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REGULAR ARTICLE

Glyphosate affects photosynthesis in first and second generation of glyphosate-resistant soybeans

Luiz Henrique Saes Zobiole · Robert John Kremer · Rubem Silvério de Oliveira Jr · Jamil Constantin

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Abstract The crop area planted to conventional soybeans has decreased annually while that planted to glyphosate-resistant (RR) soybean has drastically increased mainly due to the wide adoption of glyphosate in current weed management systems. With the extensive use of glyphosate, many farmers have noted visual plant injury in RR soybean varieties after glyphosate application. A new generation designated as "second generation—RR2" has been recently developed and these RR2 cultivars already are commercially available for farmers and promoted as higher yielding relative to the previous RR cultivars. However, little information is currently available about the performance of RR2 soybean beyond commercial and farmer testimonial data. Thus, an evaluation of different glyphosate rates applied in different growth stages of the first and second generation of RR soybeans, revealed a significant decrease in photosynthesis. In general, increased glyphosate rate and late applications (V6) pronounced decrease photosynthetic parameters and consequently decreased in leaf area and shoot biomass production. In contrast, low rate and early applications were less damage for the RR soybean plants, suggesting that with early applications (V2), plants probably have more time to recover from glyphosate or its metabolites effects regarding late applications.

Keywords Glyphosate resistant soybean (*Glycine max* L.) · Glyphosate · Photosynthesis · Biomass

Abbreviations

DAS	Days after sowing
A	Photosynthetic rate
E	Transpiration rate
gs	Stomatal conductance
Ci	Sub-stomatal CO_2
ETR	Photosynthetic electron transport rates
Fo′	Minimal fluorescence of a light adapted leaf
Fm′	Maximal fluorescence of a light adapted leaf
Fs	Steady state fluorescence of a light adapted leaf
Fv′/Fm′	Intrinsic efficiency of photosystem 2
PS2	Photosystem 2
PhiPS2	Quantum efficiencies of photosynthetic electron transport through photosystem 2

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L. H. S. Zobiole (✉) · R. S. de Oliveira Jr · J. Constantin
Center for Advanced Studies in Weed Research (NAPD),
State University of Maringá (UEM),
5790 Colombo Av.,
87020-900 Maringá, Paraná, Brazil
e-mail: lhzobiole@uol.com.br

R. J. Kremer
United States Department of Agriculture, Agricultural Research Service, Cropping Systems & Water Quality Research Unit, University of Missouri,
327 Anheuser-Busch Natural Resources Building,
Columbia, MO 65211, USA

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PhiCO2	Quantum yield based on CO_2 assimilation
qN	Non-photochemical quenching of chlorophyll fluorescence
qP	Proportion of open reaction centers
RR1	Glyphosate-resistant soybean—first generation
RR2	Glyphosate-resistant soybean—second generation
Non-RR	Conventional soybean near-isogenic parental line

Introduction

Soybean is a major crop cultivated worldwide with a global production area that continuously increases each year mainly due to the wide use of glyphosate in current weed management systems based on glyphosate-resistant or Roundup Ready® (RR) soybeans. The first generation of RR soybeans was introduced in 1996 in U.S. (Duke 2005), developed by insertion of the cp4 epsps encoding sequence derived from the common soil bacterium *Agrobacterium* sp. strain cp4 (Franz et al. 1997). This gene (cp4 epsps) directs the production of the 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.

A new generation designated as "second generation—RR2" has been recently developed based on a new technique of *Agrobacterium*-mediated gene delivery to soybean meristem, where cells were induced directly to produce shoots and give rise to transgenic plants (Martinell et al. 2002). This technique allowed direct transformation of the gene cassette into elite soybean cultivars such as Asgrow soybean variety A3244 (Paschal 1997). Using elite cultivars as the base genetics, the superior agronomic characteristic of A3244 can be introgressed to other soybean varieties through crosses with the MON 89788 insertion event containing the cp4 epsps cassette (Taylor et al. 2007). In 2008, these RR2 cultivars were commercially available for farmers and promoted higher yields relative to the previous RR cultivars.

Although a main argument for RR biotechnology is that it reduces the need for herbicides (Gianessi and Carpenter 2000), it is believed that with the introduction of RR2, farmers will use even more glyphosate as it does not damage the crop and allows a wide window of application. However, little information is currently available about the performance of RR2 soybean beyond commercial and farmer testimonial data. In addition, many farmers reported visual plant injury in some RR1 soybean varieties after glyphosate application (Zablotowicz and Reddy 2007). Such symptoms known as "yellow flashing" or yellowing of the upper leaves have been attributed to the accumulation of the primary phytotoxic metabolite aminomethylphosphonic acid (AMPA) (Reddy et al. 2004).

