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Glyphosate Can Decrease Germination of Glyphosate-Resistant Soybeans

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ABSTRACT: We investigated the effects of different concentrations of glyphosate acid and one of its formulations (Roundup) on seed germination of two glyphosate-resistant (GR) and one non-GR variety of soybean. As expected, the herbicide affected the shikimate pathway in non-GR seeds but not in GR seeds. We observed that glyphosate can disturb the mitochondrial electron transport chain, leading to H_2O_2 accumulation in soybean seeds, which was, in turn, related to lower seed germination. In addition, GR seeds showed increased activity of antioxidant systems when compared to non-GR seeds, making them less vulnerable to oxidative stress induced by glyphosate. The differences in the responses of GR varieties to glyphosate exposure corresponded to their differences in enzymatic activity related to H_2O_2 scavenging and mitochondrial complex III (the proposed site of ROS induction by glyphosate). Our results showed that glyphosate ought to be used carefully as a pre-emergence herbicide in soybean field crop systems because this practice may reduce seed germination.

KEYWORDS: *antioxidant, mitochondria, reactive oxygen species, pesticide, shikimate, Roundup*

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is one of the most important economic crop cultures worldwide, and its production was greatly leveraged by the introduction of glyphosate-resistant (GR) plants.[1] Nowadays, about 60% of the worldwide cultivated area is planted with GR soybeans.[2] GR plants are provided with an exogenous gene, *cp*4 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which encodes the bacterial version of the enzyme CP4EPSPS, conferring plants tolerance against glyphosate (*N*-phosphonomethyl glycine).[3] Non-GR plants, in contrast, are susceptible to the effects of glyphosate linked to the inhibition of EPSPS, which prevents the biosynthesis of aromatic amino acids.[4]

The primary mode of action of glyphosate is the inhibition of EPSPS; however, the herbicide has several secondary effects on plant physiology,[5] which have even been observed in GR plants.[6−8] When applied to seeds, glyphosate-based herbicides have shown either deleterious,[9,10] little, or no effect[11,12] on germination, but the exact mechanism by which the herbicide could affect the germination process is still unclear.

Although recommended for use on postemergence stages, glyphosate-based herbicides are often applied in combination with, or following, the application of pre-emergence herbicides in programs of weed control.[13] In this scenario, the use of glyphosate just before sowing can result in the exposure of crop seeds to residual concentrations of the herbicide in the soil. Little is known, however, about the effects of glyphosate on seed germination of GR plants. Recently, we have shown that glyphosate (glyphosate acid and its commercial formulation Roundup) impairs the mitochondrial electron transport chain (ETC) in *Dimorphandra wilsonii* (a non-GR Brazilian legume) seeds, with complex III as its precise target.[14] Similarly, in *Lemna minor* leaves, mitochondrial ETC complex III appeared quite sensitive to glyphosate (glyphosate acid), which resulted in the formation of reactive oxygen species (ROS) by the electron shuttling from semiquinone to oxygen.[15] The oxidative stress due to ROS accumulation was related to the herbicide toxicity to *L. minor*,[15] as also observed in some other plant species.[16−20] Unlike what was expected, however, the impairment of respiratory ETC in *D. wilsonii* seeds did not induce oxidative stress, and ROS accumulation was prevented by the activity of antioxidant enzymes such as catalase (CAT) and ascorbate peroxidase (APX). ROS provided important signals during seed germination,[21] and the ability of plants to cope with excessive ROS production is directly linked to seed germinability.[22] Therefore, we hypothesized that antioxidant systems have a central role in seeds germinating under the presence of glyphosate. Moreover, it is known that oxidative responses under stress conditions (such as the presence of herbicide) differ among plant species, organs, and tissues and even among varieties of the same species.[17,23]

In this context, the present study evaluates the effects of the glyphosate acid and the glyphosate-based commercial formulation Roundup on seed germination of three varieties of soybean. Because GR and non-GR soybean seeds differ in their antioxidant system activities,[24] by evaluating the effects of the herbicides on GR and non-GR seeds, we determined if the effect of glyphosate on soybean seed germination is linked to oxidative metabolism. Moreover, two different varieties of GR

