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Glyphosate effects on symbiotic nitrogen fixation in glyphosate-resistant soybean

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ABSTRACT

Most soybean (*Glycine max* L. Merr.) cultivars grown in the U.S. are Roundup Ready®, genetically modified for resistance to the herbicide glyphosate. The objectives of this study were to examine the impacts of glyphosate applications on symbiotic N_2 fixation in glyphosate-resistant (GR) soybean and to evaluate the responses of rhizobial isolates and *nifH* gene abundance in the rhizosphere soil to glyphosate. In two repeated greenhouse experiments, GR soybean received foliar applications of glyphosate once or twice during the study period; the untreated GR and near-isogenic conventional cultivar served as controls. Plants were harvested twice, two days after each glyphosate application. In addition to plant growth parameters, the nitrogenase activity of root nodules and the abundance of the *nifH* gene in the rhizosphere were determined using the acetylene reduction assay and quantitative PCR, respectively. Glyphosate-treated GR soybean had lower chlorophyll content, root mass, nodule mass, total plant N, and nitrogenase activity than the untreated conventional cultivar, especially for the second harvest. Without glyphosate application, few differences were observed between the two cultivars. Glyphosate inhibited the growth of rhizobia isolated from root nodules; however, the *nifH* gene abundance in the rhizosphere was not different among the treatments. Glyphosate appeared to exert stress to the GR soybean cultivar used in this study. Further research is needed to verify the greenhouse experiment findings and possible yield effects under field conditions.

1. Introduction

Glyphosate, the active ingredient of the herbicide Roundup®, has been the most widely used herbicide in U.S. agricultural crop production since 2001 (Grube et al., 2011). Glyphosate inhibits the biosynthesis of aromatic amino acids (i.e., phenylalanine, tyrosine, and tryptophan) and leads to several metabolic disturbances, including interruption of protein production, secondary product biosynthesis, and a general metabolic disruption of the phenylpropanoid pathway owing to reduction in the biosynthesis of aromatic amino acids (Franz et al., 1997). Glyphosate-resistant (GR) soybean is genetically engineered by the insertion of a transgene (*CP4 EPSPS*) from *Agrobacterium* sp. strain CP4, which encodes a glyphosate-insensitive version of 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) (Padgette et al., 1995).

Soybean (*Glycine max* L. Merr.) is the second largest crop (by value) in the U.S. (USDA, 2016). Since its commercialization in the USA in 1996, the GR soybean planting area increased from 17% of the total soybean area in 1997 to 94% in 2016 (USDA, 2016). In spite of the advantages of GR soybean adoption, concerns have been raised regarding the potential negative impact of glyphosate (Kremer and Means, 2009; Reddy, 2001). Glyphosate is known to cause a reduction in plant chlorophyll content in GR soybean (Reddy et al., 2000; Zobiole et al., 2010a, 2011; Krenchinski et al., 2017). It has also been associated with decreases in soybean shoot and root biomass as well as root nodule biomass and number (King et al., 2001; Kremer and Means, 2009; Reddy and Zablotowicz, 2003; Zobiole et al., 2010c). Previous studies have indicated that the effect of glyphosate on the N_2-fixing activity of soybean is not consistent (Zablotowicz and Reddy, 2007). The N content of GR soybean appears to be affected by glyphosate application rates, the developmental stage of soybean, and the soybean cultivar itself (King et al., 2001; Reddy and Zablotowicz, 2003).

Nitrogen fixation in soybean is the result of symbiosis between the bacterium *Bradyrhizobium japonicum* and soybean roots. Like plants, bacteria also use the shikimate pathway to synthesize aromatic amino acids (Pollegioni et al., 2011). EPSPS, a key enzyme in the pathway, can be divided into two classes. Class I EPSPS enzymes are naturally

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sensitive to glyphosate and have been found in plants and many Gram-negative bacteria (e.g., *Escherichia coli* and *Salmonella typhimurium*). Class II EPSPS are naturally tolerant to glyphosate and are found only in bacteria, including *Agrobacterium* sp. strain CP4 and *Pseudomonas* sp. PG2982 and some Gram-positive bacteria (Pollegioni et al., 2011). Although there are no reports of isolation and characterization of EPSPS from *B. japonicum*, there is evidence that the presence of sublethal concentrations of glyphosate in culture media leads to accumulation of a large amount of shikimate and reductions in the growth of *B. japonicum* (Hernandez et al., 1999; Moorman et al., 1992). However, different strains of *B. japonicum* have different sensitivities to glyphosate (Hernandez et al., 1999; Moorman et al., 1992). Some strains in the family *Rhizobiaceae* may degrade glyphosate and use it as the sole source of P in the presence of aromatic amino acids (Liu et al., 1991).

Effects of glyphosate on symbiotic N_2 fixation in soybean have been evaluated in both greenhouse and field experiments (e.g., Bohm et al., 2009; King et al., 2001; Powell et al., 2009; Zobiole et al., 2010b); however, the findings have been inconsistent with respect to effects of glyphosate on N_2 fixation. For example, King et al. (2001) found significant reductions in nitrogenase activity in GR soybean in three of the four growth chamber studies when glyphosate was applied at 21 days after emergence and suggested that N_2-fixing activity was more sensitive in the early stages of soybean development. Powell et al. (2009), however, did not observe negative effects of glyphosate on N_2 fixation in two greenhouse experiments. On the contrary, they found N_2 fixation was greater for glyphosate-treated soybean plants than for untreated plants when glyphosate was applied at the first trifoliolate developmental stage. There is a potential risk of reducing symbiotic N_2 fixation in GR soybean caused by the application of glyphosate, especially for repeated applications at high rates.