Field observations in Brazil and the North Central United States also suggested that frequent applications of glyphosate induce Fe, Zn, and Mn deficiencies in RR soybeans (Huber 2006; Johal and Huber 2009). A previous study demonstrated that glyphosate reduced shoot concentrations of mineral nutrients in RR soybean as compared to nontreated RR soybean or their near-isogenic nontreated non-RR with the effect being most pronounced in the early maturity group cultivars (Zobiole et al. 2010a). The effect on decreased shoot mineral concentration could be attributed to reductions in photosynthetic parameters as a result of direct damage of glyphosate to chlorophyll (Pihakaski and Pihakaski 1980; Kitchen et al. 1981; Reddy et al. 2004) or immobilization of essential micronutrients by glyphosate, due to the ability of glyphosate to form insoluble glyphosate-metal complexes (Jaworski 1972; Kabachnik et al. 1974; Bromilow et al. 1993; Coutinho and Mazo 2005).

Moreover Zobiole et al. (2010b), in further studies with the highly glyphosate-sensitive early maturity group cultivars, evaluated the influence of increasing glyphosate rates on photosynthesis and water use efficiency of the plants. They demonstrated that as glyphosate rates increased, all photosynthetic parameters and chlorophyll fluorescence decreased drastically, consequently demonstrating that photosynthesis, water use efficiency and biomass production of RR soybean were strongly affected by glyphosate. In fact, little data are available regarding the effects of glyphosate on RR soybean physiology, especially those related to photosynthesis, and even less information about glyphosate effects on RR2 cultivars. Thus, the objective of this research was to evaluate the photosynthesis of RR1 and RR2 soybeans treated with different glyphosate rates.

Materials and methods

Soil and growth conditions

Two experiments were carried out in a 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 (400-700 nm) and a photosynthetic photon flux density of 1,500 μmol m^{-2} s^{-1} at the top of the leaf canopy, at the University of Missouri, Columbia, MO, USA, between July and October, 2009. The experimental units for both experiments were 5 dm^{-3} clay pots filled with soil from the A horizon of a Mexico silt loam soil (fine, smectitic, mesic Aeric Vertic Epiaqualfs). Soil properties were (C_{org}: 25.2 g kg^{-1}; P: 15.87 mg kg^{-1}; K: 45.86 mg kg^{-1}; Ca: 1,782.64 mg kg^{-1}; Mg: 123.89 mg kg^{-1}; Fe: 80.70 mg kg^{-1}; Mn: 43.23 mg kg^{-1}; B: 14.06 mg kg^{-1}; Cu: 1.78 mg kg^{-1}; Zn: 9.82 mg kg^{-1}; Mo: 1.61 mg kg^{-1}; $pH_{(CaCl2)}$: 6.77). The soil was air-dried and sieved to pass through a 5 mm mesh screen. Consistent soil water (0.33 $g.g^{-1}$) contents were maintained throughout the experiment.

Seed and glyphosate application

Seeds of cv. BRS 242 RR (RR1—first generation) and seeds of the "new generation" of RR soybean cv. AG3539 RR (RR2—second generation), were sterilized for 2 min in 2% NaClO and then inoculated with 100 mL 50 kg^{-1} of seeds of a culture of *Bradyrhizobium japonicum*, strains SEMIA587 and SEMIA 5019 at a concentration of 5×10^9 rhizobia per gram. Six seeds per pot were sown at 3 cm depth and thinned to one plant per pot at the one-leaf (V1) growth stage.

Plants at different growth stages V2 (12 and 10 DAS—days after sowing), V4 (25 and 22 DAS), and V6 (32 and 35 DAS) for RR1 and RR2, respectively, were sprayed at 7:00 am using the commercially formulated potassium salt of glyphosate 540 g a.e. L^{-1} (Roundup Weather Max®, Monsanto Company) under different rates (800, 1,200 and 2,400 g a.e. ha^{-1}). Except for the higher rate (2,400 g a.e. ha^{-1}), the other rates used are according to Gazziero et al. (2008), which the label used for single glyphosate application at V4 growth stage in RR soybeans, varies around 600 to 1,200 g a.e. ha^{-1}. Spray applications were made with a moving track sprayer using an even flat-fan (Teejet, Spraying Systems Co., Wheaton, IL) nozzle tip delivering 187 $L.ha^{-1}$ at 150 kPa. The sprayed solution did not cause run-off from leaves and plants were irrigated the following day to ensure the leaf absorption of the herbicide. The pots were irrigated daily in order to keep the soil moist ensuring the consistent soil water content.

Photosynthesis analysis

At the last fully expanded trifolium (diagnostic leaf) at R1 growth stage, photosynthetic parameters as photosynthetic rate (*A*), stomatal conductance (*gs*), transpiration rate (*E*) and sub-stomatal CO_2 concentration (*Ci*) were recorded by an infrared gas analysis (IRGA; Li-Cor, LI 6400XT, Lincoln, NE, USA) and calculated using the equations of von Caemmerer and Farquhar (1981). Time to reach R1 growth stage differed slightly for each cultivar, with RR1 at 42 DAS and RR2 at 38 DAS.

