Glyphosate Effects on Phenolic Metabolism of Nodulated Soybean (*Glycine max* L. Merr.)

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Glyphosate is a herbicide that blocks the shikimic acid pathway. Three *Bradyrhizobium japonicum* strains with different sensitivities to glyphosate were used to test the effect of this herbicide on the phenolic metabolism of nodulated soybeans and on the bacteroid nitrogenase activity. Glyphosate caused an inhibition in the bacteroid nitrogenase activity that was related with the sensitivity of the nodule-forming strains. Both leaves and nodules accumulated huge amounts of shikimate and phenolic acids (mainly protocatechuic acid), indicating that the herbicide was translocated to the nodule and disturbed phenolic metabolism. However, this accumulation was not clearly related to the sensitivity of the different strains. Bacteroids from control plants were incubated with the same concentration of shikimate, and phenolic acid accumulated in glyphosate-treated plants. Despite the high levels found in nodules, they were not responsible for the decrease of the nitrogenase activity. Glyphosate by itself caused a small inhibition of the bacteroid nitrogenase activity.

Keywords: *Benzoic acids; glyphosate; Bradyrhizobium; Rhizobium; shikimate*

INTRODUCTION

Grain legumes such as soybean (*Glycine max* L. Merr.) are very important crops because of their economic importance and their ability to fix atmospheric nitrogen. This importance is expected to increase with the need to develop sustainable agricultural practices and with the development of herbicide-resistant crops. In fact, newly developed transgenic soybean plants that contain glyphosate tolerance genes are able to tolerate the glyphosate levels necessary for adequate commercial weed control (Hinchee et al., 1993). Although inherited glyphosate resistance in weeds has been recently reported (Powles et al., 1998), the low environmental impact and great efficacy of this herbicide, combined with the continued development of glyphosate-resistant agricultural crops, have made the spectrum of use for this chemical much more selective and thus more suitable for use in agricultural applications. However, although repeated applications of glyphosate could control weeds in glyphosate-resistant legume fields, it could also affect symbiotic microorganisms associated with them under natural conditions. In fact, glyphosate can alter the growth of free living cells of some nitrogen fixing species such as *Azotobacter* sp. (Santos and Flores, 1995), *Rhizobium* (Moorman, 1986), and *Bradyrhizobium* (Moorman et al., 1992). Besides, Eberbach and Douglas (1989), studying *Trifolium subterraneum*, and Mårtensson (1992), in small-seeded legumes, reported a reduction of growth and nodulation by several herbicides including glyphosate. However, there is still a lack of information about the effect of this herbicide on legumes such as soybean and rhizobia under symbiotic conditions.

Because glyphosate is readily translocated within the plant, it could reach metabolic sinks of legumes such as new developing leaves and nodules. Glyphosate acts by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Steinrücken and Amrheim, 1980), causing a reduction in the synthesis of aromatic amino acids and cinnamic acids and the accumulation of high levels of shikimate and hydroxybenzoic acids in leaves and flowers (Lydon and Duke, 1988; Becerril et al., 1989). Moorman et al. (1992) suggested that the disruption of phenolic metabolism by glyphosate could cause two effects: first, a physiological and ecological disturbance, because flavonoid-type compounds are responsible for the symbiotic interactions between legumes and *Rhizobium;* second, accumulation of phenolic acids that could interfere with the nitrogen fixation process within the nodule. By using nodulated plants we will verify the latter suggestion. Therefore, the aim of this paper was to study the collateral effects of sublethal concentrations of glyphosate on the phenolic metabolism of nodulated soybean, inoculated with several rhizobial strains showing different tolerances to the herbicide in the free living form, and to investigate the role of accumulated phenolic acid intermediates in the nitrogen fixation process. This is the first report on the effect of glyphosate on phenolic nodule metabolism.