Prokaryotic organisms carry out N_2 fixation via the nitrogenase complex consisting of subunits that are encoded by the genes *nifH, nifD and nifK* (Zehr et al., 2003). The *nifH* gene, which encodes for the nitrogenase reductase subunit (the Fe protein) of the nitrogenase complex, is the most sequenced of the three genes and has become a marker for studying the diversity and abundance of diazotrophs (Gaby and Buckley, 2012). Many studies have suggested that the abundance of the *nifH* gene depends on environmental conditions such as land use and management, N fertilizer, plant type, soil pH, soil organic matter, soil depth, and seasonal change (Colloff et al., 2008; Coelho et al., 2009; Hayden et al., 2010; Jung et al., 2012; Levy-Booth and Winder, 2010; Poly et al., 2001b). However, there has been no reported assessment of the effect of glyphosate on *nifH* gene abundance in the rhizosphere of GR soybean. As a portion of the foliar-absorbed dose of glyphosate can eventually be released into the rhizosphere (Coupland and Caseley, 1979), it may influence the diazotrophic community as well as other microbes in the rhizosphere (Kremer and Means, 2009; Cherni et al., 2015; Newman et al., 2016a). The objectives of this study were to assess the effects of glyphosate application on symbiotic N_2 fixation in GR soybean and to evaluate the responses of rhizobial isolates and *nifH* gene abundance in the rhizosphere soil to glyphosate.

2. Materials and methods

2.1. Greenhouse experiment

Two repeated experiments were performed in a climate-controlled greenhouse with the maximum temperature set at 28 °C and the minimum at 23 °C. The recorded average daily temperature was 25 °C for Greenhouse Experiment I and 26.7 °C for Greenhouse Experiment II. Natural daylight was supplemented with sodium vapor lamps to provide a total of 18 h of illumination. Soil used in the experiments was a Compass sandy loam (coarse-loamy, siliceous, subactive, thermic Plinthic Paleudult) collected at the surface layer (0–20 cm) from field plots at the E.V. Smith Research Center, Shorter, AL, USA. The soil was not recently cropped with soybean, but glyphosate had been applied. Soil pH was 6.8, which is appropriate for soybean growth. Soil test results showed that no additional P and K were needed. Soil was air-dried and passed through a 2-mm sieve prior to use. About 3.3 kg of soil were placed in high-density polyethylene pots (13 cm in diameter and 24 cm in height, Stuewe & Son Inc., OR, USA) with an approximate soil volume of 2.54 L to obtain a bulk density of about 1.3 g cm^{-3}.

Near-isogenic conventional and Roundup Ready® Prichard soybean cultivars [Maturity Group (MG) VIII] (Boerma et al., 2001) were obtained from the University of Georgia Research Foundation, Inc. Seeds were disinfected by immersing in 0.1% sodium hypochlorite for 2 min, washed several times using sterile distilled water, and air dried in a Class II biosafety cabinet. At planting, disinfected seeds were mixed with the peat-based Rhizo-Stick® inoculant (Becker Underwood Inc., Ames, IA, USA), containing 10^8 viable *Bradyrhizobium japonicum* strain TA-11 NOD+ per gram, at the recommended rate of 3.4 g inoculant kg^{-1} seeds. Three seeds were planted in each pot. At the V1 developmental stage, plants were thinned to one plant per pot. After emergence, pots were watered as needed to maintain about 50% of the soil water holding capacity. To reduce variability of light exposure in the greenhouse, pots were rotated periodically.

The experiment was a randomized complete block design with four replications. Glyphosate treatments consisted of a single application at 1.68 kg a.e. ha^{-1}, two sequential applications (1.68 kg a.e. ha^{-1} each), and a control without glyphosate application for the GR soybean cultivar (Table 1). The near-isogenic conventional (i.e., non-GR) soybean cultivar also served as a control and did not receive any herbicide. Glyphosate (Roundup Ultra®, Monsanto, St. Louis, MO, USA) was applied using a pressurized indoor spray chamber calibrated to deliver 281 L ha^{-1} at 221 kPa using a single Teejet 8002 E flat fan nozzle. Each application was a post-emergence over-the-top spray to plants. The timing of glyphosate application is shown in Table 1. Plants were harvested two days after each glyphosate application in order to capture soybean response to different cumulative glyphosate rates in different growth stages: the first harvest (Harvest 1) included the conventional and untreated GR cultivars as well as the GR cultivar receiving a single glyphosate application; the second harvest (Harvest 2) included all four treatments (Table 1).

Immediately before each harvest, chlorophyll content was measured

Table 1
Soybean cultivar and glyphosate treatments used in greenhouse experiments.

Treatment		Glyphosate rate (kg a.e. ha^{-1})	Application timing		Harvest timing (DAP)	
Soybean cultivar[a]	Glyphosate application		DAP[b]	Growth stage[c]	First	Second
Greenhouse Experiment I						
Prichard	None (0 x)	0	–	–	21	31
Prichard RR	None (0 x)	0	–	–	21	31
Prichard RR	Once (1 x)	1.68	19	V2-V3	21	31
Prichard RR	Twice (2 x)	1.68 + 1.68	19 and 29	V2-V3 and V5	–	31
Greenhouse Experiment II						
Prichard	None (0 x)	0	–	–	24	32
Prichard RR	None (0 x)	0	–	–	24	32
Prichard RR	Once (1 x)	1.68	22	V3-V4	24	32
Prichard RR	Twice (2 x)	1.68 + 1.68	22 and 30	V3-V4 and V5-V6	–	32

[a] Prichard is the conventional cultivar; Prichard RR is the glyphosate-resistant cultivar.
[b] DAP: days after planting.
[c] Growth stage descriptions follow Pedersen (2009).