A chlorophyll fluorometer chamber (LI6400-40) was integrated on pulse-amplitude modulated (PAM) with the LI6400XT to evaluate the fluorescence parameters. The photosynthesis system's chamber (LI6400-40) has a sampling area of 2 cm^2 and an internal red–blue LED light source that was used to obtain the desired photosynthetic photo flux density (PPFD) and take chlorophyll fluorescence measurements (Kumudini et al. 2008). For measurement of electron transport rate (*ETR*), the modulation frequency of the measuring light was 10 kHz under actinic illumination and increased to 20 kHz during saturating pulses. The saturating pulse was set for a duration of 0.8 s. The quantum yield of photosystem 2 (*PhiPS2*) photochemistry was calculated by the following equation:

$$PhiPS2 = Fm' - Fs/Fm'$$

where Fm' is the maximal fluorescence in the light during a saturating light flash and *Fs* is "steady-state" fluorescence in the light (Genty et al. 1989). The *PhiPS2* can be used to calculate *ETR* as:

$$ETR = PhiPS2{*}f{*}PPFD{*}\alpha leaf\,absorbance$$

where *f* is the fraction of absorbed quanta that is used by photosystem 2 (*PS2*) and is typically assumed to be 0.5 for C3 plants (Kumudini et al. 2008).

The coefficient of non-photochemical quenching of chlorophyll fluorescence (qP) and the coefficient of the proportion of open reaction centers (qP) were also estimated by the following equations (Krause and Weis 1991).

$$qP = Fm' - Fs/Fm' - Fo'$$

$$qN = (Fm/Fm') - 1$$

With these variables measured, the ratio Fv'/Fm' was calculated according to Demming-Adams and Adams (1992) by the equation:

$$Fv'/Fm' = Fm' - Fo'/Fm'$$

All of the photosynthetic measurements were taken at a constant air flow rate of 500 μmol s^{-1}. The concentration of CO_2 was 400 μmol CO_2 mol^{-1} air using the system's CO_2 injector (model 6400–01, Li-Cor), and the temperature was maintained at 26± 2°C. IRGA was calibrated to provide similar leaf and air temperature within the sample chamber at a constant PPFD of 1,500 μmol m^{-2} s^{-1} PARi.

SPAD readings

The SPAD sensor (Minolta SPAD-502 meter) readings were taken randomly on leaf mesophyll tissue only (with veins avoided) of the terminal leaflet of the diagnostic leaf (Singh et al. 2002; Richardson et al. 2002; Pinkard et al. 2006). Two leaves were chosen per plant in the pot and measurements were immediately taken per leaf and averaged to provide a single SPAD unit.

Leaf area and biomass

The leaf area was measure using a leaf area meter (Delta T. Devices) per entire plant per pot to obtain a total area (cm^2 $plant^{-1}$). After these assessments, shoots were clipped at the soil surface and roots were carefully removed from soil, washed under running water, packed in paper bags to dry in an air circulation oven at 65–70°C, and weighed after constant dry weight was achieved. Biomass was determined by weighing plant parts.

Data analysis and experimental design

Two experiments were designed as a completely randomized block, in a factorial scheme (3×3×2) +1 with four replicates. The first factor was the glyphosate rate (800, 1,200 and 2,400 g a.e. ha^{-1}), the second was soybean growth stage (V2, V4 and V6) and the third factor was the two cultivars of different RR generations (cv. BRS 242 RR—first generation "RR1" and cv. AG3539 RR—second generation "RR2"). The additional treatment was a non−applied glyphosate treatment. Data were subjected to analysis of variance, and when F values were significant ($P<$ 0.01), regression analysis were conducted. Data were analyzed using PROC MIXED by SAS statistical program (SAS Institute 2006) and equations were adjusted using the polynomial model $\hat{y} = a + bx + cx^{0.5}$ by SigmaPlot 10.0 statistical package (SPSS 2000).

Results

Photosynthetic parameters

In both experiments, the photosynthetic rate (A) was severely affected by glyphosate application at R1 growth stage. However, the effects were more pronounced with increased glyphosate rate and late applications (Fig. 1a). The different cultivars RR1 and RR2 were affected by glyphosate, although although A in RR2 was higher than in RR1. Considering the treatments without glyphosate, RR1 presented 16.43–17.17 μmol m^{-2} s^{-1}, while RR2 was between 26.82–26.97 μmol m^{-2} s^{-1}, around 60% more than RR1 (Table 1). These findings are in accordance with those reported by farmers, in which some glyphosate-resistant soybean varieties are visually injured by glyphosate (Zablotowicz and Reddy 2007).

In general, the stomatal conductance (gs) decreased as glyphosate increased however there was no difference among the growth stage of application on RR1. In addition, for the V4 growth stage on RR2, there was no significant different among the glyphosate rates, however all glyphosate rates decreased the gs (Fig. 1b). As stomatal conductance declined with increased rates of glyphosate, a sharp decreased was observed for transpiration rate (E) in both cultivars, but the effect was greater for RR2 than RR1 (Fig. 1c,

