

Effect of Glyphosate on Intact Bean Plants (*Phaseolus vulgaris* L.) and Isolated Cells¹

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ABSTRACT

Whole bean (var. "Eastern Butterwax") plants and isolated cells were used to investigate possible mechanisms of action of glyphosate [*N*-(phosphonomethyl)glycine]. Results showed that glyphosate was quickly absorbed by the whole plant but not by individual cells and that it caused a rapid reduction in leaf dry matter accumulation, leaf expansion, leaf angle, and stomatal aperture without affecting the water status of the plant. Glyphosate also caused a rapid reduction in cellular uptake of ⁸⁶Rb and ³²P which preceded its detrimental effects on photosynthesis, RNA and protein synthesis, and respiration of isolated cells. This reduction in ion absorption was not due to a loss of membrane integrity, decrease in energy supply or chelation of ions. It was concluded that glyphosate was directly inhibiting the ion absorption process of bean leaf cells.

MATERIALS AND METHODS

Plant Culture and Treatment. Eight-day-old bean (*Phaseolus vulgaris* L. "Eastern Butterwax") plants used in these studies were maintained in a growth chamber illuminated with a combination of fluorescent and incandescent lamps at an intensity of 475 μE/m²·s at plant level. Unless otherwise stated, herbicide treatment consisted of foliar application to intact plants of 1 mM glyphosate as the isopropylamine salt plus 0.05% (v/v) MON 0027 (a nonionic surfactant produced by Monsanto Company). All experiments described below were conducted at least twice.

Cell Isolation. In experiments where isolated cells were utilized, the procedure of Jensen *et al.* (14) as modified by Ashton *et al.* (2) was followed. Microscopic examination of the isolated cells was made to insure that they were intact. The number of cells in suspension at several different 540 nm *A* readings was determined with a Coulter counter. These values were then used to construct a standard curve of *A* at 540 nm versus cell concentration from which the number of cells used for each test could be approximated.

Absorption of Glyphosate. Absorption of radioactive glyphosate into intact bean leaves was followed over 4 h. Ten μl of 4.2 mM herbicide containing 0.08 μCi of methyl¹⁴C-labeled glyphosate (1.87 mCi/mmol) plus 0.8% (v/v) surfactant was placed in a lanolin circle on unifoliolate bean leaves. No other herbicide application was made. One, 2 and 4 h after treatment the lanolin enclosed areas were removed, the remainder of each leaf was homogenized, and the radioactivity was determined.

Absorption of radioactive glyphosate into single cells of treated leaves was also examined. One-tenth ml distilled H₂O containing 0.2 μCi (1.3 mM) of methyl¹⁴C-labeled glyphosate (0.5 mCi/mmol) plus 0.8% surfactant was evenly distributed over the surfaces of untreated bean leaves. Immediately and 1, 2, 4, and 24 h after treatment, leaves were removed and rinsed with 0.1 mM [¹²C]-glyphosate. Single cells were isolated in a medium containing 0.1 mM [¹²C]glyphosate to minimize cellular loss of any absorbed [¹⁴C]glyphosate. The washed cells were quantitatively transferred to a scintillation vial and the amount of radioactivity determined.

Bean Growth and Physiological Changes in Response to Glyphosate. Dry weight and moisture content of glyphosate-treated leaves were measured over 96 h. At each sampling time, leaves were removed from the bean plants, weighed and dried at 75 C to a constant weight. In other studies leaf area, leaf position, and stomatal resistance were measured at several sampling times during the first 24 h after herbicide application. Inasmuch as the bean leaf shape approached that of a triangle, the formula $a = 0.5 bh$ where *a* is area, *b* is the base or widest part of the leaf and *h* is midrib length was used. Leaf position was determined by measuring the acute angle made by the leaf blade and stem while stomatal resistance was measured with a ventilated diffusion porometer.

Plant water status in response to glyphosate application was determined by using a pressure bomb as described by Scholander *et al.* (22). The negative of the pressure measured was converted

The mechanism of action of glyphosate, a nonselective herbicide, has been studied in several research laboratories. Results from some of these studies have indicated that glyphosate affects the aromatic amino acid biosynthetic pathway (10, 13, 17, 21, 24) while others have reported findings inconsistent with this hypothesis (4, 8). Studies conducted with intact plants (27), bean root mitochondria (9) and pea (*Pisum sativum* L.) chloroplasts (20) indicate that respiration and photosynthesis are not the primary sites of glyphosate activity. Abu-Irmaileh *et al.* (1) have reported that production of C₂H₄ increased within 12 h and CO₂ within 24 h when bean plants were treated with glyphosate. They postulated the rise in C₂H₄ production may be responsible for glyphosate phytotoxicity.