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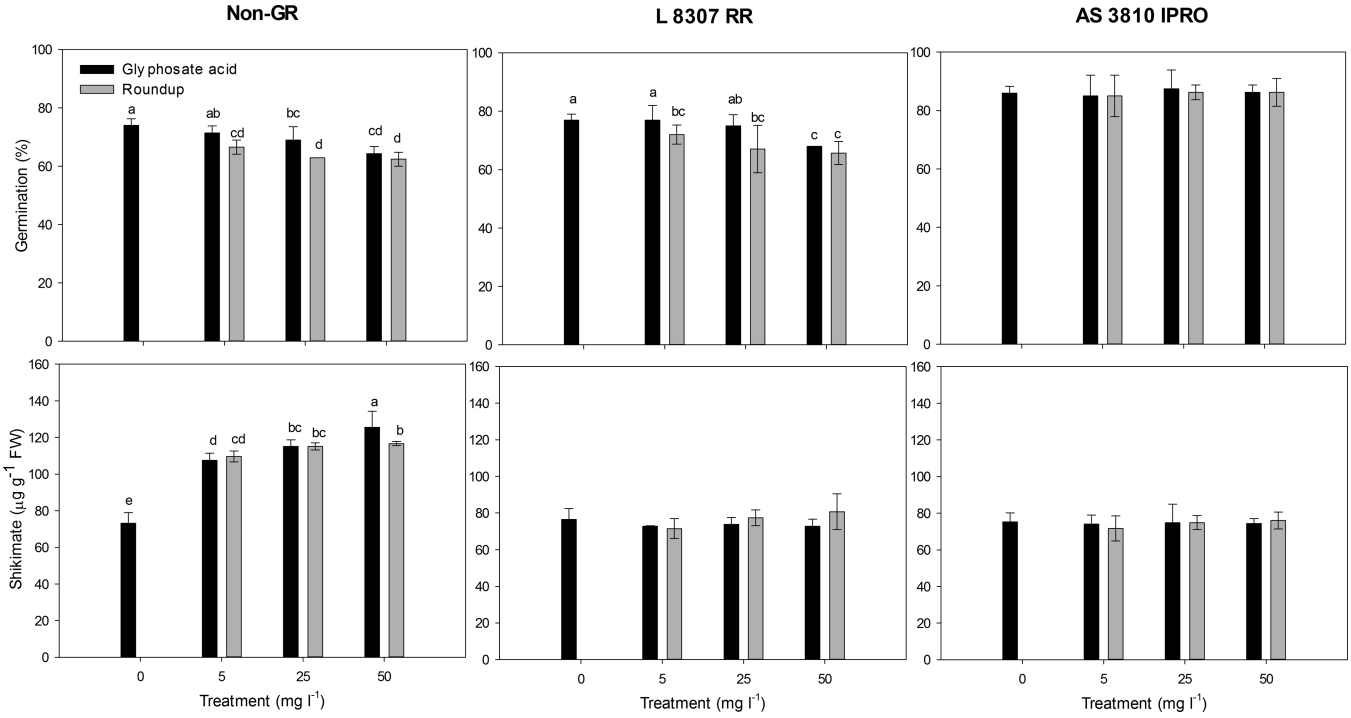

Figure 1. Germination and embryo shikimate concentration of soybean seeds exposed to different glyphosate and Roundup concentrations (0, 5, 25, and 50 mg ai L^{-1}). Bars represent means ± SD of four replicates. Letters highlight significant differences among herbicide treatment concentrations within the same variety ($P < 0.05$) by the Student t test.

soybean seeds were tested with the aim of understanding if the herbicides could have different effects on GR varieties. More specifically, we aimed at (i) understanding the mechanisms of glyphosate action on germination and (ii) verifying whether pre-emergent usage of the herbicide on soybean crops could affect seed germination, possibly reducing yields.

MATERIALS AND METHODS

Plant Material and Germination Assays. The non-GR soybean variety BRS-284 (EMBRAPA) and the GR varieties L 8307 RR (Riber-KWS) and AS 3810 IPRO (Agroeste) collected in 2016 were used in this study. The seeds were surface sterilized in 2.5% sodium hypochlorite for 5 min and thoroughly rinsed with deionized water before sowing. Germination assays were carried out by placing four replicates of 25 seeds in germination boxes lined with filter paper (Whatman no. 1) moistened with 20 mL of deionized water or with treatment solutions. The germination boxes were arranged in a completely randomized order in a growth chamber at 30 °C in the dark. Seeds were considered to have germinated when approximately 2 mm of the primary root had emerged. Seed viability was evaluated by the tetrazolium test before germination and in nongerminated seeds after germination experiments. Seeds were pre-imbibed in distilled water for 6 h and then immersed in 0.5% tetrazolium (2,3,5-triphenyltetrazolium chloride, Sigma) solution for 2 h in the dark. The initial viability of the seed lot was 70% for non-GR and 100% for GR varieties. Germination percentages were corrected by initial viability.

Analytical grade glyphosate (Pestanal grade) obtained from Sigma-Aldrich (Oakville, Canada) and the commercial glyphosate Roundup formulation (Monsanto, Brazil) were used in the experiments. Stock solutions (1000 mg L^{-1}) of glyphosate and Roundup were prepared in ultrapure water and used to obtain solutions with the desired concentrations of the chemicals used to moisten seeds (20 mL/germination box). Seeds were submitted to different concentrations of glyphosate and Roundup [0, 5, 25, and 50 mg active ingredient (ai) L^{-1}], considering a field application rate of 2.8 kg glyphosate ha^{-1} [19 g L^{-1} of active equivalent (ae)].[16] To ensure reproducibility of the obtained data, the germination experiment was repeated twice, with no statistically significant differences according to ANOVA (data not shown).

Biochemical Evaluations. Biochemical evaluations were performed in the whole seed after the extraction of the tegument, here considered as embryo (cotyledons + embryonic axe). The mean germination time (average length of time required for final germination of a seed lot)[25] for all of the varieties studied was <48 h. Therefore, we followed the physiological changes of early seed germination 24 h after treatment induction. Biochemical analyses were performed on seeds treated in parallel experiments under the same conditions used in the germination tests. Embryos of six seeds from each germination box were manually extracted and ground in liquid nitrogen, constituting one replicate (with a total of four replicates/treatment). Samples were stored at −80 °C until analyzed.

Shikimate concentrations were evaluated following the methods of Bijay and Dale[26] with modifications: 0.1 g of embryos was ground in liquid nitrogen and homogenized with 1 mL of 0.25 M hydrochloric acid. Then, the extract was centrifuged at 25000g for 15 min, and 100 μL of the centrifuged supernatant was reacted with 1 mL of 1% periodic acid solution. After 1 h, 1 mL of 1 M sodium hydroxide and 0.6 mL of 0.1 M glycine were added to the samples, and the absorbance was measured at 380 nm.