MATERIALS AND METHODS

Plant and Bacterial Growth Conditions and Herbicide Treatment. *Bradyrhizobium japonicum* strains ISJ-32, ISJ-33, and ISJ-48 were obtained from Dr. Dulce Rodriguez (SIA, Seville, Spain). Their original sources are as follows: ISJ-32 = NifTAL 376, Iowa, 1960; ISJ-33 = NifTAL 102, Florida, 1959; ISJ-48 = 3−15-B3, Gembloux, Belgium. Strains were maintained on yeast extract manitol (YEM) agar slants at 4 °C according to the method of Vincent (1970). Bacteria were grown in YEM for 7 days in 100 mL Erlenmeyer flasks on an orbital shaker (150 rpm) at 25 °C. Cells were collected and resuspended in phosphate buffer as described by Garcia-

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Table 1. Effect of Glyphosate on the Growth of Different Strains of *B. japonicum* (ISJ-32, ISJ-33, and ISJ-48)[a]

glyphosate concn (mM)	ISJ-32		ISJ-33		ISJ-48	
	k (days^{-1})	inhib (%)	k (days^{-1})	inhib (%)	k (days^{-1})	inhib (%)
control	0.960 ± 0.103	0	0.929 ± 0.138	0	0.943 ± 0.125	0
0.03	0.449 ± 0.050	53	0.773 ± 0.101	17	0.607 ± 0.082	36
0.1	0.310 ± 0.029	68	0.686 ± 0.071	26	0.475 ± 0.051	50
0.3	0.226 ± 0.025	76	0.614 ± 0.055	34	0.396 ± 0.031	58
1	0.202 ± 0.018	79	0.538 ± 0.061	42	0.336 ± 0.039	64

[a] The specific growth rate (k) was determined by the lineal 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, during the exponential growing phase. Inhibition was calculated from growth rates as a percentage of the untreated control. Estimates of k are shown with the standard error of the estimate. All regressions were significant ($p < 0.05$) with r^2 values ranging from 0.81 to 0.95. Each value is the average of at least eight determinations.

Plazaola et al. (1993), and 3 mL of suspension was used to inoculate 100 mL Erlenmeyer flasks containing 40 mL of MSR medium (Chakrabarti et al., 1981), to produce a final concentration that was 8×10^7 cells mL^{-1}. To test the tolerance of the *B. japonicum* strains to the glyphosate, non-pretreated cells were grown for 5 days at 150 rpm at 25 °C in the presence of different concentrations of glyphosate (0, 0.03, 0.1, 0.3, and 1 mM). Cell numbers were determined turbidimetrically at 680 nm each 12 h, and absorbance was correlated through experiment with colony plate count and direct count using a phase contrast microscope (Nikon Optiphot). Shikimate content on *B. japonicum* cultures was determined after 4 days in 0 and 0.3 mM glyphosate treatments.

Seeds of soybean (*Glycine max* L. Merr. cv. Fiorir) were ethanol sterilized, and three plants were planted in 2 L pots containing perlite/vermiculite (50:50%) and watered every other day with Evans nitrogen-free nutrient solution (Evans, 1981). Twelve-day-old plants were inoculated with 1 mL of a suspension of not glyphosate-treated *B. japonicum* (1.3×10^8 cells per plant) grown as described above. When non-nodulated plants were used, nutrient solution was supplemented with 10 mM KNO_3.

Plants were grown in a growth chamber with the following conditions: 25/20 °C day/night temperatures; 55−75% relative humidity; and a photoperiod of 14 h at 350 μmol m^{-2} s^{-1} PFD. When the plants were 6 weeks old, each pot was uniformly sprayed with 1 mL of a solution with 0, 5, or 10 mM glyphosate (isopropylamine salt) with 0.1% Triton X-100. On surface bases, 10 mM treatment represents about half of the field recommended dose of the commercial product. Leaves and nodules were harvested 7 days after treatment. At harvest, leaves were frozen with liquid nitrogen, lyophilized, and stored at −20 °C until HPLC analysis.

Analytical Methods. Shikimate and hydroxybenzoic acids were extracted and analyzed for bacterial cultures as described by Moorman et al. (1992) and for plants as described by Becerril et al. (1989). To determine hydroxybenzoic acids, the samples (leaves and nodules) were refluxed in 1 N HCl at 100 °C for 1 h. Refluxing was interrupted after 30 min to sonicate for 5 min and then refluxing was resumed. Hydroxybenzoic acids were extracted with ethyl acetate, filtered, and evaporated to dryness. The residue was resuspended in 2 mL of pH 2.7 phosphoric acid and analyzed by HPLC.