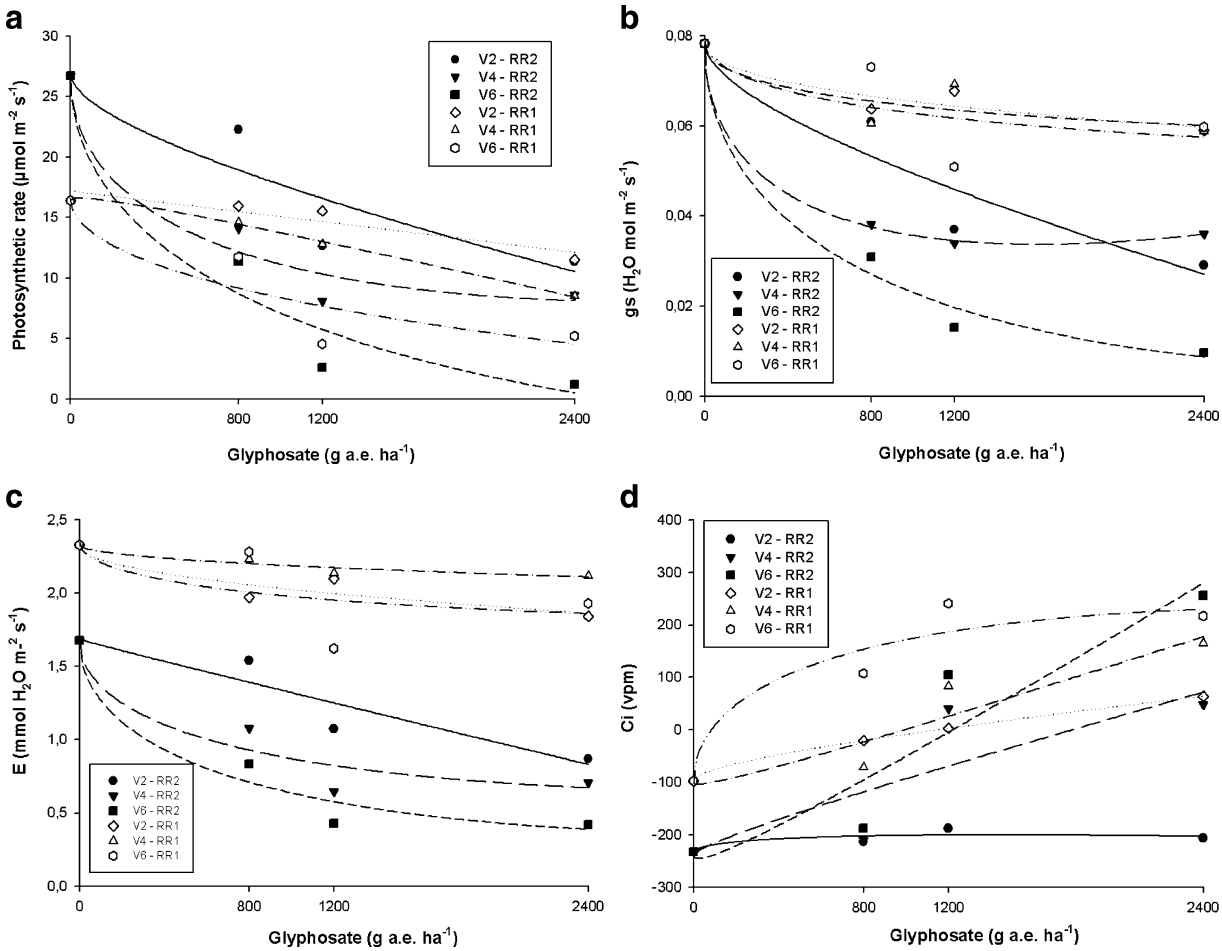

Fig. 1 Photosynthetic rate (A), stomatal conductance (gs), transpiration rate (E) and sub-stomatal CO_2 concentration (Ci) in different RR soybeans under increasing rates of single glyphosate application at different growth stages of soybean, V2, V4 and V6 (n=6, P<0.01)

Table 1). No significant differences in *E* among the glyphosate rates were found with RR1 comparing with RR2, which in RR2 *E* decreased as glyphosate increased (Fig. 1c). The late growth stage (V6) of RR2 was more sensitive to glyphosate application than the other growth stages; there was no difference in plant growth measurements between V4 and V6 for RR1, as both were lower than V2 (Fig. 3).

Magalhães Filho et al. (2008) reported that partial stomatal closure leads to decreased *gs* and increased sub-stomatal CO_2 concentration (*Ci*). Thus with increased glyphosate rate and late application, decreased *gs* and increased *Ci* was noticed for both cultivars. In fact, the CO_2 assimilation was severely decreased by glyphosate (Fig. 1d) except for the V2 growth stage of RR2 which was not notice this tendence.

All fluorometer parameters analyzed were affected by glyphosate. The photosynthetic electron transport rates (*ETR*) declined with increasing glyphosate rates (Fig. 2a). For both cultivars the late applications were more affected than the early applications. Comparing the two cultivars without glyphosate, ETR of RR2 was lower than RR1, which RR1 presented 104.90–106.37 µmol m^{-2} s^{-1}, while RR2 was between 168.59–168.79 µmol m^{-2} s^{-1}, also around 60% more than RR1 (Table 2), the same percentage of reduction of the *A* (Table 1). Queiroz et al. (2002) also found a linear relation between *gs* and *ETR*, describing a relationship in which decreases in stomatal conductance reflect an apparent reduction in photosynthetic electron transport rate (Table 3).