Hydrogen peroxide (H_2O_2) concentrations were measured following the method of Velikova et al.,[27] using 0.1 g of embryos. H_2O_2 was extracted in 2 mL of 0.1% trichloroacetic acid (TCA), and after centrifugation at 12000g for 15 min, 300 μL of the centrifuged supernatant was reacted with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI. Samples were read at 390 nm, and H_2O_2 concentrations were given on a standard curve.

Antioxidant enzymes were extracted by macerating 0.1 g of embryos in 1 mL of an extraction buffer containing 100 mmol L^{-1} potassium phosphate buffer (pH 7.8), 100 mmol L^{-1} EDTA, 1 mmol L^{-1} L-ascorbic acid, and 2% PVP (m/v). The protein contents of all the samples were determined using the Bradford method. Catalase (CAT; EC 1.11.1.6) and ascorbate peroxidase (APX; EC 1.11.1.11) activities of the embryos were determined spectrophotometrically. Catalase assay was performed following ref 28 at 25 °C in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 250 mM

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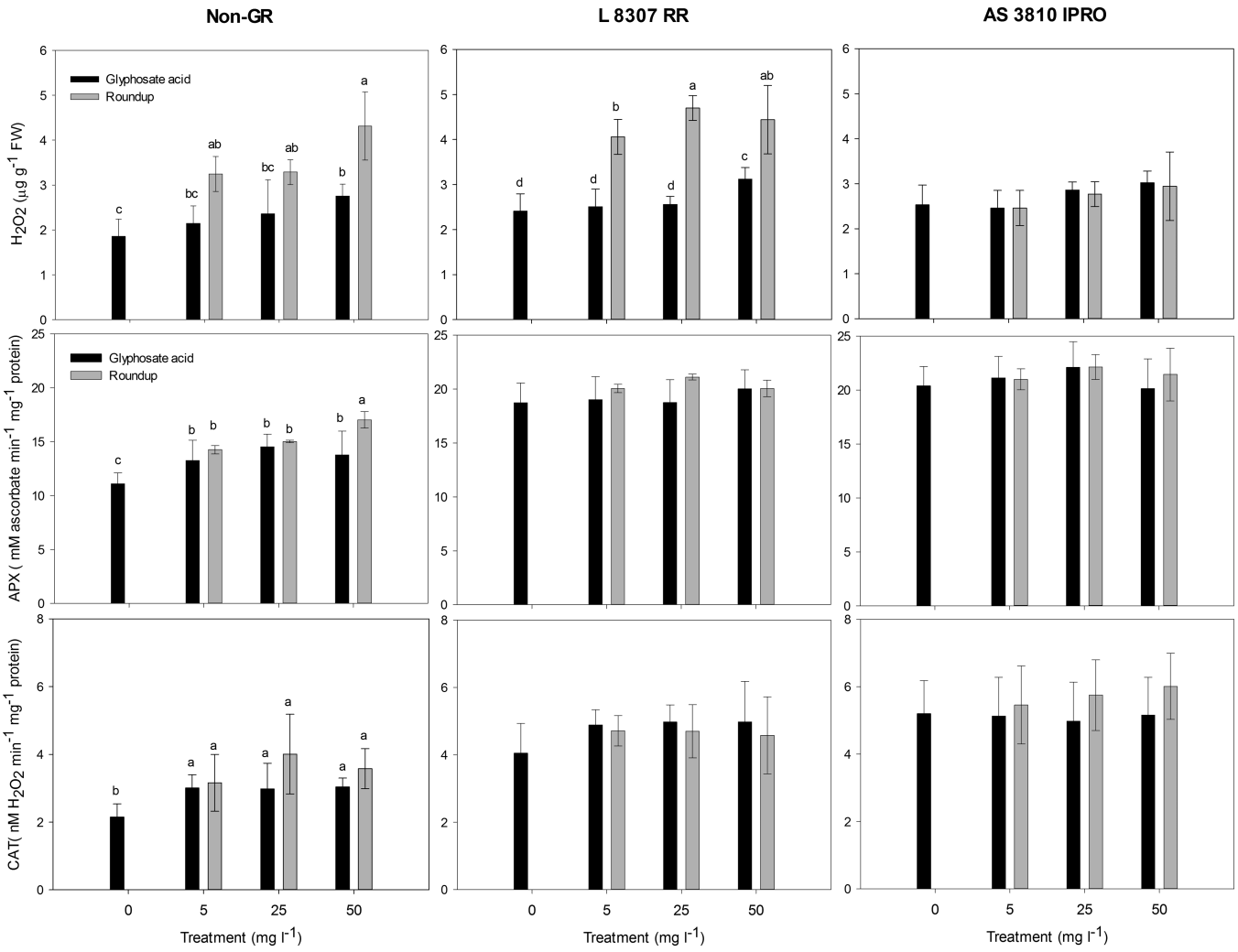

Figure 2. Hydrogen peroxide (H_2O_2) concentration and ascorbate peroxidase (APX) and catalase (CAT) activities in embryos of soybean seeds exposed to different glyphosate and Roundup concentrations (0, 5, 25, and 50 mg ai L^{-1}). Bars represent means ± SD of four replicates. Letters highlight significant differences among herbicide treatment concentrations within the same variety ($P < 0.05$) by the Student t test.

hydrogen peroxide, and distilled water. Catalase activity was determined following the decomposition of H_2O_2, for 1.5 min at 10 s intervals, monitoring changes in absorbance at 240 nm with a molar extinction coefficient of 0.0394 mM^{-1} cm^{-1}. Ascorbate peroxidase activity was determined following ref 29 at 28 °C in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 10 mM L-ascorbic acid, 2 mM hydrogen peroxide, and distilled water. The APX activity was estimated by monitoring ascorbate oxidation rate (ε = 2.8 mM^{-1} cm^{-1}) at 290 nm for 3 min at 15 s intervals.

