Glyphosate affects chlorophyll, nodulation and nutrient accumulation of “second generation” glyphosate-resistant soybean (Glycine max L.)

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1. Introduction

The “first generation” of Roundup Ready® (RR1) soybeans was commercialized in U.S. in 1996 [1]. It was developed by inserting the gene cp4 epsps derived from the soil bacterium Agrobacterium sp. strain CP4 [2]. This gene directs the production of 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS) that is less sensitive to inhibition by glyphosate compared to the endogenous EPSPS of non-transgenic soybean plants. Modification of transgenic techniques resulted in “second generation” (RR2) cultivars that were commercially available to farmers in 2008 and promoted for higher yielding traits relative to RR1 cultivars.

A benefit promoted for using RR biotechnology is the use of lower amounts of herbicides [3], however with the introduction of RR2 that is highly tolerant to potential herbicide damage, the volume and scope of glyphosate may actually increase with RR2 soybean as glyphosate-resistant weeds continue to proliferate. Many farmers report frequent visual plant injury in RR1 soybean varieties after glyphosate application [4]. The typical symptom is known as “yellow flashing” possibly due to the accumulation of the primary phytotoxic metabolite aminomethylphosphonic acid (AMPA) [5]. Although yellow flashing is usually considered non-persistent because it tends to disappear within two weeks after herbicide application [6], recent research demonstrated that glyphosate or one of its metabolites is apparently active in the soybean plant through the R1 growth stage or later, as indicated by decreased shoot mineral concentrations in leaf tissue and seed [7]. In fact, several studies concluded that glyphosate affects micronutrient nutrition of plants by chelation of metal ions by glyphosate [8,9]. This can lead to nutrient immobilization and interference with uptake and translocation by the plant [10].

Glyphosate, as a strong metal ion chelator [8,11] may immobilize essential micronutrients such as Fe [12] and Mn [13] that are required as components, co-factors or regulators of physiological functions. We recently demonstrated chelation of Ni, by glyphosate and decreased biological nitrogen fixation in RR soybeans due to reduced Ni available for hydrogenase and ureide metabolism in soybean [14]. Toxic effects of glyphosate to symbiotic microorganisms include interference with aromatic amino acid synthesis. The accumulation of intermediates of the shikimic acid pathway may represent a loss of energy and may be a significant factor responsible for reduced growth and yield in RR1 soybean [15].

Information on effects of glyphosate on mineral nutrition and symbiotic N2 fixation in RR2 soybeans is limited or absent. Thus, this research evaluated the nutrient accumulation and nodulation...
in first and second generation RR soybean cultivars at different rates of glyphosate applied at various growth stages.

2. Material and methods

2.1. Test location and soil characteristics

Growth trials were conducted between July and October, 2009 at the USDA-ARS greenhouse facility (Columbia, MO, USA), using the A horizon of a Mexico silt loam soil (fine, smectitic, mesic Aeric Vertic Epiaqualfs) with no previous herbicide use collected from Bradford Research and Extension Center, University of Missouri, in Boone County-MO. The experimental units were 5-dm \(^3\) clay pots filled with air dried soil \((C_{org} = 25.2 \text{ g kg}^{-1}; P: 15.87 \text{ mg kg}^{-1}; K: 45.86 \text{ mg kg}^{-1}; Ca: 1782.64 \text{ mg kg}^{-1}; Mg: 123.89 \text{ mg kg}^{-1}; Fe: 80.70 \text{ mg kg}^{-1}; Mn: 43.23 \text{ mg kg}^{-1}; B: 14.06 \text{ mg kg}^{-1}; Cu: 1.78 \text{ mg kg}^{-1}; Zn: 9.82 \text{ mg kg}^{-1}; Mo: 1.61 \text{ mg kg}^{-1}; pH_{calc}: 6.77) sieved through a 5-mm mesh screen. A consistent soil water \((0.33 \text{ g g}^{-1})\) contents was maintained throughout the experiment. Plants were grown in the greenhouse equipped with an evaporative cooling system \((26–30 °C; 22–26 °C day/night)\) with a 12-h photoperiod of full sunlight, midday irradiance \((400–940 \text{ nm})\), and a photosynthetic photon flux density of 1500 \(\mu\text{mol m}^{-2} \text{s}^{-1}\) at the top of the leaf canopy.

2.2. Experimental design

The experiment was repeated twice in its entirety in a randomized complete block design, using a factorial scheme \((2 \times 3 \times 3)\), replicated four times in each treatment for each experiment. The first factor was represented by cultivars \((RR1 \text{ or } RR2)\), the second was glyphosate rate \((800, 1200 \text{ and } 2400 \text{ g a.e. ha}^{-1})\), and the third was application timing within RR soybean growth stages \((V2, 12 \text{ and } 10 \text{ DAS – days after sowing; V4, 25 \text{ and } 22 \text{ DAS; or V6, 32 \text{ and } 35 \text{ DAS for RR1 and RR2, respectively.})}\). A non-applied glyphosate treatment was the control.