The minimal (*Fo'*) and maximal (*Fm'*) fluorescence of light-adapted leaves decreased as glyphosate

Table 1 Regression analyses and correlations for the variables analyzed in different RR soybean treated with different rates of glyphosate applied as a single treatment

Growth stage	Estimation of model parameters adjusted			R^2
	a	b	c	
Fig. 1a				
RR1				
V2	17.17	−0.0021		0.94*
V4	16.43	−0.0045	0.0567	0.99*
V6	16.61	0.0009	−0.2888	0.91*
RR2				
V2	26.97	−0.0025	−0.2142	0.92*
V4	26.86	0.0066	−0.7055	0.98*
V6	26.92	0.0051	−0.7871	0.98*
Fig. 1b				
RR1				
V2	0.0781	0.0001	−0.0004	0.95*
V4	0.0779	0.0003	−0.0005	0.89*
V6	0.0790	0.0004	−0.0006	0.85*
RR2				
V2	0.0788	−0.0007	−0.0007	0.95*
V4	0.0783	0.0002	−0.0022	0.99*
V6	0.0785	0.0001	−0.0024	0.99*
Fig. 1c				
RR1				
V2	2.32	0.00001	−0.0095	0.93*
V4	2.33	0.00005	−0.0048	0.96*
V6	2.35	0.00001	−0.0152	0.85*
RR2				
V2	1.68	−0.0003	−0.0009	0.94*
V4	1.68	0.0003	−0.0352	0.96*
V6	1.68	0.0004	−0.0455	0.98*
Fig. 1d				
RR1				
V2	−97.38	0.0271	1.9643	0.99*
V4	−101.10	0.1409	−1.2103	0.94*
V6	−100.92	−0.1096	12.1038	0.97*
RR2				
V2	−233.67	−0.0232	1.7628	0.89*
V4	−240.02	0.0992	1.5069	0.88*
V6	−240.03	0.2653	−2.3876	0.94*

*(n=6, P<0.01)

increased, thus the intrinsic efficiency of photosystem 2 (*Fv′/Fm′*) was also affected by increased glyphosate rates (Fig. 2b–d). This ratio *Fv′/Fm′* was more affected by late applications and in RR2 than RR1 (Fig. 2d). In addition, steady state fluorescence (*Fs*) was also affected by increased glyphosate rates (Fig. 2e) and consequently the quantum efficiencies of photosynthetic electron transport through photosystem 2 (*PhiPS2*), quantum yield based on CO_2 assimilation (*PhiCO2*), the coefficient of non-photochemical quenching of chlorophyll fluorescence (*qP*) and the coefficient of the proportion of open reaction centers (*qP*) were also affected by glyphosate (Fig. 2f–i). However, RR2 was more damaged than