The enzymatic activities of the mitochondrial ETC complexes I–IV were determined by spectrophotometry on embryo homogenates (200 mM phosphate buffer, pH 7.5) using 30 mg of protein in each assay from seeds exposed to 0 or 50 mg L^{-1} of the tested herbicides. Complex I (NADH:ubiquinone oxidoreductase) and complex II (succinate dehydrogenase) assays were performed following ref 30, and their activities were calculated by monitoring the rates of NADH (ε = 5.5 mM^{-1} cm^{-1}) and ubiquinone (ε = 9.6 mM^{-1} cm^{-1}) decreases per microgram of protein. Complex III (ubiquinol-cytochrome c reductase) activity was determined following the method of Birch-Machin et al.,[31] monitoring cytochrome cIII reduction rates (ε = 19 mM^{-1} cm^{-1}). Complex IV (cytochrome c oxidase) activity was determined following ref 32 by calculating the rate of increase in absorbency caused by the oxidation of cytochrome cII to cytochrome cIII (ε = 19 mM^{-1} cm^{-1}). All activities were expressed as per microgram of protein (which was determined by the Bradford method).

To identify if mitochondria complex III enzyme is the site of ROS (hydrogen peroxide) induction by glyphosate herbicides in seeds of soybean,[14,15] seeds were exposed to 0 or 50 mg L^{-1} solutions (glyphosate acid or Roundup) with 15 μM rotenone (an inhibitor of mitochondrial ETR complex I) or 15 μM antimycin A (an inhibitor of mitochondrial ETR complex III Qo site).[15,33] Seed germination was recorded, and H_2O_2 concentrations in embryos (24 h after exposure) were assessed.[27]

Statistical Analyses. Statistical analyses were performed using JMP 7.0 software (SAS Institute Inc.). The results were expressed as the averages of four replicates. The data were tested for normality (Shapiro–Wilk) and homogeneity (Bartlett) and then statistically evaluated. Germination, shikimate, oxidative stress marker, and assays involving mitochondrial ETR inhibitors were evaluated using three-way analysis of variance (ANOVA). Interactions between herbicide (glyphosate acid and Roundup), concentrations (0, 5, 25, and 50 mg ai L^{-1})/inhibitor (rotenone and antimycin) and varieties (non-GR, L 8307 RRn and AS 3810 IPRO) were included in the model. Meanwhile, two-way ANOVA was used in evaluations of the activity of mitochondrial ETC complexes. In these evaluations, the interaction between herbicide and varieties was included in the model. When differences were detected by ANOVA, means were compared by a post hoc Student t test (significance at $P < 0.05$) for all pairwise comparisons.

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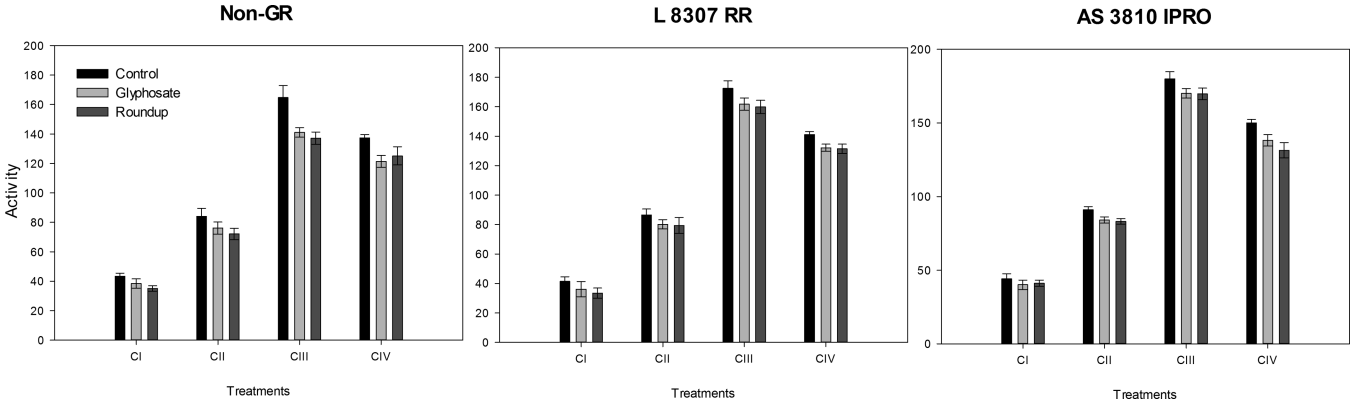

Figure 3. Complex I (NADH:ubiquinone oxidoreductase; mmol NADPH mg^{-1} protein s^{-1}), complex II (succinate dehydrogenase; mmol ubiquinone mg^{-1} protein s^{-1}), complex III (ubiquinol-cytochrome *c* reductase; mmol cytochrome *c*III mg^{-1} protein s^{-1}), and complex IV (cytochrome *c* oxidase; mmol cytochrome *c*III mg^{-1} protein s^{-1}) activities in embryos of soybean seeds exposed to different glyphosate and Roundup concentrations (0 and 50 mg ai L^{-1}). Bars represent means ± SD of four replicates.

