Plant Physiol. (1991) 97, 1109–1114
0032-0889/91/97/1109/06/$01.00/0

Received for publication May 22, 1991
Accepted July 15, 1991

Effect of *N*-(Phosphonomethyl)glycine on Carbon Assimilation and Metabolism during a Simulated Natural Day[1]

Wen-Jang Shieh, Donald R. Geiger*, and Jerome C. Servaites
Department of Biology, University of Dayton, Dayton, Ohio 45469-2320

ABSTRACT

The effects of *N*-(phosphonomethyl)glycine (glyphosate) on the regulation of carbon assimilation, metabolism, and translocation were studied in leaves of sugar beet (*Beta vulgaris* L., Klein E-type multigerm) under a light regimen that began with gradually increasing irradiance as generally occurs on a natural day. Soon after application, glyphosate caused a marked increase in ribulose bisphosphate and a decrease in phosphoglyceric acid. The response is most simply explained by direct inhibition of ribulose bisphosphate carboxylase activity. The extent of inhibition was small, and the carbon assimilation rate did not decrease. As predicted, photosynthesis declined within an hour after glyphosate was applied to leaves under gradually increasing light. Inhibition resulted from a decrease in ribulose bisphosphate due to depletion of carbon from the photosynthetic carbon reduction cycle. Photoinhibition, a light-dependent limitation of photosynthetic capacity, appeared to be necessary for marked glyphosate-induced inhibition of photosynthesis. As a result, photosynthesis rate increased with irradiance until it exceeded 400 micromoles per square meter per second but then declined as the light level increased beyond 500 micromoles per square meter per second. Glyphosate changed the allocation of newly fixed carbon between starch and sucrose for export. Changes in the levels of ribulose bisphosphate and phosphoglyceric acid produced important effects on the regulation of carbon assimilation and metabolism.

It is well documented that the herbicide glyphosate[2] inhibits the enzyme 5-*enol*pyruvyl shikimic acid-3-phosphate synthase (EC 2.5.1.19) (1, 17) and, as a result, increases entry of carbon into the shikimate pathway (1, 2, 17). Increasing the amount of carbon that is diverted from the PCR cycle to shikimate-3-phosphate (12, 17) decreases levels of PCR cycle intermediates, particularly RuBP and PGA, in sugar beet leaves (4, 21). In addition, increasing entry of carbon into the shikimate pathway may cause an excessive amount of energy to be dissipated by the increased use of ATP and phospho*enol*pyruvate (12, 17).

The well-defined effects of glyphosate on the PCR cycle are useful for studying regulation of photosynthesis, carbon metabolism, and translocation in sugar beet leaves (8, 21). Application of glyphosate to leaves causes the level of RuBP to decrease (4, 21), and, under RIL, this causes inhibition of NCE after several hours (5, 8, 10). Glyphosate also markedly changes allocation of recently fixed carbon between starch and sucrose. As soon as NCE begins to decrease, the rate of starch accumulation slows to near zero, but sucrose synthesis and translocation continue undiminished (8). The nearly complete inhibition of starch synthesis coincides with a decline in PGA (21) and likely results from reduction in adenosine diphosphoglucose pyrophosphorylase (EC 2.7.7.27) activity (9).

Recently, we observed that the diurnal time courses of Rubisco activation state and RuBP and PGA levels in sugar beet leaves are consistently different under GIL from what they are under RIL (7). Under RIL, the RuBP level is about twice as high as it is at midday under GIL, but the Rubisco activation state is only half of what it is under GIL. The ability to change the Rubisco activation state and the levels of RuBP and PGA by altering the light regimen and by glyphosate treatment provides an opportunity to study interaction of these factors in the regulation of carbon assimilation (23). The questions that we were particularly interested in examining were the level of RuBP that is needed for maintaining photosynthesis rate and how the level of RuBP interacted with Rubisco activation state. We predict that the lower RuBP level present in leaves under GIL will likely result in a more rapid inhibition of NCE following glyphosate application (21) and, consequently, could cause other related effects on carbon allocation.

When glyphosate inhibits NCE, it disrupts the balance between the absorption of light and its use for carbon assimilation. These conditions can result in photoinhibition, the light-dependent lowering of photosynthetic capacity (13, 14, 16). We have seen evidence of time-dependent deterioration under steady high light conditions (8). The gradual increase in irradiance that occurs under GIL provides a means to determine whether increasing light level is a factor in the inhibition of NCE and, if so, to identify the threshold level at which photoinhibition begins. Furthermore, the shorter period at full light under GIL should produce a less severe response.