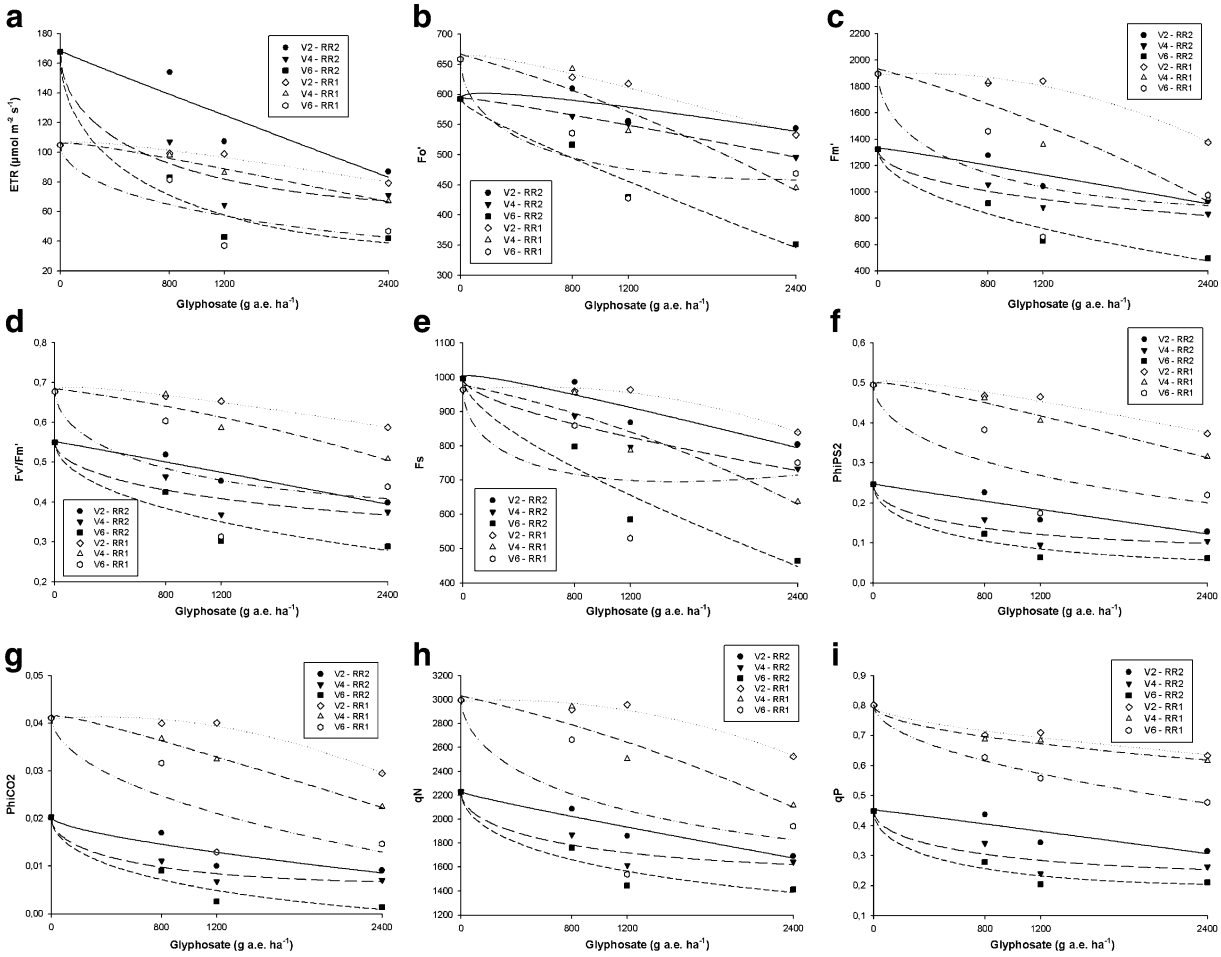

Fig. 2 Photosynthetic electron transport rates (ETR), minimal fluorescence of a light adapted leaf (Fo′), maximal fluorescence of a light adapted leaf (Fm′), steady state fluorescence of a light adapted leaf (Fs), intrinsic efficiency of photosystem 2(Fv′/Fm′), quantum efficiencies of photosynthetic electron transport through photosystem 2 (PhiPS2), quantum yield based on CO_2 assimilation ($PhiCO_2$), non-photochemical quenching of chlorophyll fluorescence (qN) and proportion of open reaction centers (qP) in different RR soybeans under increasing rates of single glyphosate application at different growth stages of soybean, V2, V4 and V6 (n=6, P<0.01)

RR1 and, as noted previously, the late application decreased these parameters more than early applications.

SPAD, leaf area and biomass production

Plants under glyphosate treatment showed chlorotic symptoms, resulting in different values among the treatments as determined by SPAD analysis (Fig. 3a). Trends for proportional decreases in photosynthetic and chlorophyll parameters related to glyphosate rate were also observed for SPAD measurements. In general, RR2 also was more affected than RR1, and early application presented less interference than late applications.

Leaf area and shoot biomass production were extremely affected by glyphosate with RR2 more sensitive than RR1, and late applications causing greater reductions than early applications (Fig. 3b and c). However, in contrast to the other variables analyzed, the decreases among the glyphosate rates presented fewer differences, but both glyphosate rates affected shoot biomass production. Regarding root dry weight, response to glyphosate was reflected by a different graphic behavior, in which the growth stages at application were more influenced than the glyphosate rate. Early application (V2), was more affected than late applications (V4 and V6), illustrated by significant decreases in root dry weight (Fig. 3d).

Table 2 Regression analyses and correlations for the variables analyzed in different RR soybean treated with different rates of glyphosate applied as a single treatment

Growth stage	Estimation of model parameters adjusted			R^2
	a	b	c	
Fig. 2a				
RR1				
V2	104.90	−0.0187	0.4049	0.99*
V4	105.24	−0.0222	0.2936	0.99*
V6	106.37	0.0084	−1.7151	0.89*
RR2				
V2	168.79	−0.0338	−0.0925	0.95*
V4	168.79	0.0302	−3.5564	0.97*
V6	168.59	0.0388	−4.5506	0.98*
Fig. 2b				
RR1				
V2	658.56	−0.0806	1.4221	0.99*
V4	661.21	−0.1407	2.3242	0.97*
V6	662.18	0.0842	−8.2857	0.94*
RR2				
V2	594.02	−0.0480	1.2231	0.84*
V4	592.32	−0.0492	0.4489	0.99*
V6	594.20	−0.0750	−1.3955	0.98*
Fig. 2c				
RR1				
V2	1,892.24	−0.4779	13.1817	0.98*
V4	1,906.97	−0.6289	10.4994	0.97*
V6	1,921.36	0.3216	−36.7188	0.88*
RR2				
V2	1,329.92	−0.1963	1.0210	0.94*
V4	1,328.01	0.0491	−12.8223	0.98*
V6	1,330.33	0.0067	−17.7717	0.98*
Fig. 2d				
RR1				
V2	962.99	−0.1376	4.2889	0.98*
V4	967.81	−0.2358	4.5409	0.96*
V6	975.22	0.1844	−14.3569	0.81*
RR2				
V2	999.86	−0.1160	1.4807	0.93*
V4	998.82	−0.0358	−3.7956	0.98*
V6	1,001.66	−0.0921	−6.7785	0.97*
Fig. 2e				
RR1				
V2	0.6778	−0.00007	0.0017	0.99*
V4	0.6798	−0.0001	0.0022	0.96*
V6	0.6873	0.00007	−0.0092	0.85*
RR2				