For shikimic acid extraction, the samples (*B. japonicum* culture, leaves and nodules) were refluxed in the same manner as above, followed by filtration and evaporation to dryness. The residue was resuspended in 20 mL of methanol, sonicated, filtered, and evaporated again. The residue was resuspended in 2 mL of pH 2.7 phosphoric acid. HPLC analysis was performed on a Tracer 250 × 4.6 mm (i.d.) Spherisorb 5 μm ODS-I reversed phase column connected with an 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^{-1} for 10 min. The solvent gradient for hydroxybenzoic acids was 3.33 mM phosphoric acid (solvent A) and methanol (solvent B) at a flow of 1.6 mL min^{-1}. 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. HPLC detection was made with a UV detector (Waters) with external standards.

Acetylene reduction activity (ARA) in bacteroids was measured with a Shimadzu GC-9 gas chromatograph with a flame ionization detector, as described in García-Plazaola et al. (1996). Bacteroids were isolated as described Stephens and Neyra (1983) and incubated at 25 °C in 25 mL of a medium containing 0.3 M sucrose and 10 mM sodium succinate in 50 mM phosphate buffer (pH 6.8). All of the isolation procedures were conducted in anaerobic conditions, under an N_2 atmosphere. The incubation assay atmosphere was 1% (v/v) oxygen, 10% (v/v) acetylene, and 89% (v/v) nitrogen as described by Stephens and Neyra (1983). These conditions allow oxygen diffusion to bacteroids and avoid possible ARA decline described when using intact nodules (Minchin et al., 1983). Ethylene production in all treatments was lineal along incubation time. Bacteroid respiration was determined in a suspension of bacteroids with a Clark-type oxygen electrode as described by Arrese-Igor and Aparicio-Tejo (1992). The concentration of bacteroids was 50 μg of protein mL^{-1}. Protein contents were measured according to the method of Bradford (1976) after disruption of the bacteroids by sonication, and bovine serum albumin was used as a standard.

Results were examined by one- and two-way (strain and treatment) analysis of variance (ANOVA). All results discussed in this paper are significant at $p < 0.05$ with Fisher's least significant difference (LSD) tests between means.

RESULTS

Growth of three *B. japonicum* strains was reduced when glyphosate was present in the medium at concentrations ranging from 0.03 to 1 mM (Table 1). The herbicide affected this growth in two ways: it first caused an increase in the *lag* period (not shown), and second a gradual decline of growth rate with increasing concentration of the herbicide. The strain ISJ-32 was very sensitive; the I_{50} value for growth rate was ∼30 μM glyphosate. The most tolerant strain was ISJ-33, with an $I_{50} > 1$ mM. Bacterial growth could not be inhibited completely for any strain at herbicide concentrations up to 1 mM. All of the rhizobial strains were affected by 0.3 mM glyphosate and accumulated high amounts of shikimate in the cultures (Table 2). Shikimate accumulation did not correlate with interstrain differences on glyphosate sensitivity, so a partially resistant strain such as ISJ-33 excreted more shikimate than a more sensitive strain such as ISJ-32.

To compare the effects of the herbicide on the phenolic metabolism of nodules, soybean plants were inoculated with each of the strains having a different sensitivities to glyphosate on free living form, and these plants were foliarly treated with glyphosate. From Table 3, it is obvious that no conclusion can be drawn from the accumulation pattern of shikimate-derived compounds in leaves when nodulated versus nitrate-watered plants are compared or even among the strains on nodulated plants. Shikimate levels on control plants were negligible, but herbicide-treated plants accumulated very

Table 2. Effect of Glyphosate on Growth and Shikimate Production by Free Living Cells of *B. japonicum* (Strains ISJ-32, ISJ-33, and ISJ-48) after 4 Days[a]

treatment	glyphosate concn (mM)	final population (10^8 cells mL^{-1})	shikimate in medium (μM)	shikimate per cell (nmol $\times$ 10^8 $cell^{-1}$)
ISJ-32	0	13.11 ± 0.39	24.2 ± 2.1	1.9 ± 0.2
	0.3	1.59 ± 0.11	611.3 ± 28.8	382.7 ± 16.0
ISJ-33	0	13.41 ± 0.32	35.2 ± 3.1	2.7 ± 0.2
	0.3	5.59 ± 0.22	2666.4 ± 272.9	476.1 ± 48.9
ISJ-48	0	12.78 ± 0.50	23.4 ± 1.2	1.8 ± 0.4
	0.3	2.45 ± 0.18	827.9 ± 66.5	342.0 ± 23.9