[1] This work was supported by grants from the Monsanto Agricultural Company, the National Science Foundation (DCB 8915789), and the Ohio Board of Regents/Research Challenge Program.

[2] Abbreviations: glyphosate, *N*-(phosphonomethyl)glycine; PCR cycle, photosynthetic carbon reduction cycle; RuBP, ribulose bisphosphate; PGA, phosphoglyceric acid; RIL, light regimen under which irradiance increases rapidly to a maximal level at the beginning of the light period; NCE, net carbon exchange; GIL, light regimen that begins with a gradual increase in irradiance as under natural daylight; PRK, phosphoribulokinase (EC 2.7.1.19).

MATERIALS AND METHODS

Plant Material

Sugar beet plants (*Beta vulgaris* L., Klein E-type multigerm) were grown in a plant growth room as described earlier (7). Plants were maintained under a 14-h photoperiod at 25°C, 60% RH day and 17°C, 75% RH night. Irradiance was increased and decreased in three steps to a maximum of 800 μmol m^{-2} s^{-1} at canopy level as described previously (7).

Measurement of NCE and Transpiration

Typically, two recently matured sugar beet leaves, leaves 4 and 5, of a 6-week-old plant were used for photosynthesis and gas exchange measurements. All experiments were carried out under a GIL light regimen in which the photoperiod began with gradually increasing irradiance similar to what occurs during natural daylight, as described previously (7). To measure gas exchange, fourth and fifth leaves were enclosed in separate chambers connected in parallel to a closed apparatus for circulating the atmosphere and measuring the rates of CO_2 exchange (3). NCE rates were measured under GIL on the first day without adding glyphosate, and, on the following day, a solution of 17 mM glyphosate, in the range used in practical applications, was applied in 0.01% Tween 20 at 50 mL m^{-2} to leaves 6 h after the beginning of the light regimen. Measurements of the effects of glyphosate on NCE and other processes were done on at least three plants with similar results. In each case, representative data for a single plant are given because averaging data from several plants is known to mask the effects being described even when the timing of the events differ only slightly.

An IR gas analyzer (MSA model 3000, Pittsburgh, PA) was used to measure CO_2 concentration. The NCE rate was determined by recording the inflow of CO_2 with a computer-controlled mass flow controller (Tylan model FC 260, Carson, CA). Flux was controlled to supply CO_2 at the rate required to maintain it at a set level in the atmosphere around the leaves.

Export and Sucrose Synthesis Rates

Export and sucrose synthesis rates were calculated according to the method of Geiger *et al.* (5). Source leaves were labeled with $^{14}CO_2$ of constant specific radioactivity maintained at a constant CO_2 concentration. Accumulation of radioactivity was monitored with a GM tube located beneath the source leaf. Export rates were calculated from the difference of the cumulative photosynthesis less the labeled carbon accumulated in the source leaves.

Enzyme and Metabolite Measurement

Leaf samples were taken from adjacent leaves outside the leaf chambers during gas exchange measurements. Leaf tissue was rapidly frozen in place by clamping tissue between brass cylinders cooled to liquid nitrogen temperature as described by Servaites *et al.* (21). The initial Rubisco activity and the leaf levels of RuBP and PGA were determined in leaf extracts as described previously (18, 19). For *in vitro* measurement of the inhibition of Rubisco activity, Rubisco was purified from sugar beet leaves by the method of Hall and Tolbert (11). Reactions were initiated by adding RuBP and various amounts of glyphosate to vials that contained fully activated sugar beet Rubisco for assaying total Rubisco activity according to the method of Servaites *et al.* (19).

RESULTS

Photosynthesis Rate

The rate of NCE decreased within 1 h following application of glyphosate (Fig. 1). The exact timing of the decline was difficult to ascertain because NCE also decreased in control leaves as irradiance gradually decreased under GIL. Throughout the remainder of the light period, the rate of NCE in glyphosate-treated leaves remained approximately 2 μmol m^{-2} s^{-1} lower than the control rate (Fig. 1). On the following day, the NCE rate increased with irradiance for the first 2 h and then, when irradiance reached 400 μmol m^{-2} s^{-1}, the increase began to slow. By 3 h, when irradiance reached 500 μmol m^{-2} s^{-1}, the NCE rate reached its maximum which was only 45% of the control, and thereafter, it decreased gradually even though irradiance continued to increase. By midday, NCE was only about 25% of the control rate.

