Production of Hydroxybenzoic Acids by *Bradyrhizobium japonicum* Strains after Treatment with Glyphosate

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Glyphosate [*N*-(phosphonomethyl)glycine] effects on the growth and metabolism of the nitrogen-fixing soybean (*Glycine max*) symbiont *Bradyrhizobium japonicum* were investigated. In higher plants glyphosate inhibits the shikimic acid pathway, causing accumulations of shikimic acid and certain benzoic acids, such as protocatechuic (PCA) and gallic acid. We sought to determine whether glyphosate also causes the accumulation of shikimic acid-derived benzoic acids in *B. japonicum*. Three strains (USDA 110, 123, and 138) were grown in defined media lacking aromatic amino acids. Glyphosate at concentrations of 0.5 and 1 mM was inhibitory but not lethal. Cell death occurred in response to 5 mM glyphosate, and rapid death was observed at 10 mM. Accumulations of 1 mM PCA were observed in the culture media of strains 123 and 138. Only trace amounts of PCA accumulated in untreated controls or in strain 110 cultures. All strains were able to metabolize PCA in the absence of glyphosate. The high levels of PCA produced by *B. japonicum* strains may alter the symbiotic interactions between this bacterium and glyphosate-resistant varieties of soybean.

INTRODUCTION

Glyphosate [*N*-(phosphonomethyl)glycine] is an effective nonselective herbicide that inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Duke, 1988). Aromatic amino acid synthesis is blocked as a consequence of EPSP synthase inhibition. The shikimate pathway becomes deregulated in some organisms, resulting in the uncontrolled flow of carbon in this pathway (Jensen, 1986). Massive amounts of shikimate, a substrate of EPSP synthase, accumulate in glyphosate-treated plant tissues [e.g., Amrhein et al. (1980)] and microorganisms (Amrhein et al., 1983; Bode et al., 1984). Elevated concentrations of shikimate-derived benzoic acids such as protocatechuate and gallate have been found in plants following treatment with glyphosate (Becerril et al., 1989; Cãnal et al., 1987; Lydon and Duke, 1988).

Glyphosate-resistant crop plants, such as soybean, are currently being developed using biotechnology that relies on the introduction of genes coding for resistant forms of EPSP synthase (Duke et al., 1991; Hinchee et al., 1988; Kishore and Shah, 1988; Shah et al., 1986). The introduction of this type of glyphosate resistance may have unforseen consequences for the symbiotic microorganisms associated with soybeans. Glyphosate is translocated readily to metabolic sinks (Honneger et al., 1986), where it accumulates because it is not metabolized by higher plants (Duke, 1988). Thus, important metabolic sinks, such as root nodules and the surrounding rhizosphere (Barber and Martin, 1976; Minchin and McNaughton, 1984), of glyphosate-resistant soybeans could be expected to accumulate high levels of herbicide. Low levels of glyphosate have been detected in the rhizosphere exudate of treated plants (Coupland and Caseley, 1979; Rodrigues et al., 1982). Significant amounts of hydroxybenzoic acids may also accumulate in these sinks in response to the high concentrations of glyphosate. In addition, the initial steps of the nodulation process appear to involve bacterial recognition of plant-produced flavonoid compounds that are derived from the shikimic acid pathway (Nap and Bisseling, 1990). Subsequent bacteroid development for N_2 fixation requires considerable protein synthesis by the bacterium. These processes could be affected by the accumulation of glyphosate and/or hydroxybenzoic acids.

Bradyrhizobium japonicum is sensitive to glyphosate when grown in media free of aromatic amino acids (Jaworski, 1972) and would be expected to respond in the same manner to glyphosate translocated by a resistant plant. Nothing is known of the effects of glyphosate on shikimate and shikimate-derived benzoic acids in *Bradyrhizobium*. Therefore, the effects of sublethal concentrations of glyphosate on the growth and synthesis of shikimic acid (SA) and protochatechuic acid (PCA) in three strains of *Bradyrhizobium* were determined.

MATERIALS AND METHODS

Bacterial Culture. *B. japonicum* strains USDA 110, USDA 123, and USDA 138 were originally obtained from the USDA Nitrogen Fixation Laboratory culture collection in Beltsville, MD. Strains were maintained on yeast-extract mannitol (YEM) slants. Cells for experiments were grown in a buffered (1.3 g/L HEPES and 1.1 g/L MES) basal salts medium (Cole and Elkan, 1973) containing additional growth factors (designated MSR medium). The growth factors used in MSR, mannitol (25 mM), glutamine (2 mM), biotin (5 mg/L), and thiamin hydrochloride (125 mg/L), were filter-sterilized and added to the autoclaved basal salts media. Cultures were incubated at 25 °C on a rotary shaker in darkness. Bacteria were grown for 4–5 days in 500 mL of MSR, at which time cultures were in late-lag phase. Cells were centrifuged at 10000*g*, washed once with sterile 0.0125 M phosphate buffer (pH 7), and then suspended in 100 mL of buffer. Cell numbers were determined using plate-count procedures on YEM agar amended with Congo Red for detection of potential contaminants. Inoculum was stored at 4 °C until just before use.

