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Running title: Physiological effects of glyphosate

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Impairment of carbon metabolism induced by the herbicide glyphosate

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Summary

The herbicide glyphosate reduces plant growth and causes plant death by inhibiting the biosynthesis of aromatic amino acids. The objective of this work was to determine whether glyphosate-treated plants show a carbon metabolism pattern comparable to that of plants treated with herbicides that inhibit branched-chain amino acid biosynthesis. Glyphosate-treated plants showed impaired carbon metabolism with an accumulation of carbohydrates in the leaves and roots. The growth inhibition detected after glyphosate treatment suggested impaired metabolism that impedes the utilization of available carbohydrates or energy at the expected rate. These effects were common to both types of amino acid biosynthesis inhibitors. Under aerobic conditions, ethanolic fermentative metabolism was enhanced in the roots of glyphosate-treated plants. This fermentative response was not related to changes in the respiratory rate or to a limitation of the energy charge. This response, which was similar for both types of herbicides, might be considered a general response to stress conditions.

Keywords: glyphosate; herbicide, carbohydrates, fermentation, herbicide action.

Abbreviations: ADH, alcohol dehydrogenase; AEC, adenylate energy charge; ALS, acetolactate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase, PDC, pyruvate decarboxylase; RC, redox charge.

Introduction

Glyphosate (*N*-(phosphonomethyl) glycine) is a wide-spectrum, non-selective postemergence herbicide that is currently the most popular herbicide; since its commercial introduction in 1974, glyphosate has become the dominant herbicide worldwide. Glyphosate inhibits the biosynthesis of aromatic amino acids, which takes place in chloroplasts via the shikimate pathway. Glyphosate specifically inhibits the activity of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) (Steinrücken and Amrhein, 1980) by acting as an analog of the second substrate, phospho *enol* pyruvate.

Although the primary target site of this herbicide is widely known, it is not entirely clear how glyphosate-induced inhibition of the shikimate pathway actually kills plants. In contrast with contact herbicides, the phytotoxic symptoms of systemic glyphosate injury often develop slowly. Actual plant death can require several days or even weeks (Gruys and Sikorski, 1999). Nevertheless, specific biochemical effects can be observed much more rapidly (Foley et al., 1983). Among these effects is the accumulation of massive levels of shikimate in plant tissues because inhibition at the level of EPSPS deregulates the carbon flow through this pathway by decreasing feedback inhibition (Duke, 1988). Some authors have supported the view that this increased carbon flow results in shortages of carbon for other essential pathways (Siehl, 1997). In contrast, many authors assume that aromatic amino acid production at a level insufficient to maintain necessary protein synthesis is the main effect, and this mechanism is consistent with the slow development of symptoms (Duke and Powles, 2008). More recently, the effects of glyphosate have been studied using new molecular methods such as transcriptional comparison (Yu et al., 2007; Zhu et al., 2008; Das et al., 2010), proteomic approaches (Ahsan et al., 2008), and metabolomic profiling (Trenkamp et al., 2009) but the full picture of the sequence of metabolic disturbances after EPSPS inhibition is not yet clear.

Inhibitors of acetolactate synthase (ALS, EC 4.1.3.18; also termed acetohydroxyacid synthase) are another class of herbicides that inhibit amino acid biosynthesis. ALS is the first enzyme in the biosynthesis of the three branched-chain amino acids (valine, leucine and isoleucine). These inhibitors have become one of the most important herbicide groups because of

their wide-spectrum weed control activity, high crop selectivity, low application rates and low mammalian toxicity (Zhou et al., 2007). More than 40 structurally different active ingredients have ALS as their primary target.

Although the primary effect of these herbicides is widely known, as it is for glyphosate, it is not fully understood how plants actually die after the inhibition of ALS. Many important effects following treatments with these herbicides have been described. Among them, ALS inhibitors have been reported to alter the carbon metabolism of treated plants. Carbohydrates are accumulated in the leaves of treated plants due to a decrease in sink strength, and carbohydrates are also accumulated in the roots of treated plants (Zabalza et al., 2004). In the roots of treated plants, carbon consumption is diverted to the low-efficiency fermentative pathway and the alternative respiratory pathway (Zabalza et al., 2005; Gaston et al., 2002; Gaston et al., 2003). This impairment indicates that the effect of these herbicides on primary metabolism has broader physiological consequences than solely a lack of branched-chain amino acids.

Both types of herbicides, the EPSPS inhibitor and ALS inhibitors, have been reported to arrest the growth of treated plants; the growth arrest is followed by a slow death (Wittenbach and Abell, 1999; Gruys and Sikorski, 1999). Moreover, several physiological effects common to both glyphosate and ALS inhibitors have been reported recently (Orcaray et al., 2010); these mainly include a general increase in total free amino acid content with a transient decrease in the proportion of the amino acids whose pathways are specifically inhibited. In addition, both types of inhibitors caused quinate accumulation. Considering this evidence, similarities at the level of carbon metabolism might be expected in plants treated with glyphosate and ALS inhibitors, but there have been no exhaustive studies on the effect of glyphosate on these parameters.