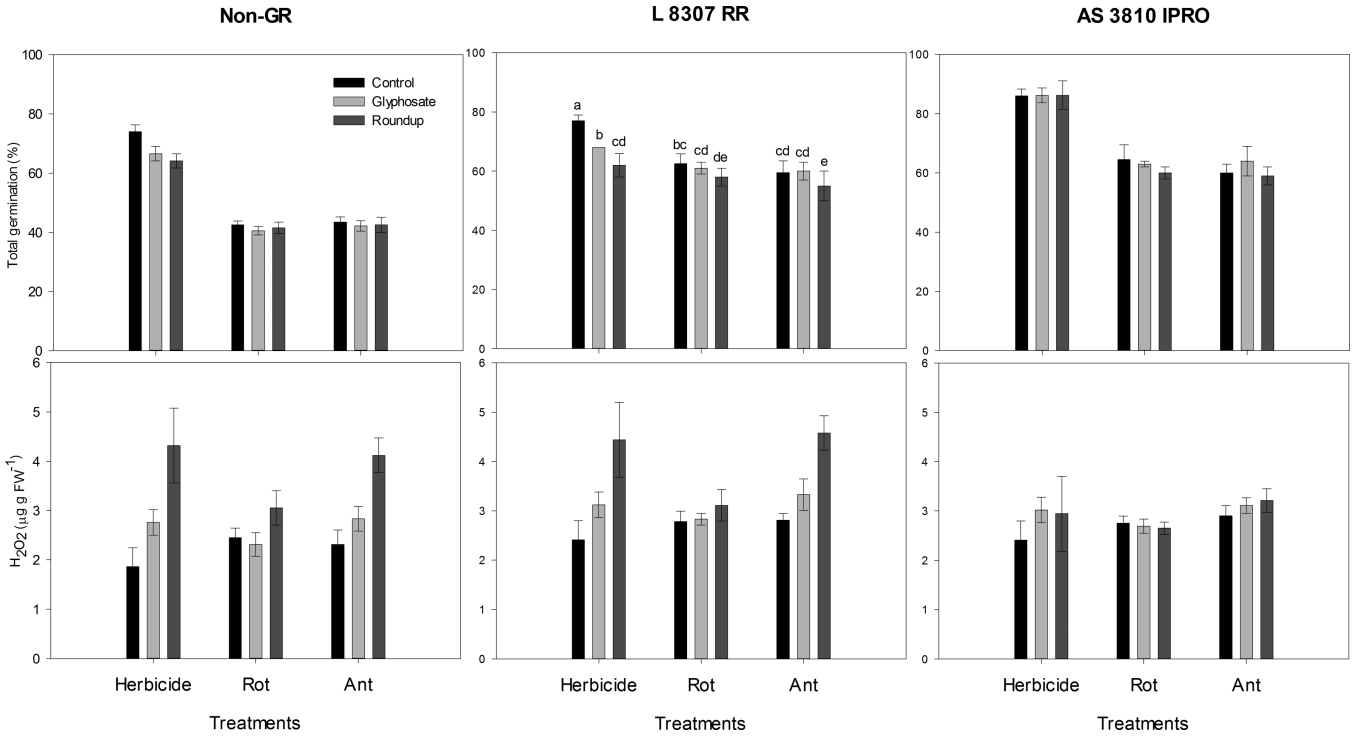

Figure 4. Germination and hydrogen peroxide (H_2O_2) concentration in embryos of soybean seeds exposed to the solution of 0 and 50 mg ai L^{-1} of glyphosate and Roundup with 15 μM rotenone (Rot) or 15 μM antimycin A (Ant). Bars represent means ± SD of four replicates. Letters highlight significant differences among herbicide treatment concentrations within the same variety ($P < 0.05$) by the Student *t* test.

RESULTS

Seed Germination, Viability, and Shikimate Concentrations. Germination percentages were significantly different among the varieties ($P < 0.0001$), being higher in AS 3810 IPRO (83.0%), followed by GR L 8307 RR (71.5%) and then the non-GR variety (68.5%). Significant interactions between variety and concentration as well as between variety and herbicide type were observed ($P < 0.05$). In the absence of herbicide treatment (0 mg L^{-1}), higher germination was observed in AS 3810 IPRO seeds (Figure 1). Moreover, these seeds were insensitive to the herbicide treatments (86.2 and 85.8% for glyphosate and Roundup, respectively; $P > 0.05$). The herbicides, however, decreased seed germination of non-GR (70.2 and 66.8% for glyphosate and Roundup, respectively) and GR-variety L 8307 (74.2 and 68.7% for glyphosate and Roundup, respectively) ($P < 0.05$; Figure 1). Glyphosate acid exposure resulted in a greater decrease ($P < 0.05$) in seed germination of non-GR (70.2%) than of GR-L 8307 (74.2%), whereas the effects of Roundup were similar between these two varieties (68.7 and 66.8%, respectively, $P > 0.05$).