[a] Each value is the average of at least four determinations ± SE.

high levels of this intermediate. In fact, this was the main compound accumulated far above of the concentration of all hydroxybenzoic acids taken together (Table 3) and represented up to 2−3% of the plant dry weight. Protocatechuic acid (PCA) was the main hydroxybenzoic acid accumulated in leaves, and its levels greatly increased upon exposure to the herbicide.

The glyphosate initially applied to leaves is rapidly translocated to other metabolic sinks of the plants, affecting phenolic metabolism in nodules (Table 4). Although on control plants nodules had higher levels of shikimate and hydroxybenzoic acids than those found in new developing leaves, in response to glyphosate application the concentration of shikimate in young leaves was higher than that found in nodules and indicates a greater phenolic metabolism disturbance in the leaves compared with nodules. Nodule shikimate and PCA concentrations did not differ substantially among strains; except for the lower glyphosate dose (5 mM), soybean plants nodulated with the most tolerant strain, ISJ-33, accumulated only 3-fold less shikimate and PCA content than plants inoculated with the two other strains. At the highest herbicide dose, the concentration of shikimate in nodules was 10, 13, and 18 times control values for plants inoculated with ISJ-33, ISJ-48, and ISJ-32, respectively.

To study the effect of the herbicide in the microsymbiont, bacteroids extracted from soybean plants treated with 0, 5, and 10 mM glyphosate were incubated in the absence of glyphosate in a medium with succinate, without a limitation of carbon skeletons. The ARA in bacteroids from glyphosate-treated plants was inhibited (Figure 1), showing the inhibition of nitrogen fixation in glyphosate-treated legume plants. The most sensitive strain was ISJ-32 and the most tolerant ISJ-33.

To detect the sensitivity of nodule metabolism to the huge amounts of shikimate or hydroxybenzoic acids caused by glyphosate treatments, bacteroids of each strain extracted from nontreated plants were incubated in the presence of the same concentrations of shikimate and PCA found in glyphosate-treated soybean nodules inoculated with each strain (Table 4). None of the compounds at any concentration produced a significant reduction of bacteroid activity (measured as respiration)

Table 3. Shikimic, Protocatechuic, Gallic, Gentisic, 4-Hydroxybenzoic, Vanillic, and Syringic Acid Contents (Micromoles per Gram of Dry Weight) in Leaves of Nodulated (with *B. japonicum* Strains ISJ-32, ISJ-33, and ISJ-48) and Nitrate-Watered Soybean Plants That Were Foliarly Treated with 0, 5, and 10 mM Glyphosate[a]