The objective of this work was to evaluate the physiological effects of glyphosate on the carbon metabolism of treated plants to determine whether the secondary effects of this herbicide are similar to those of branched-chain amino acid biosynthesis inhibiting-herbicides. To achieve this goal, glyphosate was applied to pea plants, and its effects on carbohydrate content, energy status, redox charge, root respiration and fermentation were assessed because all of these parameters are altered after ALS inhibition.

Materials and methods

Plant material and treatment application

Pisum sativum L. cv Snap Sugar Boys was grown in aerated hydroponic culture as described in in Zabalza et al. (2005). Plants were 12-days old when glyphosate herbicide (commercial formula, Glyfos, BayerGarden, Valencia, Spain) was applied to the nutrient solution (to half of the plants) at a concentration of 0.23 mM (53 mg active ingredient l^{-1}). In similar previous studies, we found this concentration to arrest plant growth and to cause plant death after 20 days (Orcaray et al., 2010). For carbohydrate analysis, leaf and root samples were taken 5 h after the beginning of the photoperiod at 0, 1, 3, and 7 days after after glyphosate treatment. Leaf and root samples were taken and immediately frozen in liquid nitrogen and stored at – 80 ºC for analytical determinations.

In other set of experiments, glufosinate was added to the nutrient solution. Preliminary experiments were conducted to determine a dose with effects on growth similar to those caused by 0.23 mM glyphosate. Finally, glufosinate (commercial formula, Finale, BayerCropscience, Valencia, Spain) was applied at 280 μM (5.56 mg active ingredient l^{-1}).

Metabolite content determination

The extraction of amino acids and determination of their concentrations using capillary electrophoresis was performed as described previously (Orcaray et al., 2010). Shikimate was extracted and analyzed by HPLC as described before (Orcaray et al., 2010). The glucose, fructose and sucrose contents were determined in ethanol-soluble extracts, and the ethanol-insoluble residue was extracted for starch analysis. Starch and soluble sugar concentration determinations were performed using high-performance capillary electrophoresis as described in Zabalza et al. (2004).

The adenylate energy charge (AEC=(ATP+0.5 ADP)/(ATP+ADP+AMP)) was calculated after measuring adenylates by capillary electrophoresis as described by Galvez et al. (2005). The

redox charge (RC=($NADH^+$+NADPH)/(NAD^++NADH +$NADP^+$+NADPH)) was calculated after measuring pyridine nucleotide contents as described previously (Zabalza et al., 2011).

Respiration and fermentation measurements

Respiratory oxygen consumption was measured using Clark-type electrodes (Rank Brothers, Bottisha, UK) using small (5-10 mm) root pieces as described in Zabalza et al. (2009).

Pyruvate decarboxylase (EC 4.1.1.1) and alcohol dehydrogenase (EC 1.1.1.1) activities were assayed in desalted extract as described Gaston et al. (2002). Western blots were produced according to standard techniques as described by Zabalza et al. (2009).

Statistical analysis

Unpaired student t-test was used as test of significance. Each mean value was calculated using samples from different single plants as replications. In figures, an asterisk (*) indicates a significant difference between control and treated plants ($p \leq 0.05$) at a given day of treatment using the t-test.

Results and Discussion

The application of 0.23 mM glyphosate to the nutrient solution of pea plants caused a rapid inhibition of plant growth. Plant death required 20 days from the onset of herbicide treatment (Orcaray et al., 2010). The measurements carried out in this work at the initial phase of the treatment (one week) allowed the evaluation of the effects on carbon metabolism in the short term.

The roots of glyphosate-treated plants showed a noticeable increase in the free amino acid pool and accumulation of shikimate (Fig. 1). These two physiological effects have been also detected in leaves of the same type of plants (Orcaray et al., 2010). The glyphosate concentration used in our experiment caused similar effects in the growth arrest, shikimate accumulation and amino acid content increase described in pea plants in previous studies (Lydon and Duke, 1988; Becerril et al., 1989; Hernandez et al., 1999; Moldes et al., 2008).

This glyphosate supplementation of the nutrient solution led to soluble carbohydrate accumulation in both the leaves and roots (Figs. 2 and 3). Sucrose and glucose accumulation were significant from days 3 and 1, respectively (Figs. 2B and C). Starch content was not affected by the herbicide (Fig. 2D). The soluble carbohydrate accumulation in the leaves of treated plants might be related to an increase in photosynthesis or a lack of translocation of photoassimilates. Photosynthesis and conductance inhibition after glyphosate treatment have already been reported. Depending on the plant tested, such inhibition has been reported to be precipitous, as in sugar beets (Geiger et al., 1986) and barley (Olesen and Cedergreen, 2010),[27] or progressive, as in velvetleaf (Fuchs et al., 2002) and peas (Orcaray et al., 2010).