An induction of phenylalanine ammonia-lyase (PAL) activity in maize (*Zea mays* L.) and soybeans (*Glycine max* [L.] Merr.) has been observed as a result of glyphosate treatment (4, 5). The authors concluded that the observed PAL induction may cause the growth effects observed after glyphosate application.

Campbell *et al.* (3) reported that glyphosate caused partial to complete disruption of the chloroplast envelope in quackgrass (*Agropyron repens* [L.] Beauv.) with eventual disintegration of the chloroplasts within 24 h of herbicide application. They indicated that this may be the result of glyphosate enhancement of the senescence process by altering membrane permeability and subsequently causing changes in osmotic potentials. Nilsson (17) has also suggested that glyphosate enhances senescence.

The studies reported here were conducted to characterize further the effects of glyphosate on specific metabolic systems in relation to observed glyphosate toxicity symptoms.

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to bars and was used as water potential.

Effect of Glyphosate on Isolated Cell Metabolic Processes. The procedures described by Jensen *et al.* (14) and Ashton *et al.* (2) were used to measure the effect of glyphosate on protein and RNA synthesis, respiration and photosynthesis.

A second method was employed to measure respiration. Twenty-four h after glyphosate application 2 ml of cell suspension plus incubation cofactors were placed in a sealed chamber, and the dark O₂ consumption was monitored for 1 h with a Clark O₂ electrode (Yellow Springs Instrument Co.).

Effect of Glyphosate on Ion Absorption by Single Cells. Isolation of single cells from bean leaves was begun 1.5 h after herbicide application. One mM glyphosate was included in maceration and assay media to ensure constant herbicide exposure. Two ml of cell suspension were placed in 25-ml Erlenmeyer flasks along with either 5 μ Ci of ⁸⁶RbCl (3.45 mCi/mg) or 5 μ Ci of [¹⁴C]juracil. After a 30-min incubation period (a total of 3 h after the initial glyphosate treatment to the intact leaves), absorption of the labeled materials was measured using the procedure of Ashton *et al.* (2).

A second method was employed to study the effect of glyphosate on ion absorption. Single cells isolated from untreated leaves were suspended in 0.35 M sorbitol plus 50 mM Mes buffer (pH 5.8) and were subsequently treated with 1 mM glyphosate for 1 h. Two ml of cell suspension were then placed in 25-ml Erlenmeyer flasks containing either 1 mM KH₂PO₄ labeled with 5 μ Ci of ³²P, 1 mM KCl labeled with 2 μ Ci of ⁸⁶Rb or 5 μ Ci of [¹⁴C]juracil. Absorption was measured after a 30-min incubation.

Effect of Glyphosate on Plant Cell Integrity. Electrolyte leakage from leaves treated with 10 mM glyphosate plus 0.5% (v/v) surfactant, 1 mM paraquat (1,1'-dimethyl-4,4'-bipyridinium ion) or 1 mM DNP³ was measured. Eight h after herbicide application leaves were excised and thoroughly washed with distilled H₂O before being placed in a beaker with 75 ml of fresh distilled H₂O. After the beakers had been on an oscillating shaker for 12 h, the conductivity of the distilled H₂O bathing solution was measured using a conductivity bridge.

The leakage of ⁸⁶Rb from glyphosate-treated tissue was measured utilizing excised bean leaves exposed for 12 h to 15 ml of a solution containing 0.5 mM CaSO₄, 50 mM Mes buffer (pH 5.8) and 5 μ Ci of ⁸⁶Rb. After ⁸⁶Rb exposure, the leaves were thoroughly washed with 0.1 mM RbCl and were placed in 50-ml beakers with 20 ml of 0.5 mM CaSO₄, 50 mM Mes (pH 5.8) and 10 mM glyphosate for 6 h. Fifteen-mm diameter discs were cut from the leaves and were floated on 5 ml of distilled H₂O. Four hours later, the radioactivity of a 1-ml aliquot of the bathing solution was determined. The labeled leaf discs were then solubilized with 0.5 ml hyamine hydroxide and bleached for 24 h with benzoyl peroxide. Dioxane scintillation fluid was added, and radioactivity determined.

Effect of Glyphosate on ATP Formation. The procedure of Moreland *et al.* (16) was followed to extract ATP from leaf tissue treated for up to 3 h with 10 mM glyphosate. The luciferase technique (7) was used to determine the ATP content of the extracts.