2.3. Seeds and inoculation treatment

Seeds of cultivars BRS 242 RR \((RR1)\) and AG3539 RR \((RR2)\), were sterilized for 2 min in 2% NaClO, rinsing in sterile water and blotted dry before inoculating with 100 mL 50 \(\text{ kg}^{-1}\) of a culture of \(Bradyrhizobium japonicum\) \((\text{strains SEMIA587 and SEMIA 5019})\) at a concentration of \(5 \times 10^8\) Rhizobia per gram. The \(B. japonicum\) inoculation rate was approximately \(1.2 \times 10^6 \text{ cells per soybean seed}\) based on dilution-plate counts \([16]\). Four seeds per pot were sown at 3-cm depth and thinned to one plant per pot at V1 growth stage.

2.4. Glyphosate applications

Treatments were sprayed with a moving track sprayer using an even flat-fan nozzle tip \((\text{Teejet, Spraying Systems Co., Wheaton, IL})\) delivering 187 L ha \(^{-1}\) at 150 kPa. The applications were at 7:00 am using the commercially formulated potassium salt of glyphosate \(540 \text{ g a.e. L}^{-1}\) \((\text{Roundup Weather Max\textsuperscript{®}, Monsanto Company})\) at different rates \((800, 1200 \text{ and } 2400 \text{ g a.e. ha}^{-1})\) and application timings \((V2, V4 \text{ and V6 growth stage.})\). Based on Gazziero et al. \([17]\), the label used for single glyphosate application at V4 growth stage in RR soybeans, varies around 600–1200 g a.e. ha \(^{-1}\). The sprayed solution did not cause run-off from leaves and plants were irrigated the following day to ensure leaf absorption of the herbicide. The pots were irrigated daily in order to keep the soil moist ensuring consistent soil water content.

2.5. Chlorophyll content

At R1 growth stage \((42 \text{ and } 38 \text{ DAS, for RR1 and RR2, respectively})\), SPAD measurements were performed with a SPAD-502 meter \((\text{Minolta Co., Osaka, Japan})\) to measure absorption at 650 and 940 nm wavelengths to estimate chlorophyll concentration \([18]\). The SPAD sensor was placed randomly on leaf mesophyll tissue only to avoid the veins. Three SPAD readings were taken per leaflet of the terminal leaf of the last fully expanded trifolium \((\text{diagnostic leaf})\) and averaged to provide a single SPAD unit from which chlorophyll content was estimated. The chlorophyll content was calculated using the SPAD unit by the equation of Arnon \([19]\) and expressed as milligrams of chlorophyll per cm \(^{-2}\) of leaf tissue by the equation of Markwell et al. \([20]\).

2.6. Nutrient accumulation

Immediately after collecting the SPAD evaluations at the R1 growth stage, leaves of the upper portion of each plant, including the diagnostic leaf, were collected from each pot. Leaves were washed in deionized water, packed in paper bags to dry in an air circulation oven at 60–70°C, and weighed after a constant dry weight was achieved. The mineral composition \((P, K, Ca, Mg, S, Zn, Mn, Fe, Cu)\) of the leaves was determined by complete perchloric nitric digestion \((6:1)\); B concentration was obtained after dry digestion \([21]\). All elements, except N, were measured using an ICP \((\text{inductively coupled plasma})\) spectrophotometer \((\text{AES Perkin Elmer, Norwalk, CT, USA})\). Nitrogen was determined using sulfuric acid digestion and measured by the micro-Kjeldahl method \([22]\).

2.7. Nodulation and biomass

After leaf collection, shoots were clipped at the soil surface and roots were carefully removed from soil, washed under running water and nodules were removed from each plant and counted. Immediately after the nodule counting, the roots, shoots and nodules were placed in separate paper bags and transferred to an air circulation oven to determine dry weights.

2.8. Statistical analyses

The data errors passed the normality test of Shapiro and Wilk \([23]\) and, because there was homogeneity of error variances, the
data for the two experimental repeats were combined and no transformations were necessary. Data were subjected to ANOVA using PROC MIXED by SAS statistical program [24]. When F values were significant ($P < 0.01$), regression analysis were conducted and equations were adjusted using the polynomial model (Eq. (1)) by SigmaPlot 10.0 statistical package [25].

$$y = a + bx + cx^{0.5}$$

(1)

3. Results and discussion

3.1. Chlorophyll content

Glyphosate significantly decreased chlorophyll content (Fig. 1) compared with the non-glyphosate control. The reduction was more pronounced as the glyphosate rate increased and application delay (V6). These findings are consistent with reports by farmers