Previous studies have shown that this concentration of glyphosate (0.23 Mm) caused a significant photosynthesis inhibition from day 3 (Orcaray et al., 2010). Thus, the soluble carbohydrate accumulation can be only due to a lower translocation to sink tissues as soluble carbohydrate accumulation occurred before (Fig. 2) than phytosynthetic decline.

Despite the carbohydrate accumulation in leaves, there was no carbohydrate shortage in the roots. Indeed, carbohydrates also accumulated in the roots of glyphosate-treated plants. Sucrose accumulation was significant from day 3 (Fig. 3A), and starch content was increased by day 1 but then decreased to control values by day 7 (Fig. 3B).

Although changes in glycolysis have been reported in glyphosate-treated leaves (Zhu et al., 2008), few studies have evaluated the carbohydrate content in both the leaves and sinks of plants treated with glyphosate. The simultaneous study of the carbohydrate content pattern allows an evaluation of the effect on phloem transport. The increase in the sucrose and starch contents in the sinks suggests that sucrose is transported from the leaves to the roots at a higher rate than the sinks are able to use it. Under these conditions, the gradient of sugars required for long-distance transport is abolished; phloem transport is inhibited by a decrease in sink strength. A similar physiological effect has been reported after ALS-inhibiting herbicide treatment (Zabalza et al., 2004). Nevertheless, the leaves of plants treated with ALS inhibitors showed starch accumulation and glyphosate-treated plants did not, as described previously (Geiger et al., 1986). Despite the different starch patterns, it can be concluded that both ALS and EPSPS inhibitors share another aspect in their mode of action: carbohydrates accumulate in the leaves due to a decrease in sink strength.

Both types of herbicides stopped plant growth, but this inhibition was not due to a lack of respiratory substrates, as carbohydrates accumulated both in source and sink organs. The growth arrest detected after the inhibition of the biosynthesis of amino acids suggests impaired metabolism that does not allow the utilization of the available carbohydrates at the expected rate.

The effect of glyphosate on the general redox charge (Fig. 4) and energy state (Fig. 5) in leaves and roots of the treated plants was assessed. These ratios are important measurements of the cellular energy status, and deviations from the standard values are considered to be sensitive indicators of metabolic disturbances. Fig. 4 shows the redox charge (RC) defined as (NADH+NADPH)/(NAD^++NADH+$NADP^+$+NADPH), and Fig. 5 shows the adenylate energy charge (AEC) calculated as (ATP + 0.5 ADP)/(ATP+ADP+AMP). The leaves of the treated plants showed no modification of the RC or AEC (Figs. 4A and 5A). Glyphosate caused a significant decrease in RC in the roots from day 1 that lasted throughout the treatment (Fig. 4B). The AEC showed that energy was not a limitation at any time point because the AEC of treated plants was even higher than that of control plants during the study period (Fig. 5B). Similarly, growth inhibition with no associated energy restriction was recently shown after ALS herbicide treatments (Zabalza et al., 2011). As discussed before, the growth inhibition detected after glyphosate treatment suggests

impaired metabolism that does not allow the utilization of the available carbohydrates at the expected rate. Moreover, this finding should be considered together with the fact that the available energy is also not being consumed at the expected rate. These circumstances were common to both the ALS- and EPSPS-inhibiting herbicides.

To evaluate other key parameters of carbon metabolism in the roots, ethanolic fermentation and respiration rates were determined. After 3 and 7 days from the supply of glyphosate to the nutrient solution, the induction of pyruvate decarboxylase (PDC) (Fig. 6A) and alcohol dehydrogenase (ADH) (Fig. 6B) activity was detected, respectively. These activities correlated well with the induction of PDC and ADH protein expression by the herbicide treatment (Fig. 6D). Therefore, the induction of the catalytic activities of PDC and ADH is a result of the overexpression of the proteins. Previous studies have also reported induction of aerobic fermentation after ALS inhibition due to an increase in the amount of fermentative enzymes (Zabalza et al., 2005), providing evidence that both types of herbicides have similar effects on the fermentative pathway.

The increased free amino pool detected after ALS and EPSPS inhibition (Fig. 1A; Orcaray et al., 2010) has been proposed to be derived from an increase in protein turnover (Rhodes et al., 1987). Indeed, it was shown that although protein synthesis occurs after ALS inhibitor treatment, the amino acids that comprise these proteins do not contain newly incorporated nitrogen; they contain nitrogen that is mainly scavenged from protein degradation (Zabalza et al., 2006). It is noticeable that plants treated with both classes of herbicides had increased levels of PDC and ADH protein synthesis in a situation in which amino acids have to be scavenged from existing proteins. This re-use of existing proteins in the synthesis of fermentative enzymes suggests that fermentation plays an important role in the plant's response to both types of herbicides and therefore in their mode of action.