Initial Rubisco Activity and Levels of RuBP and PGA

Within 15 min after application, glyphosate caused a rapid increase in the level of RuBP and a corresponding decrease in the PGA level (Fig. 2, B and C). After more than doubling in the first one-half hour, RuBP then decreased sharply. By 2 h after application, the level of PGA began to decrease along with the RuBP concentration. Both metabolites then remained at a low levels for the remainder of the day. On the day following glyphosate application, NCE, RuBP, and Rub-

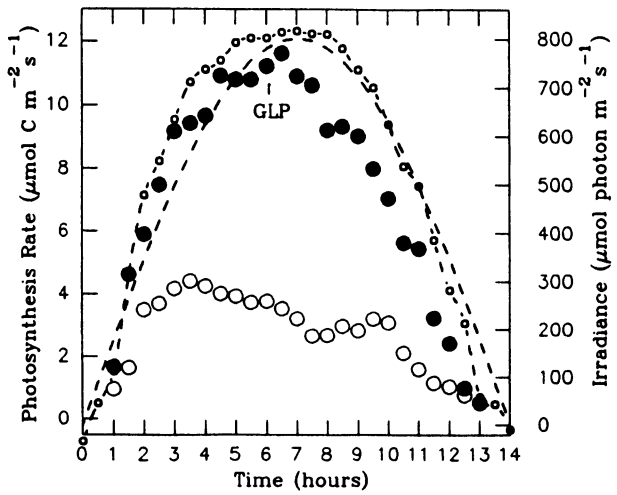

Figure 1. Time course of sinusoidal irradiance and of NCE rate in sugar beet leaves during GIL. ---, Irradiance time course. NCE rates: O, for the control day, before treatment; ●, the day when glyphosate (GLP) was applied, O, the day following glyphosate application. Glyphosate was applied (arrow) at 6 h.

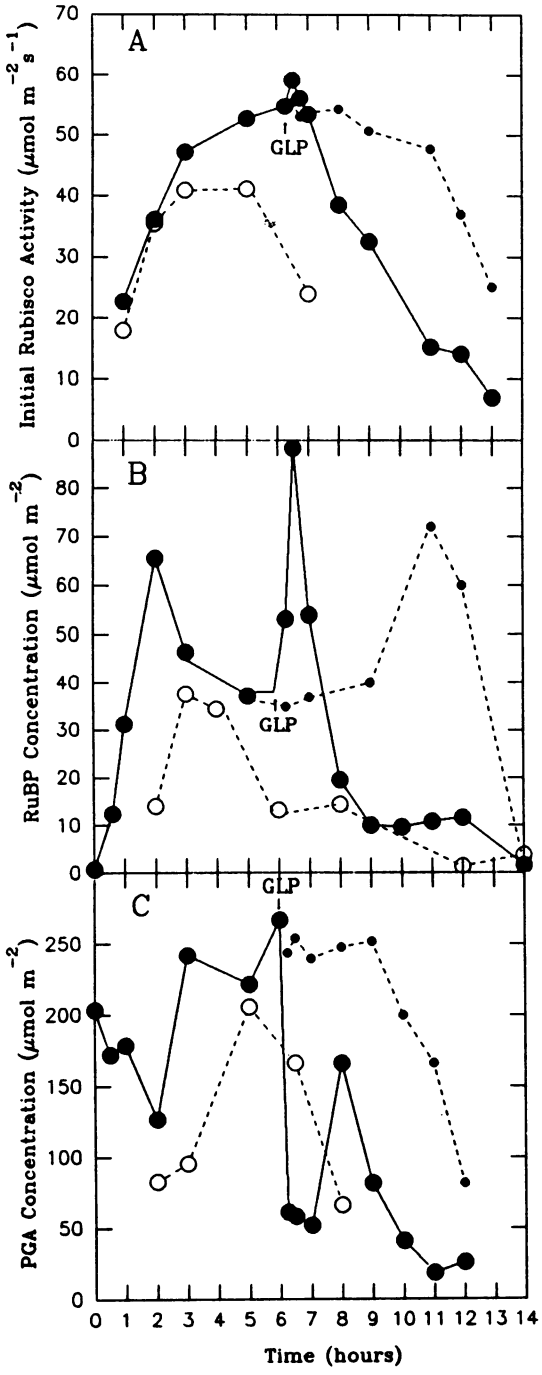

Figure 2. Effect of glyphosate (GLP) on initial Rubisco activity and levels of RuBP and PGA in sugar beet leaves. Values for initial Rubisco activity (A) and levels of RuBP (B) and PGA (C) are given for the day of glyphosate application (●) and the day following glyphosate application (○). Values for a control leaf during the last half of the day are shown for comparison (small ●). Arrow, Time of application of glyphosate.

isco activity increased during the morning and then declined before midday (Figs. 1 and 2).