Dose–Response Experiments. Strains were inoculated into 100 mL of MSR medium in Erlenmeyer flasks at a level of approximately 10^7 cells/mL. Initial populations were verified

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by plate counts. This relatively high level of inoculum was chosen to enhance the detection of hydroxybenzoic acids. Filter-sterilized solutions of the isopropyl amine salt of glyphosate (97% purity, Chem Services, West Chester, PA) were added to MSR to obtain concentrations of 0, 0.5, 1, and 5 mM. Three replicate flasks from each glyphosate level were removed from the shaker at 1, 2, and 3 days after inoculation. Cell numbers were determined by serial dilutions onto YEM agar, and the remainder of each culture was frozen until analyzed for PCA or SA.

Shikimate Production. Initial dose–response experiments showed shikimic acid levels to be low in glyphosate-treated cultures. After development of a more sensitive analysis for SA, an additional experiment was performed. Cultures of strains 138 and 110 were prepared as described above with the glyphosate added at 1 mM. Cell numbers were followed over a 4-day period using the methods described above and the concentrations of SA determined by HPLC.

Protocatechuate Degradation. A filter-sterilized solution of PCA (Sigma Chemical Co., St. Louis, MO) was added to 100 mL of MSR to obtain a concentration of 1 mM and inoculated with each strain at approximately 10^7 cells/mL. Three replicate cultures of each strain were analyzed for PCA concentration daily for 4 days.

Analytical Methods. Analysis for PCA was performed according to the methods of Lydon and Duke (1988) with modifications. The cultures (cells and medium) were brought to 1 N by adding concentrated HCl and refluxed for 1 h at 100 °C. PCA was extracted with ethyl acetate, concentrated, and prepared for HPLC. Gradient HPLC analyses for PCA and *p*-hydroxybenzoic, gallic, gentisic, vanillic, and syringic acids were made using a programmable UV detector with external standards.

Shikimic acid was extracted by refluxing the culture for 1 h at 100 °C followed by filtration and evaporation to dryness. The residue was sonicated in 20 mL of MeOH for 5 min and centrifuged at 30000*g* for 10 min. The supernatant was evaporated to dryness and the residue resuspended in 2 mL of pH 2.7 phosphoric acid. Shikimate was analyzed by HPLC with a Bio-Rad (Richmond, CA) Aminex HRX-87H column (300 × 7.8 mm i.d.) and a photodiode array detector under isocratic conditions. Phosphoric acid (pH 2, 24 mM) was the mobile phase, and the flow rate was 0.6 mL/min. The immediate substrate of EPSP synthase, shikimate 3-phosphate, was hydrolyzed to shikimic acid in this procedure.

Reversal Experiments. Experiments were undertaken to reverse the effects of glyphosate by the addition of the amino acids L-tryptophan, L-phenylalanine, and L-tyrosine (Sigma) to cultures of strain 138. Aqueous filter-sterilized solutions of these amino acids were added to 100 mL of MSR to achieve concentrations of 0.25 mM. Equivalent amounts of sterile water were added in place of the amino acid solutions to media not receiving glyphosate. Glyphosate was added as previously described at concentrations of 1 and 2 mM. Cultures were incubated for 3 days and populations determined as described previously.

RESULTS

Growth of all three *B. japonicum* strains was affected by glyphosate at concentrations ranging from 0.5 to 5 mM (Table I). The 5 mM level of glyphosate resulted in a gradual decline in cell numbers, whereas the lower concentrations allowed some growth. Glyphosate at 10 mM resulted in rapid death (<0.1% survival) of all three strains during a 4-day period. The sublethal concentrations of glyphosate (0.5 and 1.0 mM) caused the greatest accumulations of PCA (Figure 1). The pattern of PCA accumulation was similar in the cultures inoculated with strains 123 and 138, but only small amounts of PCA were produced by strain 110. Only minute amounts of PCA were detected in any of the cultures not treated with glyphosate. Other experiments, in which cells were separated by centrifugation and filtration from culture medium treated with 1 mM glyphosate, showed that only a small fraction (<1%) of the PCA was cellular, indicating that PCA was being excreted into the medium (data not shown).