In the affected varieties (non-GR and L 8307), glyphosate acid and Roundup concentrations had different effects on germination percentage of seeds ($P < 0.0001$; Figure 1). Detrimental effects of glyphosate acid on seed germination of both varieties were observed only at the highest concentration (50 mg L^{-1}) ($P < 0.05$). In contrast, all of the studied concentrations of Roundup led to decreases on germination percentage of these seeds ($P < 0.05$). Moreover, by comparing the effects of the same herbicide concentration on each variety,

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it was noted that Roundup induced a greater decrease in germination than glyphosate acid ($P < 0.05$)—with the exception of seeds exposed to 50 mg L^{-1} ($P > 0.05$). None of the concentrations of glyphosate acid or Roundup decreased the viability of the soybean seeds of the three varieties tested ($P > 0.05$).

Shikimate was greater in embryos of non-GR seeds (104.55 $\mu g\ g^{-1}$ FW) than in GR seeds (74.70 and 68.61 $\mu g\ g^{-1}$ FW in L 8307 RR and AS 3810 IPRO, respectively) ($P < 0.0001$). A significant interaction was observed between concentration and variety ($P < 0.0001$). In non-GR seeds treated with both glyphosate acid and Roundup, shikimate concentrations increased in embryos regardless of the herbicide concentration used (Figure 1); in both GR varieties, however, shikimate concentrations did not differ among treatments ($P < 0.05$; Figure 1).

Oxidative Stress Markers. Hydrogen peroxide (H_2O_2) concentrations were greater in embryos of seeds treated with Roundup ($P < 0.0001$) and in embryos of GR (2.53 and 2.41 $\mu g\ g^{-1}$ FW in L 8307 RR and AS 3810 IPRO, respectively) than in non-GR seeds (1.6 $\mu g\ g^{-1}$ FW). Significant interactions were observed between herbicide, concentration, and variety ($P = 0.0006$). In non-GR seeds and in the L 8307 RR variety, all Roundup treatments greatly increased H_2O_2 concentrations in embryos, which was only observed in seeds treated with 50 mg glyphosate acid L^{-1} (Figure 2).

Ascorbate peroxidase (APX) and catalase (CAT) activities were lower in embryos of non-GR-seeds (13.75 mM ascorbate min^{-1} mg^{-1} protein and 3.02 nM H_2O_2 min^{-1} mg^{-1} protein, respectively) ($P < 0.0001$; Figure 2). Between GR varieties, APX and CAT activities in embryos were greater in AS 3810 IPRO (21.01 mM ascorbate min^{-1} mg^{-1} protein and 4.98 nM H_2O_2 min^{-1} mg^{-1} protein, respectively) than in L 8307 RR (19.56 mM ascorbate min^{-1} mg^{-1} protein and 4.59 nM H_2O_2 min^{-1} mg^{-1} protein, respectively) seeds ($P < 0.0001$; Figure 2). Significant interactions were observed between concentration and variety ($P < 0.0001$) for the activity of both enzymes. Within the same concentrations, APX and CAT activities were greater in embryos of GR seeds. Moreover, in non-GR seeds, herbicide treatment increased both enzyme activities in embryos (Figure 2).

Activity of Mitochondrial Electron Transport Chain Enzymes. The activity of complex I enzyme was greater in embryos of seeds from the AS 3810 IPRO than in the non-GR variety (41.82 and 37.00 μmol NADPH mg^{-1} protein s^{-1}, respectively; $P < 0.05$). Non-GR seeds showed the lowest activity of complex II–IV enzymes, which was greater in AS 3810 IPRO ($P < 0.05$; Figure 3). In all seed varieties, the enzyme activities decreased after the treatment with glyphosate acid or Roundup ($P < 0.05$; Figure 3).

Possible Site of Herbicide-Induced ROS Formation in Seeds. To identify the possible site of herbicide-induced ROS formation, the effects of rotenone and antimycin on seed germination and embryo H_2O_2 were investigated (Figure 4). Germination was decreased by antimycin and rotenone in all varieties ($P < 0.001$; Figure 4). Significant interaction between variety, herbicide, and inhibitor was observed ($P < 0.05$). L 8307 RR seeds exposed to glyphosate acid or Roundup and treated with rotenone or antimycin showed decreased germination when compared with their respective controls (50 mg L^{-1} glyphosate acid or Roundup) ($P < 0.05$; Figure 4).

A significant interaction between herbicide and inhibitor was observed for H_2O_2 concentration in embryos ($P < 0.001$). In the absence of herbicide treatments (0 mg L^{-1}) both mitochondrial inhibitors led to increased H_2O_2 concentrations. In the presence of glyphosate acid or Roundup (50 mg L^{-1}), however, embryos of seeds treated with rotenone (50 mg L^{-1} glyphosate acid/Roundup + Rot) showed decreased H_2O_2 concentrations.