strain	glyphosate concn (mM)	Shik	PCA	Gall	Gent	4-HB	Van	Syr
ISJ-32	0	0	0.08 ± 0.01	0.045 ± 0.024	0.134 ± 0.028	0.111 ± 0.016	0.162 ± 0.021	0.202 ± 0.055
	5	62.0 ± 6.2	1.77 ± 0.17	0.073 ± 0.017	0.151 ± 0.041	0.136 ± 0.014	0.229 ± 0.016	0.298 ± 0.062
	10	109.2 ± 14.3	3.11 ± 0.69	0.083 ± 0.029	0.193 ± 0.065	0.150 ± 0.021	0.364 ± 0.057	0.308 ± 0.065
ISJ-33	0	0	0.31 ± 0.07	0.065 ± 0.006	0.119 ± 0.024	0.119 ± 0.030	0.167 ± 0.015	0.347 ± 0.126
	5	117.8 ± 8.3	2.24 ± 0.12	0.050 ± 0.021	0.195 ± 0.025	0.166 ± 0.069	0.366 ± 0.091	0.377 ± 0.124
	10	157.9 ± 15.7	3.70 ± 0.65	0.157 ± 0.053	0.342 ± 0.088	0.157 ± 0.039	0.324 ± 0.113	0.248 ± 0.063
ISJ-48	0	0	0.27 ± 0.14	0.044 ± 0.022	0.108 ± 0.012	0.168 ± 0.123	0.228 ± 0.053	0.264 ± 0.061
	5	100.2 ± 2.2	1.82 ± 0.10	0.037 ± 0.011	0.156 ± 0.069	0.086 ± 0.016	0.231 ± 0.052	0.293 ± 0.032
	10	184.4 ± 2.6	4.78 ± 1.30	0.038 ± 0.009	0.107 ± 0.016	0.127 ± 0.014	0.319 ± 0.125	0.417 ± 0.182
NITRATE	0	0	0.27 ± 0.12	0.022 ± 0.011	0.027 ± 0.004	0.165 ± 0.060	0.343 ± 0.181	0.189 ± 0.013
	5	98.0 ± 0.2	2.05 ± 0.90	0.023 ± 0.008	0.024 ± 0.003	0.139 ± 0.022	0.222 ± 0.013	0.211 ± 0.015
	10	137.6 ± 13.3	3.79 ± 0.71	0.021 ± 0.013	0.036 ± 0.005	0.120 ± 0.009	0.350 ± 0.099	0.260 ± 0.019

[a] Each value is the average of three to five determinations ± SE. Shik, shikimic acid; PCA, protocatechuic acid; Gall, gallic acid; Gent, gentisic acid; 4-HB, 4-hydroxybenzoic acid; Van, vanillic acid; Syr, syringic acid.

Table 4. Shikimic, Protocatechuic, Gallic, Gentisic, 4-Hydroxybenzoic, Vanillic, and Syringic Acid Contents (Micromoles per Gram of Dry Weight) in Nodules of Nodulated (with *B. japonicum* Strains ISJ-32, ISJ-33, and ISJ-48) Soybean Plants That Were Foliarly Treated with 0, 5, and 10 mM Glyphosate[a]

strain	glyphosate concn (mM)	Shik	PCA	Gall	Gent	4-HB	Van	Syr
ISJ-32	0	2.16 ± 0.19	0.76 ± 0.11	0.132 ± 0.041	0.061 ± 0.024	0.242 ± 0.009	1.23 ± 0.24	0.263 ± 0.015
	5	27.13 ± 2.36	2.81 ± 0.34	0.192 ± 0.011	0.028 ± 0.005	0.195 ± 0.012	0.91 ± 0.10	0.330 ± 0.029
	10	39.17 ± 2.90	5.74 ± 0.55	0.281 ± 0.109	0.048 ± 0.003	0.179 ± 0.018	0.97 ± 0.09	0.402 ± 0.152
ISJ-33	0	3.46 ± 0.17	0.68 ± 0.40	0.109 ± 0.031	0.029 ± 0.010	0.350 ± 0.023	1.03 ± 0.07	0.417 ± 0.051
	5	11.76 ± 2.25	1.29 ± 0.08	0.191 ± 0.049	0.060 ± 0.007	0.311 ± 0.034	1.06 ± 0.13	0.524 ± 0.038
	10	33.55 ± 2.41	4.91 ± 0.30	0.241 ± 0.068	0.041 ± 0.003	0.398 ± 0.032	1.05 ± 0.05	0.494 ± 0.069
ISJ-48	0	3.32 ± 0.83	0.45 ± 0.11	0.189 ± 0.102	0.027 ± 0.014	0.243 ± 0.072	0.96 ± 0.06	0.236 ± 0.043
	5	32.26 ± 1.83	3.27 ± 0.16	0.242 ± 0.022	0.036 ± 0.012	0.134 ± 0.017	0.54 ± 0.23	0.254 ± 0.034
	10	44.00 ± 3.49	2.98 ± 0.70	0.191 ± 0.027	0.041 ± 0.005	0.173 ± 0.014	1.34 ± 0.10	0.342 ± 0.143

[a] Each value is the average of three to five determinations ± SE. Shik, shikimic acid; PCA, protocatechuic acid; Gall, gallic acid; Gent, gentisic acid; 4-HB, 4-hydroxybenzoic acid; Van, vanillic acid; Syr, syringic acid.