Table I. Effect of Glyphosate on the Growth of *B. japonicum* Strains in a Medium Containing No Aromatic Amino Acids

strain	glyphosate, mM	rate, k, day^{-1}	inhibition, %
110	0	1.766 ± 0.099[a]	0
	0.5	1.044 ± 0.040	41
	1.0	0.938 ± 0.055	47
	5.0	−0.221 ± 0.182	100
123	0	1.063 ± 0.181	0
	0.5	0.955 ± 0.112	10
	1.0	0.936 ± 0.125	12
	5.0	−0.684 ± 0.254	100
138	0	1.075 ± 0.146	0
	0.5	0.910 ± 0.129	15
	1.0	0.873 ± 0.165	19
	5.0	−0.899 ± 0.578	100

[a] The specific growth rate (k) was determined by the linear regression $\ln X = \ln X_0 + kt$, where X and X_0 are the populations at time (t) and at the initial time (t_0), respectively. Estimates of k are shown with the standard error of the estimates. Negative values resulted from net population declines during the 3-day incubation. All regressions were significant ($P < 0.05$) with r^2 values ranging from 0.73 to 0.98. Inhibition was calculated from the growth rates as a percentage of the untreated control.

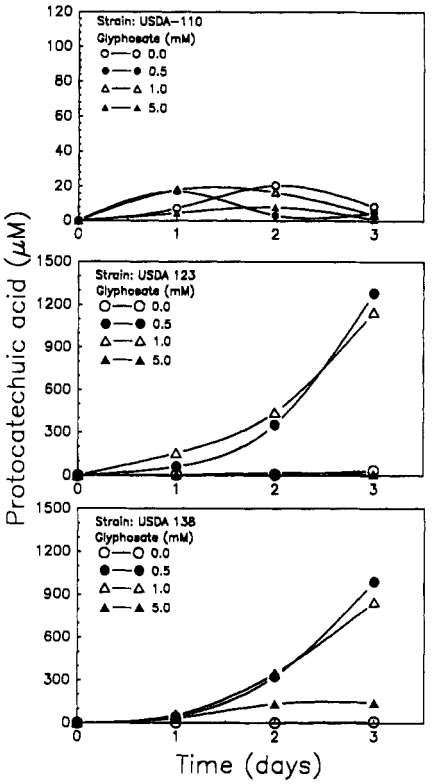

Figure 1. Effects of glyphosate on the accumulation of protocatechuic acid in the culture media of three *B. japonicum* strains. Note the different scales used for strain 110 data.

Shikimic acid levels (combined cells and medium) for strains 110 and 138 were increased by glyphosate when expressed on a per cell basis (Figure 2). However, the concentrations of shikimic acid were substantially lower than those of PCA. This suggests that the shikimate accumulations within the bacteria are reduced by direct conversion into PCA. Alternatively, shikimic acid could be converted to dehydroshikimate and in turn to gallate and then PCA. This latter possibility seems unlikely since HPLC analyses showed that levels of gallate were not elevated in glyphosate-treated cultures. The relative concentrations of *p*-hydroxybenzoic, gentisic, syringic, and

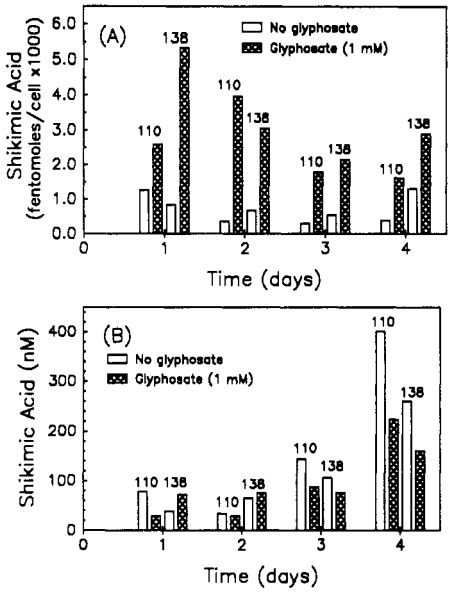

Figure 2. Effect of glyphosate on the accumulation of shikimate, expressed on a per cell basis (A) or as total shikimate accumulated in cells and media (B) by cultures of *B. japonicum* strains 110 and 138.

