds for carbon accumulation in recovering plants.

EXPERIMENTAL

Plant material. Seeds of velvetleaf were planted in 1.2-l pots containing 2/3 Jiffy-mix (Ball Jiffy) and 1/3 perlite–vermiculite and watered every other day with a soln of Peters 20–20–20 (N-P-K), general purpose fertilizer (0.25g/l) or H_2O. The plants were grown in a greenhouse in which the temperature varied from 20 to 30°. When the plants were 25-days-old, they were treated with 0, 1 and 5 mM glyphosate (isopropylamine salt) in 0.1% Triton X-100 by applying a 0.1 ml drop of the treatment soln over the entire adaxial surface of the fourth leaf from the apex.

Leaves were harvested at 6, 12 and 18 days after treatment. When harvested, leaves were separated into treated leaves that received the treatment soln and non-treated leaves from above the fourth leaf of the plants. All samples were triplicated. At harvest, leaves were dipped into liquid N_2, lyophilized, and stored at −20° over silica gel.

Extraction of hydroxybenzoic and cinnamic acids. Hydroxybenzoic acids were extracted as before [6] by refluxing 150 mg dry wt of tissue in 25 ml 1 M HCl at 100° for 1 hr. Refluxing was interrupted after 30 min to sonicate for 5 min and then refluxing resumed. The resulting extract was filtered through Whatman No. 1 paper, the pH adjusted to 2.5 with concd NaOH, and the vol. brought to 50 ml with deionized H_2O. A 2 ml aliquot was filtered through a 0.2 μm PTFE syringe filter and analysed by HPLC for shikimate. The rest of the extract was washed twice with 25 ml HPLC grade EtOAc, the EtOAc fractions combined, evapd to dryness at 35° with a rotorary evaporator, redissolved in 2 ml 3.3 mM phosphoric acid (pH 2.7), filtered through a 0.2 μm syringe filter and analysed by HPLC for hydroxybenzoic acids.

Cinnamic acids and benzaldehydes were extracted by refluxing 100 mg of dry tissue for 15 min with 25 ml of deoxygenated (with N_2) 1 M NaOH at 100°. Further base hydrolysis of the extract under these conditions resulted in a gradual decomposition of cinnamates with time. The extract was cooled, adjusted to pH 2.5 with concd HCl, centrifuged at 12000 *g* for 20 min and filtered through Whatman No. 1 paper to prevent the formation of an emulsion which formed during liquid–liquid extraction when particulate matter was present. The cinnamic acids and benzaldehydes were extracted with EtOAc following the same procedure as for hydroxybenzoic acids.

HPLC analysis was performed on a [Alltech 250 × 4.6 mm (i.d.)] Spherisorb 5 um ODS-I reversed phase column connected with a Bio-Rad ODS-5S guard column. The HPLC solvent used in the shikimate assay was 3.33 mM phosphoric acid (pH 2.7)

with a flow of 0.6 ml/min for an isocratic run of 10 min. The injection volume was 25 μl.

The solvent gradient for hydroxybenzoic acids was similar to that used previously [6, 20]. It was composed of 3.33 mM phosphoric acid (solvent A, pH 2.7) and HPLC-grade MeOH (solvent B) at a flow rate of 1.6 ml/min. The gradient was as follows: 5% B in A at 0–10 min; a linear transition from 5 to 20% B in A from 10 to 25 min; 20% B in A from 25 to 42 min. The injection volume was 25 μl.

Cinnamic acids and benzaldehydes were assayed using the same solvents described above for benzoic acids at a flow rate of 1 ml/min and with the solvent gradient as follows: 30% B in A from 0 to 15 min; a linear transition from 30 to 35% B in A from 15 to 45 min; and 40% B in A from 45 to 60 min. The injection volume was 25 μl. After each sample, the column was washed with MeOH and re-equilibrated with the original soln.

Shikimate detection was performed from 200–280 nm with a photodiode array and the integration detector made at 215 nm. Hydroxybenzoate and cinnamate samples were scanned from 200 to 360 nm and integrations were made at 215 nm for hydroxybenzoates and 290 nm for cinnamates and benzaldehydes. The compounds were identified by comparison of R_t and UV spectral characteristics with those of standards. R_ts (in min) of the compounds analysed were: shikimate, 8.55; *p*-hydroxybenzaldehyde, 11.85; caffeate, 12.87; vanillin, 15.81; syringylaldehyde, 20.10; *p*-coumarate, 22.09; ferulate, 26.82; cinnamate, 53.63; gallate, 9.05; protocatechuate, 16.44; gentisate, 21.69; 4-hydroxybenzoate, 24.09; vanillate, 31.54; and syringate, 39.79.

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