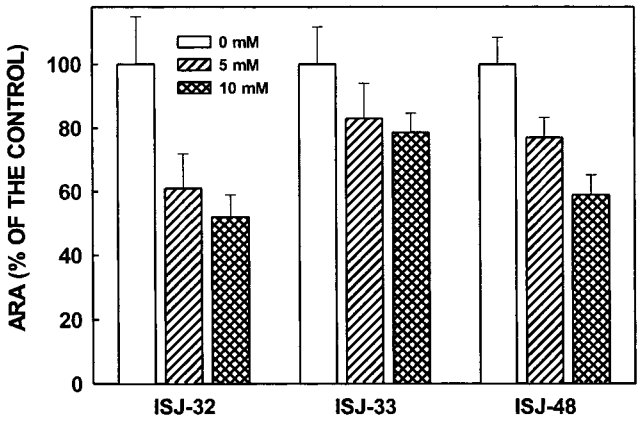

Figure 1. Inhibition of ARA in bacteroids of *B. japonicum* (strains ISJ-32, ISJ-33, and ISJ-48) extracted from plants 7 days after application of 1 mL of a solution of 0, 5, or 10 mM glyphosate. Values are expressed as a percentage of each control. Control values of ARA were 0.208, 0.222, and 0.247 μmol of C_2H_4 mg^{-1} of protein h^{-1} for ISJ-32, ISJ-33, and ISJ-48, respectively. Each value is the average of at least six determinations ± SE.

Table 5. Effect of Shikimate or Protocatechuate on ARA and Respiration (Resp) of Bacteroids of *B. japonicum*[a]

	ISJ-32		ISJ-33		ISJ-48	
treatment	ARA	Resp	ARA	Resp	ARA	Resp
shikimate						
3.0 mM			87 ± 7	96 ± 2		
6.0 mM	81 ± 10	99 ± 7				
7.5 mM					95 ± 5	103 ± 4
8.0 mM			84 ± 8	95 ± 3		
10.0 mM	65 ± 15	99 ± 7			92 ± 5	104 ± 4
protocatechuate						
0.75 mM	103 ± 8	103 ± 3				
1.0 mM			98 ± 5	106 ± 3		
1.5 mM					100 ± 4	107 ± 5

[a] Results are expressed as percentage of control for each strain. Control values of ARA were 0.208, 0.222, and 0.247 μmol of C_2H_4 mg^{-1} of protein h^{-1} for ISJ-32, ISJ-33, and ISJ-48, respectively. Control values of respiration were 1.928, 2.868, and 2.086 μmol of O_2 mg^{-1} of protein h^{-1} for ISJ-32, ISJ-33, and ISJ-48, respectively. Each value is the average of at least six determinations ± SE.

or nitrogenase activity (measured as ARA), except a slight reduction with 10 mM shikimate on ARA activity from the sensitive ISJ-32 strain (Table 5). In the same way, bacteroids from nodules of control plants were extracted and incubated in the presence of several glyphosate concentrations to test the direct effect of the herbicide on N_2 fixation ability (Figure 2). Glyphosate produced a slight reduction in the ARA activity of bacteroids. This reduction varied for each strain, so the most tolerant strain was ISJ-48 with a 7% ARA reduction, whereas ISJ-32 and ISJ-33 reductions were 25−30% at 1 mM concentration. Higher concentrations did not increase significantly the inhibition of this activity.

DISCUSSION

All of the strains were affected by the presence of glyphosate irrespective of its concentration. The data from Table 1 and those of others (Jaworski, 1972; Moorman et al., 1992) indicate that sensitivity to glyphosate is an interstrain characteristic of *B. japonicum*. Therefore, ISJ-32 could be distinguished as a very sensitive strain, ISJ-33 as a tolerant strain, and ISJ-48 as an intermediate strain in terms of growth rate inhibition. Such a discrimination was sustained by the