Initial Rubisco activity increased with increasing irradiance before application of glyphosate and reached nearly full activation by midday (Fig. 2A). Immediately following application of glyphosate, the initial Rubisco activity appeared to increase slightly and, about 1 h after glyphosate application, began a gradual decrease. Initial Rubisco activity decreased faster than it did under control conditions, diminishing in parallel with the decline in NCE (Figs. 1 and 2A). On the second day, initial Rubisco activity increased for the first 3 h as in control leaves but, after irradiance reached about 400 μmol m^{-2} s^{-1}, activity stopped and then began to decrease as light continued to increase.

Glyphosate was found to be an inhibitor of purified sugar beet Rubisco *in vitro* (Fig. 3). At a glyphosate concentration of 10 mM and a RuBP concentration of 30 μM, Rubisco was inhibited about 17%.

Starch Synthesis

The time course of accumulation of transient starch in control leaves coincided with that for cumulative NCE, except for the first and last 2 to 3 h of the day when irradiance was low and starch was degraded (Fig. 4A). Approximately 40% of recently assimilated carbon was allocated to accumulation of starch during the middle of the day. When glyphosate was applied, the rate of starch accumulation decreased in proportion to NCE for several hours (Fig. 4B), and then accumulation stopped and starch began to degrade as NCE declined.

Export

The time course for export in untreated leaves also coincided with that for NCE, except for the time at the beginning and end of the day when it was supplied by starch degradation (Fig. 5). During the middle of the day, approximately 50% of recently assimilated carbon was allocated to export. Following

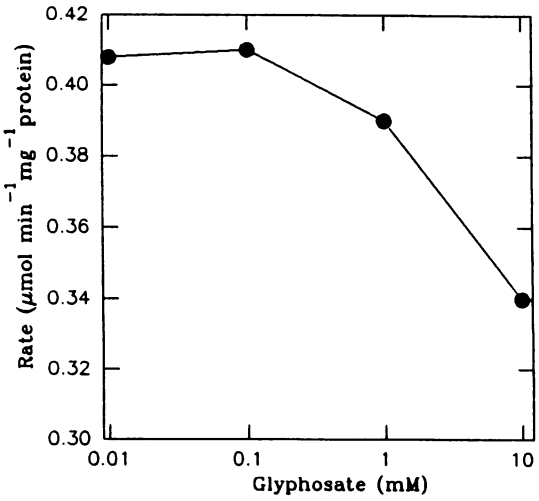

Figure 3. *In vitro* inhibition of total Rubisco activity by glyphosate at various concentrations. RuBP concentration in assay solution was 30 μM.