Table 2 (continued)

Growth stage	Estimation of model parameters adjusted			R^2
	a	b	c	
V2	0.5509	−0.00006	0.0001	0.97*
V4	0.5521	0.00002	−0.0050	0.94*
V6	0.5526	0.00001	−0.0064	0.96*
Fig. 2f				
RR1				
V2	0.4944	−0.00008	0.0019	0.99*
V4	0.4960	−0.0001	0.0013	0.99*
V6	0.5013	0.00004	−0.0081	0.89*
RR2				
V2	0.2482	−0.00004	−0.0001	0.94*
V4	0.2482	−0.00004	−0.0052	0.96*
V6	0.2479	0.00005	−0.0067	0.98*
Fig. 2g				
RR1				
V2	0.0410	−0.00001	0.0003	0.98*
V4	0.0411	−0.00001	0.0001	0.99*
V6	0.0416	0.00005	−0.0006	0.90*
RR2				
V2	0.0250	−0.00001	−0.0002	0.91*
V4	0.0204	0.00004	−0.0005	0.98*
V6	0.0204	0.00003	−0.0006	0.98*
Fig. 2h				
RR1				
V2	2,991.64	−0.4209	11.4480	0.97*
V4	3,030.50	−0.2587	−0.00005	0.96*
V6	3,031.61	0.2134	−35.0441	0.88*
RR2				
V2	2,234.74	−0.1921	−2.0108	0.97*
V4	2,236.65	0.1660	−20.7165	0.96*
V6	2,237.66	0.1483	−24.5873	0.97*
Fig. 2i				
RR1				
V2	0.8011	−0.00005	−0.0026	0.98*
V4	0.8015	−0.00002	−0.0037	0.99*
V6	0.8032	−0.00004	−0.0065	0.99*
RR2				
V2	0.4514	−0.00006	0.0002	0.91*
V4	0.4518	0.00005	−0.0068	0.94*
V6	0.4507	0.00008	−0.0094	0.98*

*(n=6, P<0.01)

Table 3 Regression analyses and correlations for the variables analyzed in different RR soybean treated with different rates of glyphosate applied as a single treatment

Growth stage	Estimation of model parameters adjusted			R^2
	a	b	c	
Fig. 3a				
RR1				
V2	47.40	0.0036	−0.4679	0.99*
V4	47.49	0.0001	−0.4077	0.99*
V6	47.81	0.0003	−0.7545	0.97*
RR2				
V2	47.82	0.0003	−0.4165	0.97*
V4	48.09	0.0004	−0.6662	0.93*
V6	47,95	−0.0006	−0.7581	0.97*
Fig. 3b				
RR1				
V2	1,123.44	0.2532	−20.4416	0.99*
V4	1,124.03	0.1880	−20.6840	0.99*
V6	1,124.83	0.3442	−31.3526	0.99*
RR2				
V2	921.38	0.2752	−22.2286	0.99*
V4	920.93	0.2824	−22.6457	0.99*
V6	920.43	0.2902	−24.3698	0.99*
Fig. 3c				
RR1				
V2	7.54	0.0014	−0.1403	0.99*
V4	7.51	0.0018	−0.1698	0.99*
V6	7.52	0.0021	−0.2064	0.99*
RR2				
V2	6.16	0.0018	−0.1541	0.99*
V4	6.15	0.0020	−0.1664	0.99*
V6	6.15	0.0020	−0.1832	0.99*
Fig. 3d				
RR1				
V2	2.05	0.0003	−0.0429	0.99*
V4	2.05	0.0003	−0.0432	0.99*
V6	2.05	0.0003	−0.0390	0.97*
RR2				
V2	1.64	0.0004	−0.0387	0.99*
V4	1.65	0.0004	−0.0356	0.99*
V6	1.64	0.0004	−0.0300	0.99*

*(n=6, P<0.01)

Discussion

Visual injuries are likely to happen in RR soybeans after glyphosate application. They are usually considered non-persistent as the yellow flashing tends to disappear within the first two weeks after herbicide application (Reddy and Zablotowicz 2003). However in this study the symptoms persisted until the R1 growth stage, demonstrating that the photosynthetic parameters and chlorophyll fluorescence were affected by single glyphosate application (Figs. 1 and 2). Measurements of leaf gas exchange and chlorophyll fluorescence have been used in combination to provide more detailed information about photosyn-