The addition of glyphosate to the nutrient solution did not affect root respiration measured polarographically as oxygen consumption of intact root pieces (Fig. 6C), as has been reported before for suspension-cultured cells (Gout et al., 1992; Aubert et al., 1996). Consequently, the increased fermentative activity was not associated with changes in oxygen consumption. Usually, under low-oxygen conditions, changes in the AEC or RC correlate well with the induction of PDC and ADH. A decrease in ATP availability has been proposed as an indirect low-oxygen sensing

mechanism in plants (Bailey-Serres and Chang., 2005; Zabalza et al., 2009). Nevertheless, in glyphosate-treated roots, the induction of fermentation did not correlate with a decrease in the AEC of the tissue (Fig. 5B). On the contrary, although fermentation was induced, the AEC of these plants was higher than that of the control plants Recently, another case of induction of fermentation with higher AEC values was reported after ALS-inhibiting herbicide treatment (Zabalza et al., 2011).

Taken together, these results lead us to propose that after these herbicide treatments, the resulting metabolic imbalances prevent the optimal utilization of available carbohydrates and energy; however, less efficient metabolic pathways are activated.

The decrease in the redox charge detected in glyphosate-treated plants from day 1 (Fig. 4B) provides evidence of a higher rate of consumption of pyridine nucleotides in the reduced form. This increased consumption cannot be explained by an increase in ADH activity only, as ADH was only induced at the end of the treatment. Nicotinamide nucleotides function as co-substrates for a variety of enzyme reactions in biological oxidation-reduction processes; therefore, any change in their ratio cannot be easily explained by the activity of a single enzyme.

This is the first study to report aerobic fermentation after glyphosate application (Fig. 6). Because this effect was common to ALS inhibitors (Zabalza et al., 2005; 2011), it can be established that both types of amino-acid biosynthesis inhibitors elicit fermentation in the roots. It remains to be determined whether other types of amino acid biosynthesis inhibition share the same physiological effects on treated plants. Glufosinate inhibits amino acid biosynthesis by specifically inhibiting glutamine synthetase in the biosynthetic pathway of glutamine (Leason et al., 1982). The application of glufosinate to pea plants resulted in ethanolic fermentation in pea roots, similar to what was observed after the application of glyphosate (Fig. 7). The specific activities of both PDC and ADH were increased after 6 and 10 days from the onset of the glufosinate treatment. Western blotting showed that these increases corresponded to increases in the amounts of the respective proteins (Fig. 7C). This result suggests that fermentation induction is a common effect of all types of herbicides that inhibit amino-acid biosynthesis, although this effect occurred later after glutamine synthetase inhibition than after ALS or EPSPS inhibition.

Two characteristics have been proposed to be related to the induction of fermentation under aerobic conditions after ALS inhibition. First, pyruvate is a common substrate of ALS and PDC. ALS inhibition would involve an increased availability of pyruvate for use by other enzymes, such as PDC (Gaston et al., 2002). Second, some abiotic stresses not related to hypoxia have been reported to induce the expression of ADH and PDC (Dolferus et al., 1994; Kürsteiner et al., 2003). Indeed, it has been proposed that fermentation has a general function in aerobic metabolism under stress conditions and that it might be an important switch in regulating carbohydrate metabolism (Tadege et al., 1999). Fermentative induction after treatment with glyphosate or glufosinate cannot easily be related to substrate availability because neither of these herbicides inhibits a pyruvate-consuming enzyme. Thus, it seems that the fermentative response can be considered a physiological effect induced under stress. Moreover, other genes related to stress situations have been reported to be induced after glyphosate treatments. Bioinformatic analysis suggested that glyphosate-induced transcriptional alteration may be partially similar to that induced by other stress factors in plants (Yu et al., 2007). Gene expression profiling revealed an increased level of alternative oxidase after herbicide treatment (Zhu et al., 2008). Recently, alternative oxidase has been suggested to have a role in counteracting deleterious short-term metabolic fluctuations, especially under stress conditions (Rasmusson et al., 2009). Thus, alternative oxidase induction (Yu et al., 2007) and fermentative induction (Fig. 6) detected after glyphosate treatment can be considered part of a general plant response to the stress conditions caused by the herbicide treatment.

A toxic level of ethanol for the plant can be one of the consequences of the ethanolic pathway activation, although ethanol is not accumulated in many organs because it is lost by diffusion to surrounding hydroponic solution during the course of the experiment. This possibility can be considered in glyphosate-treated plants. Nevertheless, in hypoxic pea plants grown in the same hydroponic system, a similar activation of the ethanolic pathway did not induce toxic ethanol accumulation, as the imposed hypoxia was not lethal (Zabalza et al., 2009). Taking into account these results together with the similar increase in fermentative activities detected in herbicide-treated plants (Figs. 6 and 7) and in hypoxic roots (Zabalza et al., 2009), a toxic ethanol level is not expected in herbicide-treated plants.