Chelation Studies. The nuclides ⁸⁶Rb and ³²P were mixed with [¹²C]glyphosate to determine whether the herbicide was chelating the ions. A TLC technique developed by Sprinkle (26) was used for separation of glyphosate from other components and for R_F determination. Locations of ⁸⁶Rb and ³²P were determined by dividing the TLC plates into small segments, scraping the absorbant into toluene scintillation fluid and measuring the radioactivity present in each sample.

RESULTS AND DISCUSSION

Absorption and Glyphosate. Glyphosate absorption into intact bean leaves was rapid and progressed linearly for 4 h following application (Table I). Individual mesophyll cells within the treated leaves, however, did not appear to absorb the [¹⁴C]glyphosate readily (Table I).

Bean Growth and Physiological Changes in Responses to Glyphosate. A reduction in the rate of dry matter accumulation began within 30 h after treatment and became more pronounced with time (Fig. 1). In addition, bean leaf expansion was significantly inhibited within 25 h (data not shown). These overall growth reductions in response to glyphosate treatment were assumed to be the result of earlier effects on one or more specific physiological processes.

Glyphosate caused a rapid change in both bean leaf angle with the stem and in stomatal resistance. The leaf angle of treated plants (32° from vertical) was significantly different from that of control plants (42° from vertical) within 2 h of glyphosate treatment even though the leaves were under no visible water stress. Stomatal resistance increased significantly (117% greater than the control) within 1 h of application indicating that glyphosate quickly caused stomatal closure. These rapid effects on leaf angle and stomatal opening could be the result of an interference with (a) one or more processes including the K⁺ ion pump which controls leaf angle and stomatal opening by directly affecting water potential and thereby turgor pressure of pulvinus cells and guard cells; (b) energy production needed for the operation of the K⁺ pump; (c) membrane integrity, the loss of which could result in electrolyte leakage and hence reduced turgor pressure; or (d) water status of the plant resulting in an increase in ABA which can either prevent K⁺ uptake or result in increased K⁺ efflux and subsequent loss in normal cellular function (19).

The effect of glyphosate on stomatal opening and leaf angle, when considered with the observation that bean leaves wilt as one of the later symptoms of glyphosate toxicity, suggests that the initial effect of glyphosate is on plant water status. However, water potential measurements on treated bean plants showed that treated plants were under less water stress than controls (-4.2 bars for the control as against -2.5 bars for the treated plants). These results agree with those reported by Schaner (23), who observed similar effects of glyphosate on bean leaf water potential. He also found that the transpiration rate of glyphosate treated plants decreased, but not until 5-6 h after treatment or 4-5 h after a significant increase in stomatal resistance was observed. This apparent discrepancy may be due to differences in methodology. Perhaps the technique used to determine transpiration (dividing the change in weight of a cup containing an individual plant by total leaf area

Table I. ¹⁴C-Methyl Glyphosate Absorption into Entire Intact Bean Leaves and Into Single Cells Isolated from Treated Bean Leaves

Ten μ l of a solution containing 0.08 μ Ci (1.87 mCi/mmol) of methyl ¹⁴C-labeled glyphosate was placed in a lanolin ring on each treated leaf. Treated area was removed prior to radioactivity analysis. Cells were isolated from leaves treated with 0.1 ml of distilled H₂O containing 0.2 μ Ci of methyl ¹⁴C-labeled glyphosate (1.5 mCi/mmol).

Time after Glyphosate Application	Leaf Absorption		Cellular Ab- sorption
	cpm absorbed/leaf	% total	
<i>h</i>			<i>cpm absorbed/ cells released from two leaves</i>
1			8
2	4,715	3	49
4	12,899	9	60
24	28,370	20	31

³ Abbreviation: DNP: 2,4-dinitrophenol.

of that plant) was not as sensitive to changes in stomatal aperture as was the ventilated diffusion porometer used in the studies reported here.

The water potential results were further substantiated by the fact that the moisture content of treated leaves paralleled that of untreated leaves for 96 h (Fig. 1) or well after the time of observed differences in weight, expansion, leaf angle, and stomatal resistance. It appears, therefore, that glyphosate does not initially affect water status but rather some other process(es).

Effect of Glyphosate on Isolated Cell Metabolic Processes. Compared to controls, there was a significant reduction in the ability of cells isolated from glyphosate-treated leaves to absorb and metabolize labeled precursors (Table II). Absorption and incorporation of [¹⁴C]uracil was significantly affected within 3 h while absorption and incorporation of [¹⁴C]leucine and evolution of ¹⁴CO₂ from [¹⁴C]glucose were not significantly reduced until 7 h after glyphosate treatment. Significant inhibition of ¹⁴CO₂ incorporation did not occur until 7 h after treatment or approximately 6 h after changes were observed in stomatal resistance. Stomatal closure following glyphosate treatment is apparently not due to lack of photosynthate needed to maintain turgor pressure.