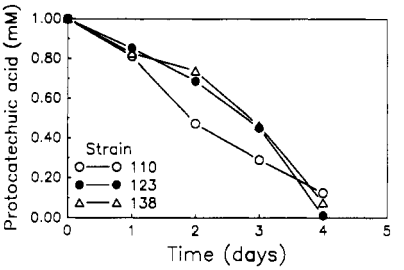

Figure 3. Concentrations of protocatechuic acid extracted from cultures of *B. japonicum* strains inoculated with approximately 10^7 cells/mL of glyphosate-free medium amended with 1 mM protocatechuic acid.

vanillic acids were not changed by treatment with glyphosate (data not shown).

All strains degraded PCA added to MSR broth cultures containing no glyphosate (Figure 3). The degradation rates appear to be approximately equivalent for the three strains. These results agree with previous reports of *Rhizobium* spp. and *Bradyrhizobium* spp. degrading PCA and other hydroxybenzoic acids (Chen et al., 1984; Hussien et al., 1974; Parke and Ornston, 1984).

The addition of the aromatic amino acids tryptophan, tyrosine, and phenylalanine did not reverse the effects of glyphosate on the growth of strain 138 (Table II). Amino acid additions had an inhibitory effect on the growth of strain 138 in the absence of glyphosate, as seen by the 23% reduction in growth rate due to amino acid addition in the absence of glyphosate. Other experiments with higher concentrations of these amino acids were performed (data not shown) with similar results.

DISCUSSION

Assessing the possible effects of glyphosate use in soybeans on *B. japonicum* depends in part on detailed knowledge of the bacterial tolerance to this herbicide. Earlier research by Jaworski (1972) established that growth of *B. japonicum* strain 71 in the presence of 1 mM glyphosate was 9% that of the glyphosate-free control, as determined by optical density after 5 days. The responses of the strains used in these experiments to 1 mM glyphosate are reasonably similar. Our results showed that population declines were induced by 5 mM, and complete death of cultures was induced by 10 mM glyphosate. Our data and those of Jaworski (1972) suggest some interstrain diversity with respect to glyphosate tolerance, although methodological differences may also account for some differences between these studies. An additional factor that may affect the toxicity of glyphosate to *B. japonicum* is the ability of members of the Rhizobiaceae to degrade glyphosate in media containing aromatic amino acids (Lui et al., 1991). The growth of these bacteria were inhibited by glyphosate concentrations above 1 mM.

Our data demonstrate that accumulations of hydroxybenzoic acids occur in *B. japonicum* in response to glyphosate. Presumably, the blockage of EPSP synthase by glyphosate causes PCA production from shikimate and shikimate 3-phosphate (Figure 4). Results with plants indicate greater levels of shikimate accumulation than were observed in these experiments, but the accumulations of PCA are generally consistent with our results (Becceril et al., 1989; Berlin and Witte, 1981; Lydon and Duke, 1988). In plant cells, shikimate is relatively rapidly sequestered in the vacuole, where it may be unavailable for transformation to other hydroxybenzoic acids, such as PCA (Holländer-Czytko and Amrhein, 1983). Production of PCA by strains 123 and 138 was greatest at the 0.5 and 1.0 mM concentrations of glyphosate, which allowed some growth.

Table II. Effect of Aromatic Amino Acids on the Inhibition of *B. japonicum* Strain USDA 138 by Glyphosate

glyphosate, mM	amino acids[a]	rate, k, day^{-1}	inhibition, %
0	none	2.000 ± 0.169[b]	0
	added	1.548 ± 0.224	23
1	none	1.546 ± 0.145	23
	added	1.392 ± 0.195	30
2	none	1.367 ± 0.122	32
	added	1.292 ± 0.192	35

[a] Amino acid mixture consisted of filter-sterilized solutions of L-tryptophan, L-phenylalanine, and L-tyrosine resulting in final concentrations of 0.25 mM each. Sterile water was added to cultures not receiving amino acids. [b] Specific growth rate determined as described in Table I. Regressions were significant ($P < 0.05$) with r^2 values ranging from 0.82 to 0.93.

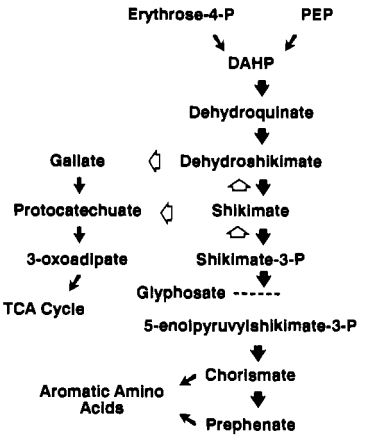

Figure 4. Shikimic acid pathway (solid arrows) and protocatechuate formation by *B. japonicum* in glyphosate-treated medium (open arrows). DAHP (3-deoxy-7-phospho-D-arabinoheptulosinic acid) is formed from erythrose 4-phosphate and phosphoenolpyruvate (PEP). The action of glyphosate on EPSP synthase is indicated by the dashed line, ultimately causing depletion of aromatic amino acids and accumulation of protocatechuate. PCA is degraded by the pathway shown in glyphosate-free medium.