Fig. 2. Macronutrient in upper leaves at R1 growth stage of RR1 and RR2 soybeans with increasing rates of glyphosate applied at different growth stages of soybean, V2, V4 and V6 ($n = 8$, $P < 0.01$).
that some glyphosate-resistant soybean varieties are visually injured by glyphosate [4]. Previous studies reported by Zobiole et al. [26], showed that the chlorotic symptoms may be related to decreased photosynthetic rates as a result of direct damage of glyphosate to chlorophyll [5,27] or immobilization of Mg and Mn (due to glyphosate cation nutrient complexes) required for chlorophyll formation and photosynthesis, respectively [28,29]. Cakmak et al. [10] reported that glyphosate can physiologically immobilize these nutrients in the tissues and compromise chlorophyll production.

Although the visual injuries that are likely to happen in RR1 soybeans after glyphosate application are usually considered to be non-persistent as the yellow flashing tends to disappear within the first 2 weeks after herbicide application [6], it was anticipated that RR2 would show little injury from glyphosate application; however, the chlorophyll content of both RR1 and RR2 was
decreased by glyphosate (Fig. 1). Accumulation of the main glyphosate metabolite, aminomethylphosphonic acid (AMPA), in the glyphosate-treated plants may have contributed to plant injury and chlorosis [5,30].

Muñoz-Rueda et al. [31], reported that decreased chlorophyll can be caused by carotenoid loss induced by sub-lethal doses of glyphosate. In this research, the rates used (800 and 1200 g a.e. ha\(^{-1}\)) are within those recommended for glyphosate application in RR soybeans (label rates used for single glyphosate applications at V4 growth stage are between 600 and 1200 g a.e. ha\(^{-1}\)). As expected, the 2400 g a.e. ha\(^{-1}\) rate caused leaf damage (Fig. 1), however plant damage resulting from an application of recommended rates for RR1 and RR2 was somewhat unexpected. Further, chlorophyll content of RR soybeans should not be affected since it is assumed that the insensitive EPSPS enzyme synthesized in the transgenic cultivars is insensitive to effects of glyphosate; however, it is not completely certain that this is the actual scenario that occurs within the transgenic RR plant after glyphosate treatment [13].

3.2. Nutrient accumulation

The macro and micronutrient accumulation in both RR cultivars was decreased by glyphosate application (Figs. 2 and 3). Decreased nutrient accumulation, in general, was affected more by late than early glyphosate application (V6 and V2, respectively). In addition, the effects were pronounced with increased glyphosate rates. These findings correlated with the decreased chlorophyll content, which was proportional to glyphosate rate (Fig. 1). Glyphosate may also prevent chlorophyll synthesis by inhibiting the formation of the porphyrin precursor δ-aminolevulinic acid (ALA) [32] or by direct inhibition of ALA synthesis [33], which is a precursor of chlorophyll and requires Fe for its function [34]. Furthermore, the chloroplast is sensitive to Mn [35] and Zn [36] deficiency, both of which are reduced by glyphosate [37]. Thus, decreased accumulation in Zn, Mn and Fe by glyphosate applications (Fig. 3A–C) could cause the decreased chlorophyll production noticed in Fig. 1.

Field observations in Brazil and the North Central United States indicate that frequent applications of glyphosate induce Fe, Zn, and Mn deficiencies in RR-soybean [13]. According to Eker et al. [9], after glyphosate is absorbed by the plant, the uptake and transport of cationic micronutrients may be inhibited by the formation of poorly soluble glyphosate-metal complexes within plant tissues. Fig. 4 illustrates the correlation with the results presented in Figs. 1–3, in which younger plants (V2 growth stage) were less injured by glyphosate than plants at a later growth stage (V6). Although leaf samples in Fig. 4 originated from RR2 plants, RR1 presented similar glyphosate effects and also showed intense nutrient immobilization with high glyphosate rates (Fig. 4).

Non-treated RR soybeans had higher nutrient accumulation than those treated with glyphosate. All macro- and micronutrients, with exception of N and K, accumulated more in RR1 than RR2 (Figs. 2 and 3 and Table 1). This result may be an individual cultivar characteristic, but it suggests that the RR2 cultivar was inefficient in nutrient uptake and translocation or was unable to rapidly recover from potential chelating effects of glyphosate [8,9].

In a recent study, Zobiole et al. [38], evaluated nutrient uptake in RR soybean under different glyphosate rates (600, 900, 1200, 1800 and 2400 g a.e. ha\(^{-1}\)), either in sequential or single applications. Single applications were applied at V4 growth stage and sequential applications at 50% of the dose at V4 and V7 growth stage. At the R1 growth stage, 50 mL samples of nutrient solution were collected from each pot, in order to determine the nutrient concentrations and electrical conductivity. The data collected provided evidence that plants subjected to increasing glyphosate rates had more nutrients remaining in solution and, therefore higher electrical conductivity to demonstrate low nutrient uptake.