We propose that the growth arrest provoked by the EPSPS inhibition is due to a metabolic alteration that impairs carbohydrate utilization, leading to carbohydrate accumulation first in the roots and then in the shoots, by phloem transport inhibition. This metabolic alteration would be derived from the inhibition of amino acid biosynthesis itself because this metabolic alteration involves inherent changes in the relative ratios of amino acids (Orcaray et al., 2010) and also induces carbon flux through the inhibition of the shikimate pathway (Lydon and Duke, 1988; Becerril et al., 1989; Hernandez et al., 1999). Both processes, although they originate from the nitrogen metabolism, can cause the mentioned effects on carbon and general metabolism.

Herbicides inhibiting branched-chain amino acid or aromatic amino acid biosynthesis showed similar short-term patterns with respect to the content of carbohydrates. The other common pattern found after ALS or EPSPS inhibitor treatment involved the induction of the ethanolic fermentative pathway. As both common effects have been detected after the application of different types of herbicides, these effects can be considered to represent physiological markers of herbicidal activity. Such markers can help in the search for new herbicidal active ingredients that are based on natural products to decrease the use of synthetic compounds. Thus, the use of physiological markers to evaluate the potential herbicidal activity of natural compounds can be very useful.

Conclusion

Glyphosate-treated plants showed impaired carbon metabolism. Carbohydrate accumulation was detected in both the leaves and roots of treated plants. Accumulation in the roots was due to a lack of utilization of available sugars as growth was arrested, which elicited soluble carbohydrate accumulation in the leaves due to a decrease in sink strength. Under aerobic conditions, ethanolic fermentative metabolism was enhanced in the roots of glyphosate-treated plants. This fermentative response was not related to a change in respiratory rates or to a decrease in the energy charge, as the energy charge was actually increased. This impaired carbon metabolism is similar to that in plants treated with herbicides inhibiting branched-chain amino acid biosynthesis, providing evidence of similar modes of action of both types of herbicides.

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Legends of Figures

Fig. 1. Total free amino acid (A) and shikimate (B) contents in the roots of control pea plants or plants treated with glyphosate. Each value is the mean ± standard error (n=5). * indicates a significant difference between the control and glyphosate-treated plants on a given day ($p \leq 0.05$).

Fig. 2. Fructose (A), glucose (B), sucrose (C) and starch (D) contents in the leaves of control pea plants or those treated with glyphosate. Each value is the mean ± standard error (n=4). * indicates a significant difference between the control and glyphosate-treated plants on a given day ($p \leq 0.05$).

Fig. 3. Sucrose (A) and starch (B) contents in the roots of control pea plants or those treated with glyphosate. Each value is the mean ± standard error (n=4). * indicates a significant difference between the control and glyphosate-treated plants on a given day ($p \leq 0.05$).

Fig. 4. Adenylate energy charge (AEC), calculated as (ATP+0.5 ADP)/(ATP+ADP+AMP) in the leaves (A) or roots (B) of control pea plants or plants treated with glyphosate. Each value is the mean ± standard error (n=3). * indicates a significant difference between the control and glyphosate-treated plants on a given day ($p \leq 0.05$).

Fig. 5. Redox charge (RC), calculated as (NADH+NADPH)/ (NAD^++NADH+$NADP^+$+NADPH) in the leaves (A) or roots (B) of control pea plants or plants treated with glyphosate. Each value is the mean ± standard error (n=3). * indicates a significant difference between the control and glyphosate-treated plants on a given day ($p \leq 0.05$).

Fig. 6. Pyruvate decarboxylase (A) and alcohol dehydrogenase (B) activities and respiratory rate (C) in the roots of control pea plants or those treated with glyphosate. Each value is the mean ± standard error (n=4). * indicates a significant difference between the control and glyphosate-treated plants on a given day ($p \leq 0.05$). D: Western blots of root pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities in roots of control (C) pea plants or those treated with glyphosate (Gly) 1, 3 or 7 days after treatment. Each lane contained 40 μg protein.

Fig. 7. Pyruvate decarboxylase (A) and alcohol dehydrogenase (B) activities in the roots of control pea plants or those treated with glufosinate. Each value is the mean ± standard error (n=3). * indicates a significant difference between the control and glufosinate-treated plants on a given day

($p \leq 0.05$). C: Western blots of root pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities in the roots of control (C) plants or those treated with glufosinate (Glu) 6 or 10 days after treatment. Each lane contained 20 μg protein.