DISCUSSION

Soil residual glyphosate in the field has been reported to be phytotoxic, decreasing yields of important crops.[34] Currently, however, the use of glyphosate as a pre-emergence herbicide for weed control[13] poses a new agricultural challenge: the exposure of crop seeds to large amounts of the herbicide. Seeds are often well protected from their surrounding environment; however, the germination process is quite sensitive to changes in the seeds' environment such as the presence of potential hazardous compounds.[11] In the present study, we observed that even GR varieties of soybean, which were developed to resist to glyphosate, showed deleterious effects of the herbicide, as seen by decreased germination of L 8307 RR seeds cultivated in the presence of glyphosate acid and Roundup (Figure 1). When exposed to the highest glyphosate acid concentration tested (50 mg L^{-1}), seed germination was reduced by 11%, whereas at all concentrations the commercial glyphosate formulation Roundup induced decreases in germination up to 14%. This loss is considerable when considering that seed germination is the first step in field production. However, no effects of the herbicides were seen in seeds of the GR variety AS 3810 IPRO. These intriguing results motivated us to investigate how the herbicides could affect the germination process in soybean seeds. For this purpose, we used a non-GR variety (BRS-284), sensitive to glyphosate, as a positive control. Similarly to L 8307 RR, these non-GR seeds experienced up to 13 and 15% of reduction in their germination when exposed to glyphosate acid or Roundup, respectively (Figure 1).

By inhibiting the EPSPs enzyme, glyphosate led to shikimate accumulation,[4] which has been used as a biomarker for glyphosate exposure. Investigating the shikimate concentrations in embryos of exposed seeds (Figure 1), we observed it to accumulate only in embryos of the non-GR variety exposed to both glyphosate acid and Roundup. Therefore, we discarded the hypothesis that seeds of L 8307 RR fail in their transgenic transformation for glyphosate resistance. Because previous studies have shown that the herbicide can alter oxidative metabolism,[15−20] we decided to investigate ROS concentrations (H_2O_2) and the activity of some ROS-scavenging enzymes (APX and CAT) in exposed seeds.

As shown in Figure 3, in non-GR seeds, exposure to both glyphosate acid and Roundup resulted in higher H_2O_2 concentrations in embryos. Although in this variety the activity of APX and CAT (both H_2O_2 scavengers) increased in herbicide-exposed seeds, these enzymatic systems failed to prevent H_2O_2 accumulation, which can become toxic and impair the germination process.[22] For instance, in non-GR and in the GR variety L 8307 RR, greater H_2O_2 concentrations were found in embryos of seeds exposed to Roundup, for which lower germination was also observed (Figures 1 and 2). H_2O_2 accumulation in embryos can cause oxidative bursts, delaying or decreasing seed germination through deterioration of cell structures and components such as fatty acids, proteins, and DNA.[35] Therefore, we hypothesized that herbicide interference with the germination of soybean seeds is linked to the induction of ROS accumulation. Reinforcing our hypothesis, no

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H_2O_2 accumulation or interference with germination was observed upon herbicide exposure of AS 3810 IPRO seeds.

Interestingly, we observed that H_2O_2 concentrations in embryos of seeds of GR varieties were greater than in non-GR varieties. For example, untreated seeds (0 mg L^{-1}) of L 8307 RR and AS 3810 IPRO had 2.41 and 2.53 μM H_2O_2 g^{-1} FW, which were 30 and 36% higher than the 1.86 μM H_2O_2 g^{-1} FW found in embryos of non-GR seeds ($P < 0.05$; Figure 2), respectively. According to Barbosa et al.,[24] the transgenic transformation by the insertion of the gene conferring resistance to glyphosate into the DNA of soybean seeds is itself a stress factor leading to higher levels of oxidative stress in GR seeds. These authors observed that even when no herbicides were used, transgenic seeds showed increased lipid peroxidation as well as higher activity of antioxidant enzymes, such as APX, CAT, and glutathione reductase (GR) than nontransgenic seeds, which indicates oxidative stress in the GR seeds. Similarly, we also observed higher APX and CAT activities in embryos of GR than non-GR seeds (Figure 2). From our point of view, the increased oxidative status of GR seeds, due to the transformation process, should not be interpreted only as a stress factor. For example, in untreated seeds, the greatest H_2O_2 concentrations in embryos of GR varieties did not prevent seed germination (Figures 1 and 2). In contrast, as mentioned above, germination was greater in GR than in non-GR seeds. Moreover, greater activities of APX and CAT in GR seeds could prevent (in the case of AS 3810 IPRO) or reduce (in the case of L 8307 RR) H_2O_2 accumulation in seeds upon herbicide exposure (Figure 2). For instance, in seeds exposed to 50 mg glyphosate acid L^{-1}, an increase of 49% in H_2O_2 concentration was seen in non-GR seeds, whereas the increase was of 29% in L 8307 RR. Similarly, under the Roundup treatment, increases in H_2O_2 concentrations ranged from 75 to 132% in non-GR seeds and from 68 to 84% in L 8307 RR. Finally, seeds of AS 3830 IPRO, which showed the highest activity of the antioxidant enzymes, did not show H_2O_2 accumulation. Therefore, in addition to favoring seed germination when exposed to glyphosate, increased oxidative status can favor GR seeds by increasing their performance under environmentally stressful conditions. For example, transgenic seeds were seen to have a greater ability to take up metals from soils and to have higher metal bioaccessibility than nontransgenic soybean seeds.[36]

Mitochondria are the major source of ROS production (such as H_2O_2) in hydrated seeds during germination,[37] and mitochondrial electron transport chain enzymes have been identified as targets of glyphosate in non-GR species.[14,15] Our study confirms these previous findings, because the activity of the enzymes related to mitochondrial ETC (complexes I–IV) decreased in seeds exposed to glyphosate acid and Roundup (Figure 3). However, it is interesting to highlight that similar to antioxidant enzymes, complex I– IV activities differ among soybean varieties. Higher activity of these enzymes indicates higher respiration in GR than in non-GR seeds—which can be related to the increased H_2O_2 concentrations in these seeds. It has been shown that GR and non-GR soybeans differ in the expression of several proteins[24,36] and, therefore, one could expect different enzyme activities between the studied soybean varieties. However, as discussed below, the differences in the activity of mitochondrial ETC enzymes in seeds of the tested varieties could be related to their differing responses to glyphosate exposure.