In the present experiment, glyphosate apparently remained active in soybean through R1 growth stage or later as indicated by decreased nutrient accumulation. It is known that glyphosate and its metabolites can remain within the plant until complete physiological maturity [30]. Nutrient balance is important because each element functions as part of a delicately balanced, interdependent physiological system within the plant [13].

3.3. Nodulation and biomass

Moorman [39] reported that reduced nodulation can be an indirect result of glyphosate injury to the plant and from direct action of glyphosate on the nitrogen-fixing symbiont, because it can be translocated to important metabolic sinks including root nodules [6] and be exuded into the rhizosphere of RR soybeans [40]. B. japonicum, possesses a glyphosate-sensitive EPSP synthase and accumulates shikimic, hydroxybenzoic and protocatechuic acids (PCA) upon exposure to glyphosate, which can inhibit growth and induces death at high concentrations [41].

Nodule dry weight is one of the minimum parameters used to quantify the efficiency of biological nitrogen fixation in soybean [42] and supports our results in which glyphosate reduced nodule dry weight and number per plant (Fig. 5) to correlate with decreased N accumulation (Fig. 2A). This reduction may be due the direct toxicity to nodule formation by glyphosate and its intermediates [41] or by decreased Ni uptake since it is an essential element for bacterial nitrogen fixation [43]. A recent study with different RR1 maturity groups showed that glyphosate decreases symbiotic N\(_2\) fixation by decreasing Ni availability to the rhizobia [14].

Field and greenhouse studies conducted over multiple years on several RR soybean cultivars found consistent and significantly
decreased nodulation on RR soybean with or without glyphosate compared with conventional cultivars with non-glyphosate or no herbicides [40,44]. Although effects may vary widely among soybean genotypes, previous research has shown that as much as 45% of the most widely-planted RR soybean cultivars grown in Brazil could be negatively affected by glyphosate resulting in
reduced nodule dry weight and numbers per plant, under one-time or sequential applications, with single applications causing most severe reductions [44]. More studies on the nitrogen fixation process in RR crops are needed in order to more fully understand the effects of glyphosate on nitrogen nutrition in these crops and to develop prudent herbicide use to minimize adverse plant effects.

We noticed a slight difference among time and rates of glyphosate applied to RR2 soybean; however, increased glyphosate rates decreased the nodule numbers in RR1, mainly by the early glyphosate application (V2 growth stage). Nodule dry weight significantly decreased with increased glyphosate rate, with both RR cultivars although there was a tendency for late applications to have less effect than early applications (Fig. 5B). Similar findings were noticed for the percentage root biomass reduction, which was more severe with glyphosate applied at V2 growth stage compared with applications at V6 growth stage (Fig. 5C).

In contrast, shoot biomass accumulation exhibited an opposite trend, with a higher percent reduction associated with late than with early glyphosate applications (Fig. 5D), suggesting that plants probably have more time to recover from glyphosate effects with early applications (V2). Previous studies also reported reduced shoot and root dry weight in RR soybeans using glyphosate at 600 and 900 g a.e. ha\(^{-1}\) (Zobiole et al. [26,38]), 1200 g a.e. ha\(^{-1}\) (Zobiole et al. [7,14]), 1680 g a.e. ha\(^{-1}\) (Reddy et al. [45]), 1800 and 2400 g a.e. ha\(^{-1}\) (Zobiole et al. [26,38]) and 6300 g a.e. ha\(^{-1}\) (King et al. [46]). Thus, the high percentage of biomass reduction in glyphosate treated RR1 and RR2 soybeans indicates that higher levels of available nutrients may actually be required to achieve physiologically sufficiency.

These results showed that the nutrient accumulations of both generations of RR soybeans are strongly affected by glyphosate rates. Non-treated plants (no glyphosate) always had higher nutrient accumulation and, in general, RR2 accumulated less nutrients than RR1. The new generation RR soybeans also showed undesirable glyphosate effects as “yellow flashing”. An approach to minimize glyphosate effects based on a more judicious use of glyphosate by treating weed populations only where and when their densities warrant application could greatly reduce glyphosate use and likely decrease glyphosate injury to RR soybean. However, management based on growth stage and weed population density will need to be considered if this strategy is undertaken.

4. Conclusions

There does not appear to be an improvement in the yield components of photosynthesis, chlorophyll content, nodulation, or nutrient sufficiency in RR2 compared with RR1 soybeans. The results suggest that management practices such as early application and lower rates might be used to minimize glyphosate injury; however, the ability to reduce the glyphosate rate may require more intensive weed management by farmers.
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