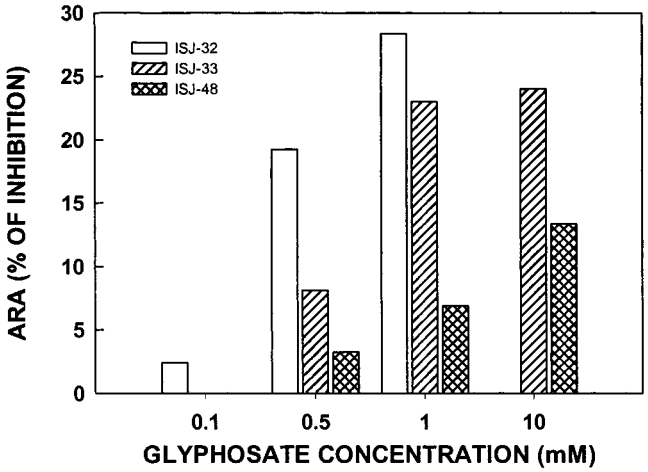

Figure 2. Inhibition of ARA in bacteroids of *B. japonicum* (strains ISJ-32, ISJ-33, and ISJ-48) extracted from control plants and incubated in the presence of 0, 0.1 (only ISJ-32), 0.5, 1, or 10 mM (only ISJ-33 and ISJ-48) of glyphosate. Values are expressed as a percentage of each untreated control. Control values of ARA were the same as in Figure 1. Each value is the average of at least four determinations.

fact that sensitive bacteria had a more reduced growth rate (Table 1) and final population (Table 2) at all herbicide concentrations used. It should be mentioned that the growth rates of the three strain cultures not treated with glyphosate were quite similar. Glyphosate blocks aromatic amino acid biosynthesis in both plants and bacteria by inhibiting the enzyme EPSP synthase, causing a deregulation on the shikimate pathway (Devine et al., 1993). As demonstrated before for plants (Becerril et al., 1989) and free living cells of *B. japonicum* (Moorman et al., 1992), all strains tested produced shikimate (Table 2) as a result of EPSP synthase inhibition. On the basis of shikimate concentration of Table 2, from 5 to 19% of the carbon available originally in the growth medium was transformed into shikimate, representing an important loss of energy and growth yield. It must be considered that although shikimate production levels in response to glyphosate were similar in the three strains (expressed per cell basis), the concentration in the medium was higher in ISJ-33 because of its greater growth. Shikimate and shikimate-derived hydroxybenzoic acids produced by *B. japonicum* are mainly excreted to the medium and <1% was cellular (Moorman et al., 1992; data not shown). Under free living conditions these compounds are diluted in the medium, where they can be degraded by *B. japonicum* or other microorganisms of soil (Moorman et al., 1992; Parke and Ornston, 1984; Parke et al., 1985). However, under symbiotic conditions the exposure of bacteroids to the herbicide could contribute to build up pools of shikimic-derived compounds within nodule tissues.

Glyphosate is rapidly transported through the phloem to the growing points, in a similar way to sucrose, responding to a source−sink relationship (Geiger and Bestman, 1990). Because metabolic degradation of glyphosate by higher plants is very slow (Devine et al., 1993), a metabolic sink for carbohydrates such as legume root nodules should accumulate high levels of the herbicide. We did not determine the concentration of glyphosate in nodules, but we could indirectly demonstrate its presence by analyzing its effects on compounds derived from the shikimate pathway of nodules. When sublethal doses of glyphosate were applied to the

leaves of nodulated soybean plants, we could determine a great disturbance on the levels of shikimate and PCA of nodules (Table 4). This effect was essentially limited to these compounds, although other hydroxybenzoic acids (i.e., gallate, gentisate, 4-hydroxybenzoate, vanillate, and syringate) were also affected. The increase in shikimic acid content in nodules was apparent, even at very low glyphosate applications. This effect could be used as a tool for assessing herbicidal activity (Becerril et al., 1989; Stasiak et al., 1991). It has been proposed that basal levels of shikimic acid could be used to predict sensitivity to glyphosate among species, so in the absence of glyphosate more tolerant *Prunus pensylvanica* showed higher shikimate levels than *Populus tremuloides* (Stasiak et al., 1991). The relative effects of glyphosate on the size of shikimate and shikimate-derived phenolics pools is an indicator of the degree of regulation of these pools within each plant (Becerril et al., 1988). This indicates that the phytotoxic effect does not correspond with shikimate concentration itself but with the degree of increase over the normal level of this compound within the tissues; usually the effect on shikimate accumulation is higher when the basal level is lower, as can be seen in new developing leaves. Assuming that shikimate production is a function of glyphosate concentration and metabolic regulation, our results provide evidence that the herbicide is mainly translocated to new developing leaves. The low levels of shikimate and hydroxybenzoic acids of control leaves indicate a great degree of regulation of phenolic pools in this tissue. Glyphosate application caused a higher increase in hydroxybenzoic acid pools of leaves (~2.4−5.7- fold over the control levels) (Table 3), whereas the accumulation in nodules ranged from 1.3- to 2.8-fold over the control levels (Table 4). On the other hand, the effects of glyphosate on the phenolic profile of leaves of nodulated and nitrate-watered plants were very similar.