Figure

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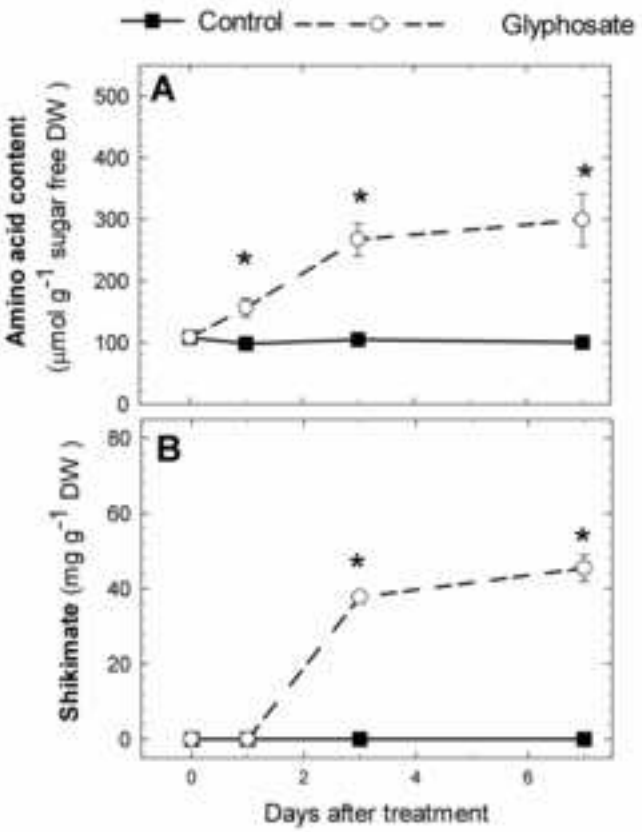

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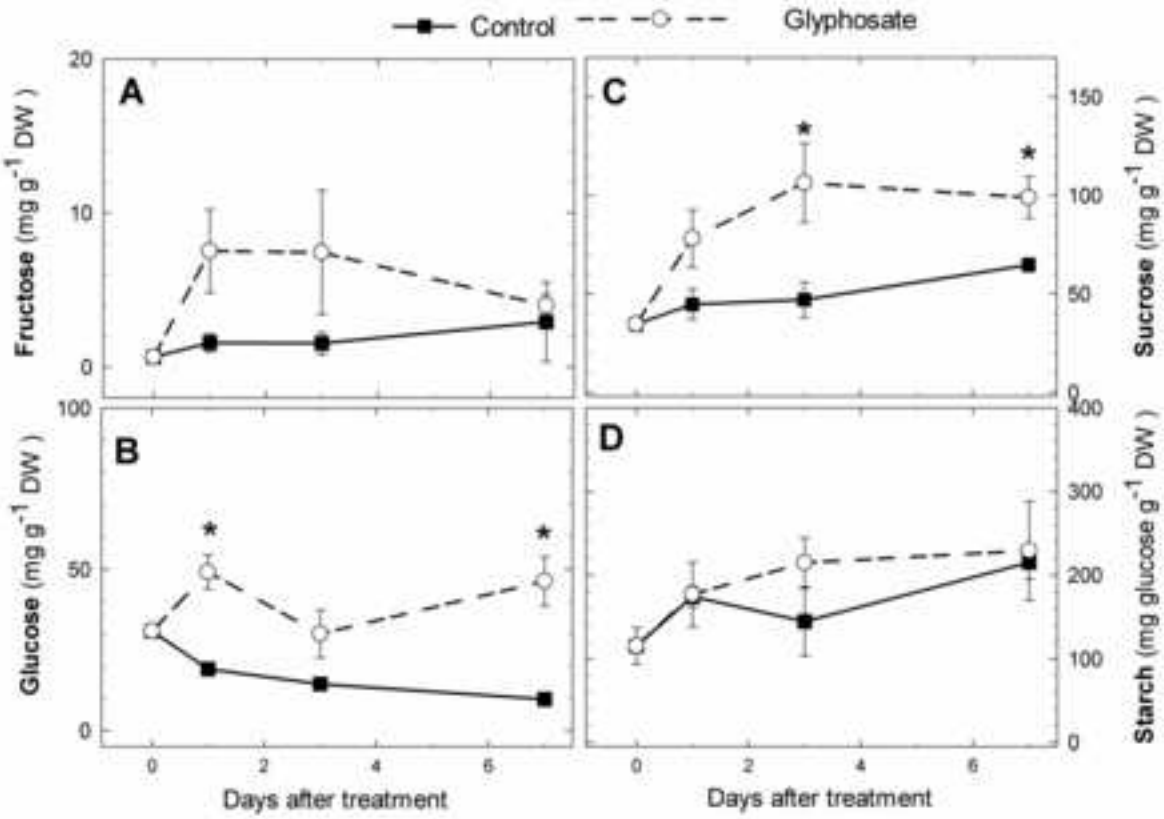