Glyphosate at 5 mM resulted in much less total accumulation or accumulations on a per cell basis than did lower glyphosate concentrations. Strain 110 produced much less PCA than strains 123 and 138, even though glyphosate reduced growth of 110 to at least the same extent as the other strains (Table I).

These *B. japonicum* strains have the enzymatic pathways necessary to degrade PCA, as do other members of the Rhizobiaceae (Parke and Ornston, 1984). No differences were found among these strains in their rate of PCA degradation. Therefore, the lower level of PCA accumulation by strain 110 (Figure 1) in response to glyphosate may not be due to enhanced PCA degradation. However, we did not assess the effects of glyphosate on the activity of this pathway. The accumulations of PCA in the presence of glyphosate may represent a balance between production and degradation. It also seems likely that glyphosate reduces the activity of the degradation pathway for aromatics to some extent due to the inhibition of aromatic amino acid synthesis and resulting metabolic dysfunction. Protocatechuate is only a weak inhibitor of plant growth (Blum et al., 1984; Schilling and Yoshikawa, 1987). However, if it were excreted into host plant cells by bacteroids, plant enzymes could transform the PCA into more potent inhibitors such as syringate, vanillate, and benzylaldehydes (Blum et al., 1984; Nicolliar et al., 1985). Sufficiently large concentrations of these compounds could disrupt nodule or root functions.

The failure of amino acids to reverse the effects of glyphosate in these short-term experiments could be explained in several ways. Fischer et al. (1986) reported that the same amino acid mixture used in these experiments reversed the inhibition of growth by glyphosate in *Escherichia coli* and *Pseudomonas aeruginosa* but not in *Bacillus subtilis*. The failure to reverse glyphosate effects in *B. subtilis* was attributed to the unregulated flow of carbon into the shikimate pathway (i.e., added aromatic amino acids do not provide feedback inhibition to stop carbon flow into the shikimate pathway). These experiments with *B. japonicum* cannot preclude the possibility that aromatic amino acids do not enter the cells in sufficient quantities or ratios to reverse inhibition. Also, the availability of amino acids in this cultural medium may change substrate utilization by *B. japonicum*, thus accounting for the reduced growth in glyphosate-free media treated with amino acids. Certainly, the accumulations of PCA by strains 123 and 138 indicate that the shikimic acid pathway is deregulated by glyphosate alone and thus support the general concept of an energy drain as carbon is cycled through the truncated shikimic acid pathway.

The levels of glyphosate used in these experiments far exceed those that would be present in soil following use at recommended rates. Glyphosate concentrations could be expected to average 1 mg/kg of soil in the surface 7 cm after a 1.12 kg/ha spray application. Glyphosate at concentrations of 20 mg/kg of soil had no effect on soil populations of antibiotic-resistant derivatives of strains 110 and 138 (Moorman, 1986). However, within the plant, concentrations in metabolic sinks may be much greater due to translocation and the lack of degradative pathways (Duke, 1988). Concentrations within the roots of two weed species ranged from approximately 0.1 to 0.5 μg/mg of root dry weight after a single application of 0.5 kg/ha of glyphosate, and plant tissue concentrations increased as the application rate increased (Honegger et al., 1986). Assuming a 90% water content for roots, these concentrations are approximately equivalent to 0.06–0.3 mM glyphosate, respectively. Gougler and Geiger (1981) reported glyphosate translocation was similar to that of sucrose in sugar beet (*Beta vulgaris*) and that glyphosate limits its own translocation by disrupting carbon metabolism (Gougler and Geiger, 1984). Concentrations of glyphosate are likely to be higher in metabolic sinks such as nodules than concentrations averaged over whole root systems, and glyphosate-resistant plants may continue to translocate glyphosate for considerably longer periods than sensitive plants. Repeated applications of glyphosate might be necessary for weed control over an extended time because glyphosate has no residual soil activity. Our results suggest that *B. japonicum* strains could be affected by repeated applications of glyphosate to glyphosate-resistant soybeans. Glyphosate-resistant strains of *B. japonicum* may be required to maximize symbiotic N_2 fixation when resistant soybean varieties are repeatedly treated with glyphosate. The potential effects of glyphosate on nodulation and N_2 fixation need to be assessed under a variety of conditions using glyphosate-resistant soybeans.

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