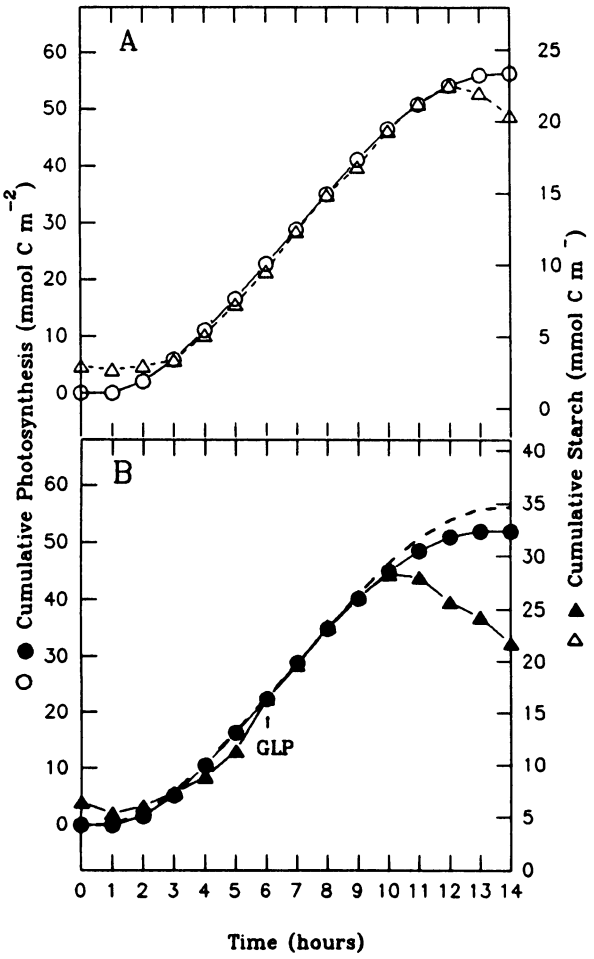

Figure 4. Time course of cumulative NCE and accumulated starch in sugar beet leaves. Values for NCE (O; ●) and starch (△, ▲) are given for the day before (A) and the day of glyphosate (GLP) application (B). The time course for cumulative NCE on the day before application (---) is shown in B for comparison.

application of glyphosate, export decreased by a greater proportion than NCE until net degradation of transient starch began 4 h later. After this point, export rate was similar to that in leaves under control conditions.

DISCUSSION

As predicted, NCE was inhibited markedly within 1 h following the application of glyphosate to sugar beet leaves under GIL (Fig. 1). Likewise, the decline in NCE occurred only when the RuBP level declined below the midday level (Fig. 2B), indicating that the level at this time was close to, but above, the level needed to sustain NCE. The fact that NCE decreased markedly with a small change in RuBP level indicates that there are mechanisms that can adjust and maintain the RuBP level within narrow limits (20).

The rapid transient increase in the substrate RuBP and the corresponding decline in the product PGA (Fig. 2, B and C) following glyphosate application is most simply explained as a weak direct inhibition of Rubisco by glyphosate, although other mechanisms may be involved. This transient decline did not occur under RIL, when the level of RuBP was high (21), suggesting that a high RuBP level prevents glyphosate inhibition of Rubisco. There was no observed effect on the NCE rate during this time, indicating that the degree of inhibition of Rubisco was slight (Fig. 1), conceivably because the transient increase in RuBP level was large enough to compensate for the inhibitory effects of glyphosate on the Rubisco reaction rate. We calculated that an inhibition of just 0.05% of the Rubisco reaction rate would be sufficient to produce the observed 0.03 μmol C m^{-2} s^{-1} rate of RuBP accumulation. This estimate was based on the measured NCE rate of 12 μmol C m^{-2} s^{-1} (Fig. 1), which required a Rubisco reaction rate of 60 μmol C m^{-2} s^{-1} to sustain it (15). Measurements of the effect of glyphosate on purified Rubisco confirmed that it is a weak inhibitor of Rubisco (Fig. 3). Under the *in vitro* assay conditions used, a glyphosate concentration of just >100 μM would be sufficient to cause a 0.05% inhibition of Rubisco. Glyphosate was applied to leaves at a concentration of 17 mM, equivalent to the recommended