According to Francki *et al.* (6), uptake and incorporation of labeled precursors in single cell suspensions often parallel each other over a wide variety of conditions. They suggest that a specific level of inhibition of a biosynthetic reaction would show a concomitant inhibition of precursor uptake. Based on this, if absorption were affected to a greater degree than incorporation, the specific effect of an inhibitor would likely be on absorption

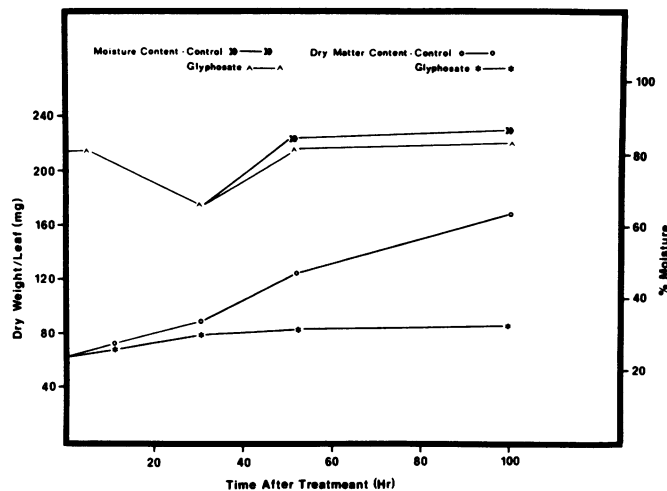


FIG. 1. The effect of 1 mM glyphosate on dry matter accumulation and moisture content of bean leaves.

Table II. Effect of Glyphosate on Absorption and Metabolism of Several Metabolic Precursors by Cells Isolated from Bean Leaves

Cells were isolated at various times after glyphosate treatment and the effect on cellular metabolism was determined.

Time after 1 mM Glyphosate Application	[¹⁴ C]Leucine		[¹⁴ C]Uracil		[¹⁴ C]Glucose		NaH ¹⁴ C-O ₃ Incorporation
	Up-take	Incorporation	Up-take	Incorporation	Up-take	Incorporation	
h	% control						
3	108	107	41 ^a	67 ^a	107	89	87
5	106	129	60 ^a	66 ^a	90	94	92
7	48 ^a	44 ^a	50 ^a	70 ^a	86	77 ^a	24 ^a

^a Values are significantly different from controls at 0.05 level as determined by the *t* test.

itself. Table II shows that glyphosate has a greater effect on [¹⁴C]uracil absorption than on [¹⁴C]uracil incorporation after 3 h, suggesting a direct effect on absorption. Later effects on absorption and incorporation of [¹⁴C]leucine, [¹⁴C]glucose respiration and ¹⁴CO₂ incorporation seem to be due to an initial reduction in precursor metabolism followed by decreased uptake. These results indicate that glyphosate causes a general slowdown of several metabolic processes rather than having a dramatic effect on specific synthetic reactions.

Glyphosate did not adversely affect respiration (as measured by O₂ consumption) required for the production of energy used for both solute absorption and incorporation (data not shown). These results agree with the report of Hanson *et al.* (9) that O₂ utilization by mitochondria isolated from bean plants was not affected 96 h after glyphosate treatment even though the plants showed visual toxicity symptoms. The results obtained using a Clark O₂ electrode to measure respiratory capacity of cells were slightly different from the results obtained with [¹⁴C]glucose. Similar discrepancies have been observed by others (12). It is generally accepted that determining O₂ consumption with the O₂ electrode is a better method of respiration measurement.

Effect of Glyphosate on Ion Absorption. Since the results of the studies on [¹⁴C]uracil uptake and incorporation by bean cells indicated that glyphosate had a rapid effect on absorption, studies were initiated to examine the effects of glyphosate on ion absorption by cells from both pretreated and untreated leaves (Tables III and IV).

Cells isolated from leaves pretreated with 1 mM glyphosate absorbed less radioactive Rb and uracil than cells from untreated tissue (Table III). The total time of exposure to glyphosate was only 3 h (1.5 h in intact leaf, 1 h during cell isolation and 0.5 h incubation in suspension media) indicating a very rapid effect. Shorter treatment times with intact leaves proved difficult; therefore, single cells from untreated leaves were exposed to glyphosate

Table III. Effects of Glyphosate on ⁸⁶Rb and [¹⁴C]Uracil Absorption by Cells Isolated From Untreated and Glyphosate-treated Leaves

Total time of glyphosate treatment was 3 h. The radiolabeled materials were present for the final 30 min of the glyphosate treatment period.