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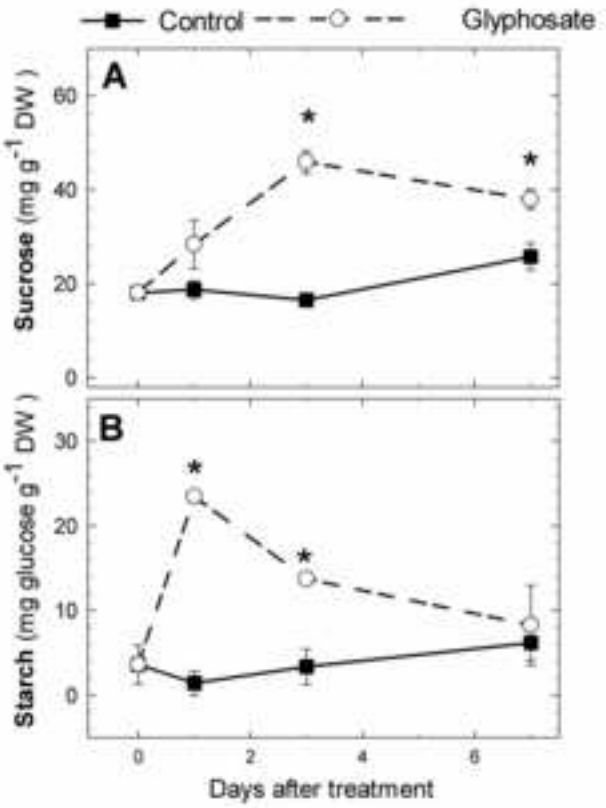

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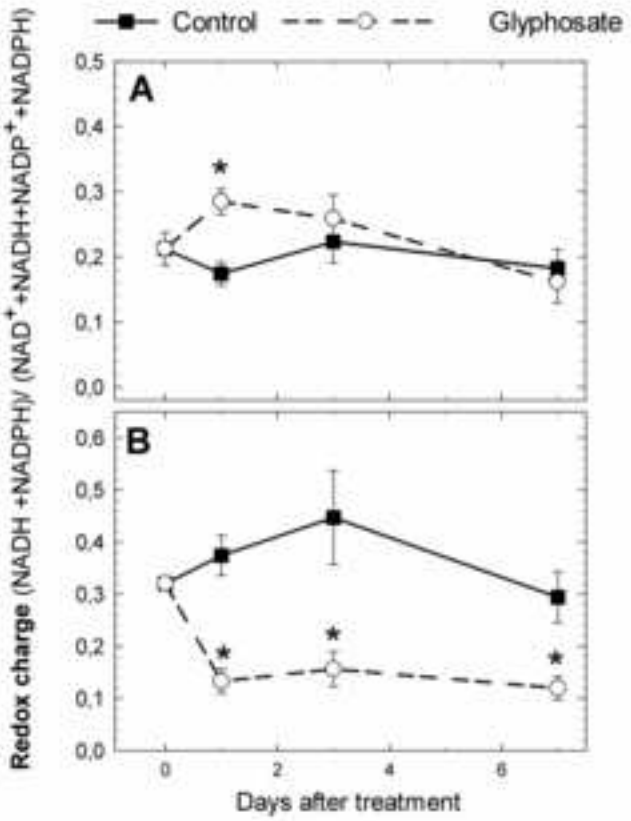

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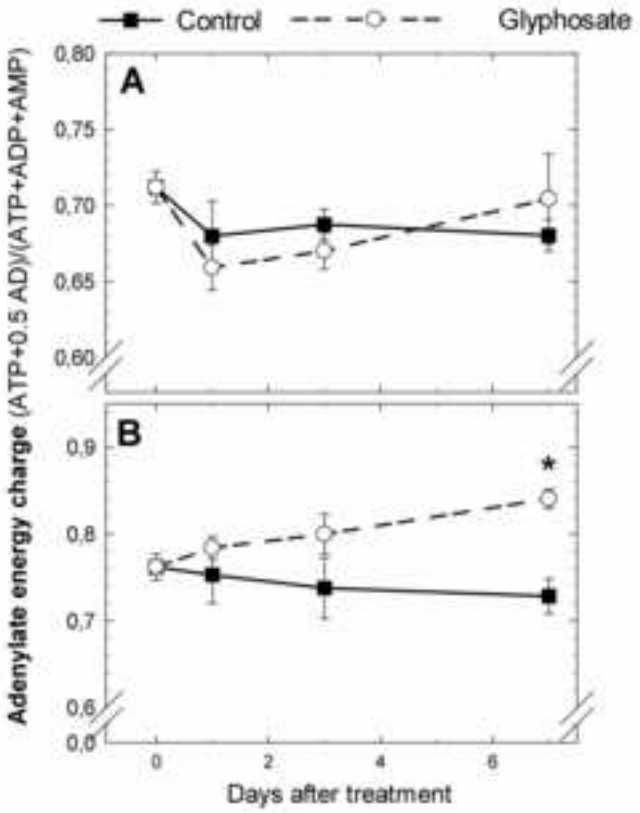