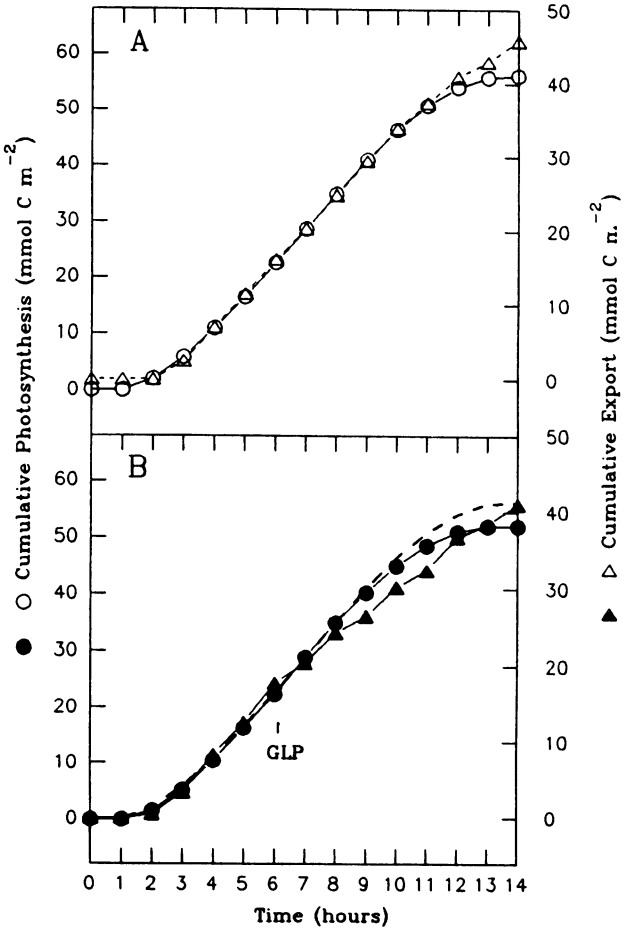

Figure 5. Time course of cumulative NCE and cumulative export in sugar beet leaves. Values for NCE (O, ●) and export (△, ▲) are given for the day before(A) and the day of glyphosate (GLP) application (B). The time course for cumulative NCE on the day before application (---) is shown in B.

agricultural dose, and it seems reasonable that at this dose there was enough glyphosate in the leaf at this time to inhibit Rubisco *in vivo*.

Inhibition of NCE (Fig. 1) was accompanied by parallel decreases in both RuBP level (Fig. 2B) and initial activity of Rubisco (Fig. 2A) showing the close interaction between the latter in regulating photosynthesis. Deactivation of Rubisco followed the glyphosate-induced decline in the level of RuBP. Streusand and Portis (22) found that a certain amount of RuBP is needed for operation of Rubisco activase *in vitro*. Rubisco also deactivates under RIL when RuBP declines to a level similar to that observed here (18), but because the RuBP level was much higher initially, deactivation occurred only after about 8 h. It appears likely that deactivation occurs when the free RuBP level declines below the level of Rubisco active sites. Hence, some threshold level of RuBP appears to be needed to maintain Rubisco activation in the leaf.

A high irradiance level also appears to be necessary for a significant degree of glyphosate-induced inhibition of NCE. Although at low levels of irradiance NCE increased with increasing irradiance on the morning following glyphosate application (Fig. 1), after irradiance exceeded 400 μmol m^{-2} s^{-1}, NCE stopped increasing with increasing irradiance and began to decline as the light level increased beyond 500 μmol m^{-2} s^{-1}. Photoinhibition, a light-dependent limitation of photosynthetic capacity, is likely to occur under these conditions because glyphosate inhibits the use of excitation energy by the PCR cycle without slowing its absorption (16). We previously observed that, several h after applying glyphosate, a time-dependent loss of photosynthetic capacity occurred when leaves were maintained under the accustomed constant high irradiance of RIL (8). A progressively smaller response of NCE to increasing CO_2 concentration (8) or light level (D. R. Geiger, unpublished results) with time under the accustomed high irradiance level indicated to us that an increasing degree of photoinhibition occurred with time following glyphosate application. In the light of these data, it seems reasonable to conclude that there was also a progressive loss of photosynthetic capacity at high irradiance during the first day (Fig. 1).

The decrease in NCE, which began about 1 h after application of glyphosate, caused a proportionate decrease in starch accumulation (Fig. 4B). As a result of the decrease in NCE and starch synthesis rate, degradation of starch began about 2 h earlier than in control leaves (Fig. 4, A and B). By contrast, under RIL (6, 8, 10), starch accumulation declined much faster than did NCE following the application of glyphosate and soon declined to near zero. During RIL, starch synthesis appeared to stop because of the glyphosate-induced lowering of the PGA level and the resulting lowering of the PGA:Pi ratio and adenosine diphosphoglucose pyrophosphorylase activity (9). The large, transient decline in PGA concentration, which occurred shortly after application of glyphosate (Fig. 4C), did not markedly inhibit starch synthesis, in contrast to what occurred at this PGA level during RIL (21).

Export from the photosynthesizing leaf was inhibited soon after application of glyphosate (Fig. 5B), in contrast to the lack of effect under RIL (8, 10). Glyphosate probably did not inhibit sucrose synthesis directly, because export returned to the control rate after NCE slowed and carbon for its synthesis came from starch rather than from newly fixed carbon (Fig. 5B). It is clear that glyphosate affects carbon allocation differently under GIL than under RIL.

ACKNOWLEDGMENTS

The authors acknowledge with gratitude the technical assistance of Robert Lentz, Rose Saluke, and James Yu and the help of Thomas Trang and Julie Buczynski in growing the plants.

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