The inhibition of EPSP synthase first causes the accumulation of shikimate and shikimate-derived hydroaromatic acids, then a deregulation of the shikimate pathway, and, ultimately, an uncontrolled flow of carbon into this pathway, producing some disruptions in other essential metabolic pathways (Devine et al., 1993). It has been speculated that glyphosate could exert part of its action because of the accumulation of phenolic acids (Moorman et al., 1992; Devine et al., 1993). Our data indicate that neither respiratory activity nor nitrogenase activity of bacteroids isolated from control plants was affected by the presence of any concentration of shikimate or PCA found in glyphosate-treated soybean nodules, except for a slight effect with the highest shikimate concentration in the most sensitive strain (Table 5). It should be mentioned that these experiments were done with buffered solutions of these intermediates. However, due to the huge amounts of these compounds found in treated plant tissues and their acid property, we cannot rule out a pH effect, if they cannot be properly compartmentalized. This effect has not been explored in higher plants.

Becerril et al. (1989) pointed out that sublethal glyphosate treatments on plants do not have long-lasting effects on phenolic acids contents, so after a few weeks, the accumulation of such compounds recovered almost control levels in leaves of velvetleaf plants. It is possible that this can also occur in other plant tissues as in nodules, particularly considering that free living forms of *Bradyrhizobium* can degrade shikimate and PCA (Moorman et al., 1992; Parke et al., 1985). However, bacteroids extracted from glyphosate-treated plants were affected in their ARA capability compared with control (Figure 1). The sensitivity of each strain to such inhibition correlated with the effect caused by the herbicide in the free living forms. Nevertheless, there was no correlation between the levels of shikimate and/or PCA accumulated in nodules and ARA inhibition of bacteroids extracted from these nodules. All of this together confirms the null role of shikimate and hydroxybenzoic acids on glyphosate toxicity on nitrogenase activity. Apart from the fact that these affected bacteroids were incubated in a medium with succinate, a limitation of carbon to the nodules of intact plants was probably not the main cause of the ARA depression but did have some effects on the bacteroid metabolism itself.

Because glyphosate is translocated continuously from leaves and likely is not further degraded, a terminal sink such as the nodules should present very high levels of the herbicide and in that form cause some interference on the nitrogen fixation process of bacteroids. To check the direct effect of the herbicide itself, bacteroids extracted from control plants were incubated with glyphosate (Figure 2). Even at concentrations as high as 1 mM ARA was only slightly affected. This limited effect of glyphosate on nitrogenase activity varied among the strains, but was correlated with neither the sensitivity showed by strains on the free living form nor the ARA inhibition of bacteroids from glyphosate-treated plants.

In conclusion, this study shows that glyphosate foliarly applied on nodulated plants is translocated to nodules, causing a disturbance of the nodule metabolism. The shikimate pathway in nodules was blocked, leading to a massive accumulation of shikimate and PCA in the nodules. However, neither these compounds nor the herbicide itself had critical effects on the nitrogen fixation process. Further studies need to be carried out to determine the effect of glyphosate on fundamental metabolic pathways of nodules sharing initial intermediates of the shikimate pathway.

ABBREVIATIONS USED

ARA, acetylene reduction activity; EPSP, 5-enolpyruvylshikimate-3-phosphate; PCA, protocatechuic acid; YEM, yeast extract manitol.

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