Treatment	Absorption ^a		Reduction Compared to Control	
	[¹⁴ C]Uracil	⁸⁶ Rb	⁸⁶ Rb	[¹⁴ C]Uracil
	cpm/10 ⁵ cells		%	
Control	1332a	6634x		
1 mM glyphosate	925b	4086y	38	31

^a Values in the same column followed by different letters are significantly different at the 0.05 level as determined by a *t* test.

Table IV. Effect of Glyphosate on [¹⁴C]Uracil, ⁸⁶Rb and ³²P Absorption by Cells Isolated from Untreated Bean Leaves

Total time of glyphosate treatment was 1.5 h. The labeled materials were present for the last 30 min of the glyphosate treatment time.

Treatment	Absorption ^a			Reduction Compared to Controls		
	[¹⁴ C]Uracil	⁸⁶ Rb	³² P	[¹⁴ C]Uracil	⁸⁶ Rb	³² P
	cpm/10 ⁵ cells			%		
Control	1857a	4912o	3675y			
1 mM glyphosate	1701a	3095p	2364z	8	37	36

^a Values within the same column followed by different letters are significantly different at the 0.05 level as determined by a *t* test.

after isolation to determine how rapidly absorption was inhibited.

Cells taken from leaves of untreated plants and exposed to 1 mM glyphosate for 1.5 h absorbed significantly less ^{86}Rb and ^{32}P (Table IV). [^{14}C]Uracil absorption was not significantly affected during this short period of exposure. These results coupled with those involving isolated cell precursor uptake and metabolism indicate that one mechanism of action of glyphosate in bean leaves involves a cellular inhibition of ion absorption which occurs before an effect on metabolic precursors is observed. Several biosynthetic reactions were generally affected after 7 h of treatment (Table II); however, only the inhibition of ion absorption could be shown as a rapidly occurring event.

If the theory that stomatal opening is controlled by a K^+ ion pump (11) is accepted, then inhibition of ion absorption would explain stomatal closure observed in response to glyphosate treatment. Inhibition of an ion pump could also account for a decrease in pulvinus cell turgor pressure resulting in changes in leaf angle of glyphosate treated leaves.

Effect of Glyphosate on Plant Cell Integrity. To determine whether reduction in ion absorption caused by glyphosate was the result of the loss of membrane integrity, its effects on electrolyte leakage were compared with paraquat and DNP. Paraquat and DNP caused a significant increase in the loss of electrolytes from bean leaves (212 and 90% increases, respectively, compared to the control) while glyphosate had little effect (7% increase). Similar results were observed by Prendeville *et al.* (18) when electrolyte leakage from bean leaves was measured 13 h after treatment with 1 mM glyphosate. In addition, floating leaves on 10 mM glyphosate did not cause any efflux of radiolabeled material from leaf tissue prelabeled with ^{86}Rb for 12 h (less than 0.2% of that present in the leaf discs). These results indicate that the initial effect of glyphosate on bean plants does not involve loss of membrane integrity and that the reduction in ion uptake caused by glyphosate is apparently due to other effects on the ion absorption processes such as reducing the ATP supply required for active transport or directly affecting some ion uptake mechanism.

Effect of Glyphosate on ATP Formation. ATP contents of glyphosate-treated plants range from 7 to 15% greater than controls during the first 3 h after treatment (data not shown), the same time period when a significant reduction of [^{14}C]uracil, ^{86}Rb and ^{32}P absorption occurred. These results verify that glyphosate has a more direct effect on solute absorption than through an effect on energy production.

Chelation Studies. TLC studies revealed that both ^{86}Rb and ^{32}P , in the presence of high levels of glyphosate, had R_F values similar to those obtained when the ions were applied individually to TLC plates ($R_F = 0.65$ and 0.57 , respectively, alone; $R_F = 0.72$ and 0.62 , respectively, when mixed with glyphosate). These results suggest that the effects of glyphosate on ion absorption are not due to chemical chelation.

The results reported here indicate that glyphosate causes rapid physiological changes in bean plants probably as a result of a decrease in cellular ion uptake. It is postulated that glyphosate directly affects ion absorption by bean leaf cells, perhaps by inhibiting an ATPase associated with the plasma membrane which

mediates ion absorption. Such an ATPase has been shown to be involved in the K^+ absorption in animal cells (25) and has also been implicated in higher plant ion transport (15).

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