Treatment of plant tissues with inhibitors of mitochondrial ETC are known to increase the generation of ROS,[15,33,38] as we also observed in seeds without herbicide exposure (Figure 4). By inhibiting complex I, rotenone increases the formation of ubisemiquinone, which in turns drives electrons to oxygen, leading to superoxide generation.[38] Similarly, antimycin A blocks complex III, leading to the accumulation of semiquinone and its side reaction with oxygen.[39] The concomitant exposure of non-GR and L 8307 RR seeds to herbicide and mitochondrial ETC inhibitors allowed us to identify possible ROS production sites in soybean seeds induced by herbicide. Embryos from glyphosate acid- and Roundup-treated seeds exposed to rotenone had decreased H_2O_2 concentrations compared to their respective controls (seeds treated with herbicides only). Antimycin A, however, did not significantly affect H_2O_2 concentrations in the embryos of herbicide-treated seeds. Rotenone, therefore, blocks the glyphosate-induced ROS production pathway, which was not blocked by antimycin A. These results indicate that in soybean seeds, glyphosate must act by impairing complex III, as proposed by Gomes et al.,[15] or upstream of this enzymatic complex.

Gomes et al.[15] reported greater decreases in complex III compared to complex I activity in glyphosate-treated plants. In our study, in turn, similar reductions in complex I (11 and 18%) and complex III (14 and 17%) were observed in non-GR seeds exposed to glyphosate-acid and Roundup, respectively (Figure 3). In L 8307 RR seeds, however, the reduction in complex I (12 and 19%) was greater than of complex III (6 and 7%). Interestingly, whereas non-GR and L 8307 RR seeds showed similar complex I activities (and similar herbicide-induced decreases in its activity), complex III activity was greater in L 8307 RR seeds (Figure 3). L 8307 RR seeds also showed less H_2O_2 accumulation upon herbicide exposure. Therefore, we propose that glyphosate could act as a complex III blocker, and the greater activity of complex III in L 8307 may indicate less inhibition and, therefore, decreased ROS accumulation than in non-GR seeds. Supporting this hypothesis, the greatest complex III activity and the least reduction in complex III activity upon herbicide exposure (5%) were observed in AS 3810 IPRO seeds, in which H_2O_2 accumulation was not detected.

Although developed to resist the herbicidal effect of glyphosate in the shikimate pathway, GR seeds are faced with a new scenario in which glyphosate can trigger oxidative stress by glyphosate interference with mitochondrial activity. By disrupting the mitochondrial electron transport chain, glyphosate can induce H_2O_2 accumulation in seeds during imbibition, leading to oxidative stress and decreased germination. However, in addition to its resistance to glyphosate, transgenic transformation apparently increases the oxidative status in GR seeds, making these seeds less vulnerable to the oxidative stress induced by glyphosate (as compared with non-GR seeds). The differences in the responses of the studied GR varieties to the presence of glyphosate were related to their differences in enzymatic activity related to H_2O_2 scavenging and complex III (the proposed site of ROS induction by glyphosate).

In accordance with our previous study,[14] Roundup was more toxic to seeds than pure glyphosate, which must be related to the presence of other components in the commercial formulation, such as trace elements and surfactant. Overall, we conclude that the use of a glyphosate-based herbicide as pre-emergence herbicide[13] could result in yield losses in soybean

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cultures, because the germination of some GR and non-GR seed varieties can be affected by the herbicide residue in the soil.

Glyphosate has a half-life of 30−40 days under laboratory conditions,[40−42] although its half-life in the field soils can vary from 2 to 197 days,[43] with a mean of about 30 days.[44] Field applications of 17.8 L Roundup ha^{-1} (corresponding to 6.4 kg of ae/ha of glyphosate) as pre-emergence followed by an early postemergence application and a late postemergence application of 2.34 L Roundup ha^{-1} (0.84 kg of ae/ha of glyphosate) were reported.[45] Considering the proposed half-life of glyphosate in soils, a cycle of about 185 days for soybean culture, and the observed effects from 5 mg Roundup L^{-1} on germination of non-GR and sensitive GR varieties, the amounts of glyphosate expected on soils may be sufficient to affect germination of subsequent cultures. For instance, residual glyphosate in the field has been observed in soil[34,46] and was reported to reduce yields in crops, such as field pea, canola, and wheat.[34] After its introduction into the market in the 1970s, glyphosate has become the most widely used herbicide worldwide, and this success was related to the genetic modification of plants to improve productivity and tolerance to the herbicide. In the face of the new scenario in which glyphosate (an inductor of oxidative stress) is not solely used as a postemergence herbicide and the fact that soil residue of the herbicide reduces crop yields, the search for new and improved soybean varieties with higher tolerance to oxidative stress during the germination process should be a focus. Moreover, we also suggest that companies consider performing germination tests for glyphosate sensitivity before introducing new soybean varieties to the market. Finally, physical−chemical properties, such as texture, mineralogy, phosphate content, and microbial activity, may affect the fate of glyphosate on soils, and therefore its effects on seed germination are claimed.

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Notes

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