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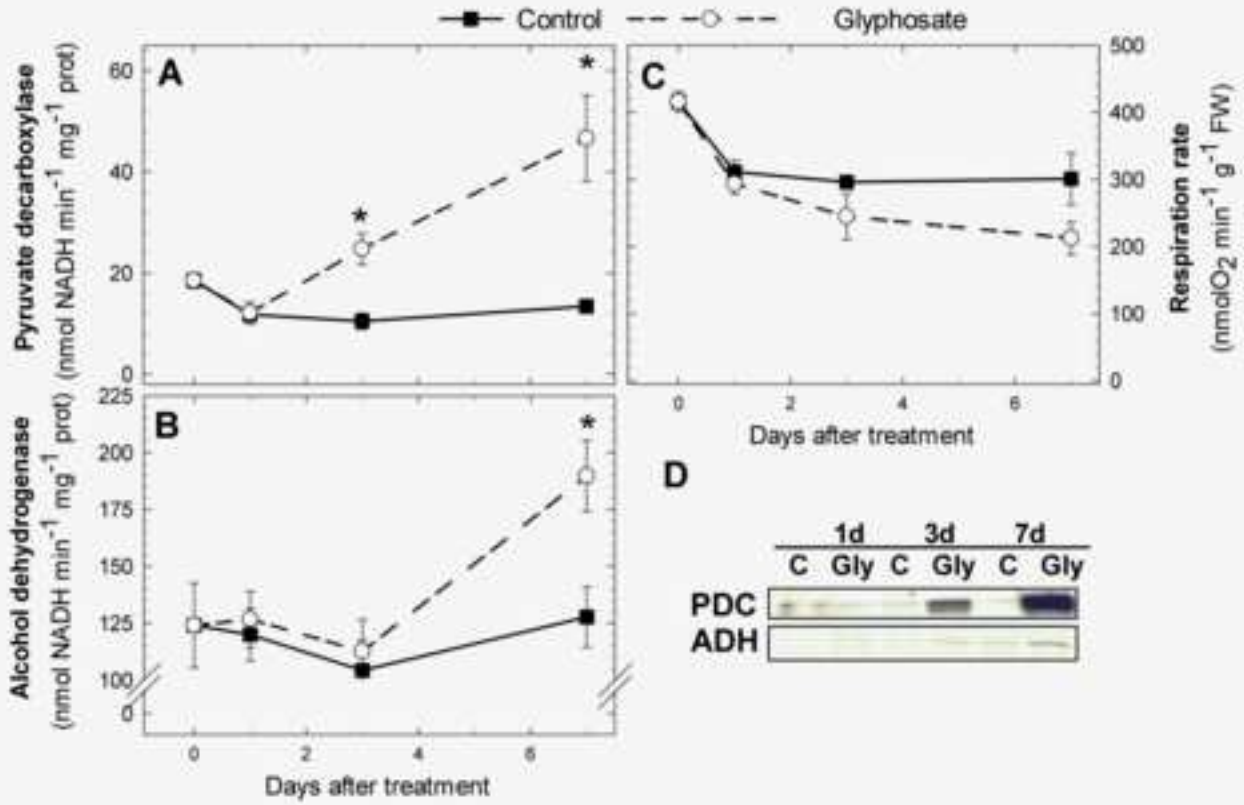

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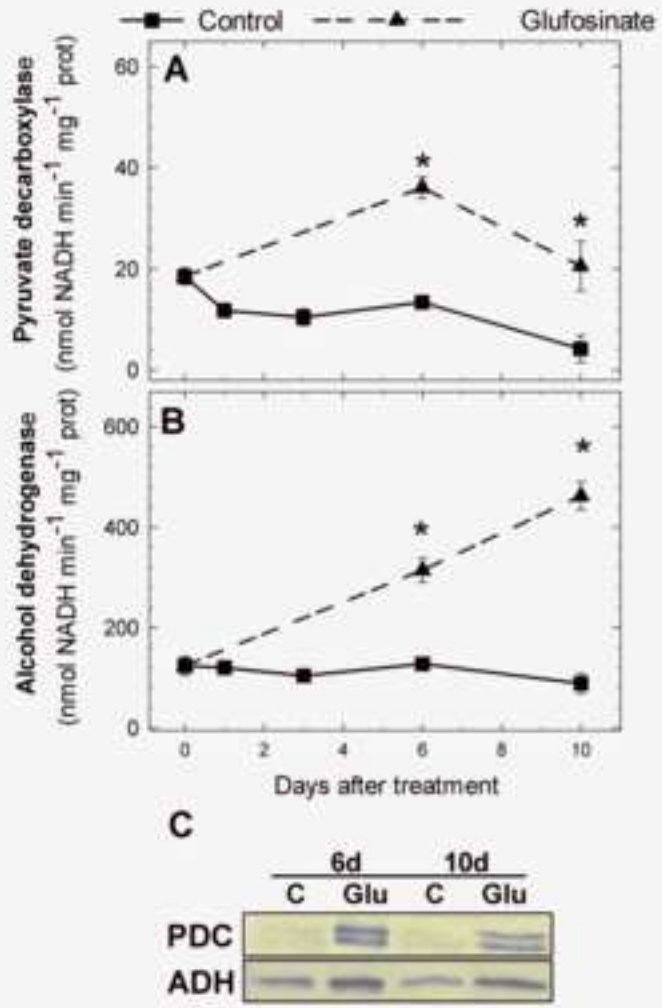

Figure 7
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