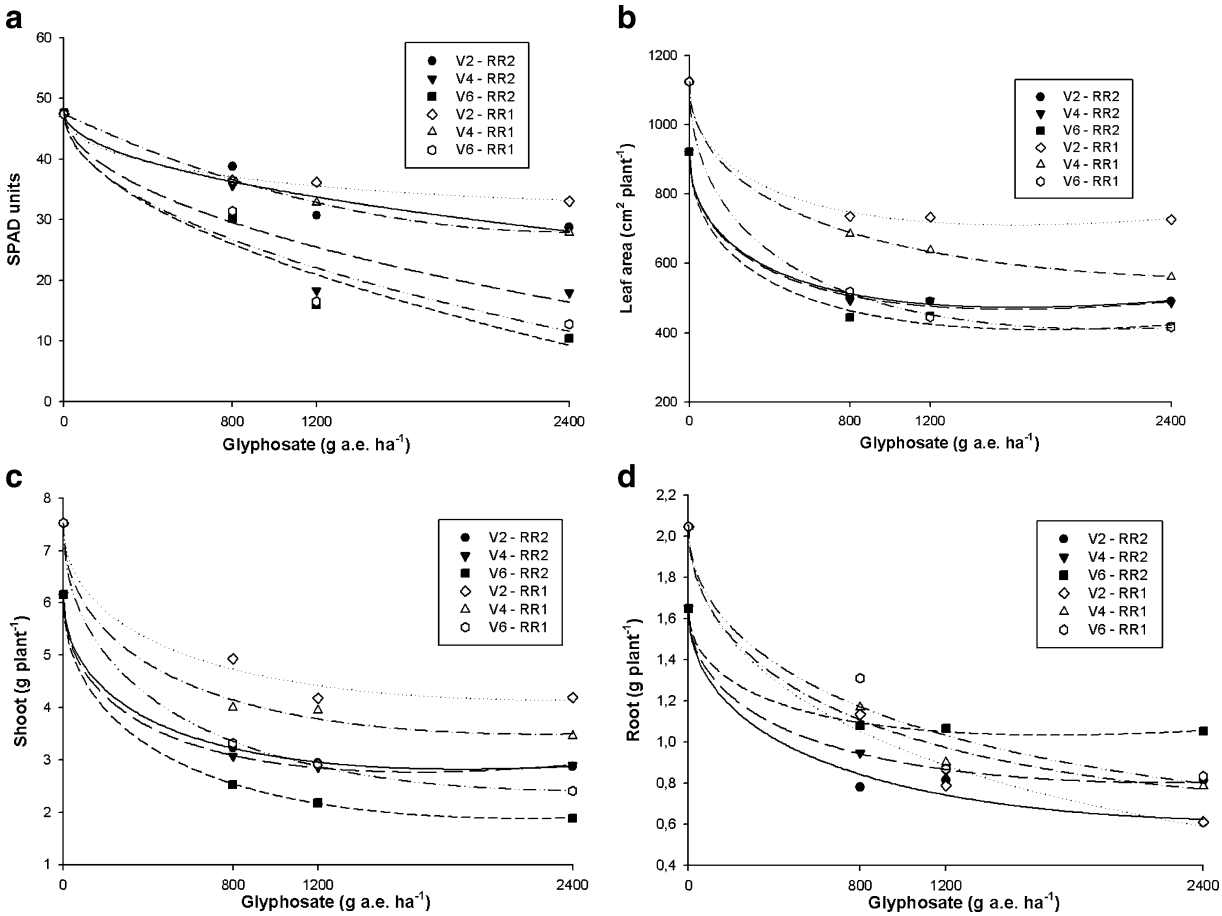

Fig. 3 SPAD units, leaf area, shoot and root dry weight in different RR soybeans under increasing rates of single glyphosate application at different growth stages of soybean, V2, V4 and V6 (n=6, P<0.01)

thetic processes than is possible with either technique used in isolation (Long and Bernacchi 2003).

Zobiole et al. (2010a) reported that a single glyphosate application (1,200 g a.e. ha^{-1}) had less effect on the photosynthetic parameters and biomass production than sequential application (600+600 g a.e. ha^{-1}). Similar results were reported by Zobiole et al. (2010b) during evaluation of different glyphosate rates (600 to 2,400 g a.e. ha^{-1}) applied either as single or sequential application in RR soybeans. They reported that at R1 growth stage, A for a single glyphosate application was lower than for sequential applications and ranged between 11 and 5 μmol CO_2 m^{-2} s^{-1} for the single application (600 to 2,400 g.a. ha^{-1}) and 11–8 μmol CO_2 m^{-2} s^{-1} for sequential application (600 to 2,400 g.a. ha^{-1}), respectively.

Da Matta et al. (2001) determined A and maximum photosynthetic rate (A_{max}) in soybean under saturating CO_2 and found A of 18.2 μmol m^{-2} s^{-1} and A_{max} of 25 μmol m^{-2} s^{-1}. Kumudini et al. (2008) also found A around 26 μmol m^{-2} s^{-1} at R3 growth stage for cv. Asgrow 3905. Results in the present study agree with these findings, since treatments without glyphosate were photosynthetic superior to those with glyphosate (Fig. 1a); however the A for RR2 was higher than RR1, therefore suggesting higher photosynthetic activity.

Zobiole et al. (2010a) reported that A, gs and E were reduced by glyphosate, possibly due to direct damage by glyphosate to chloroplasts (Campbell et al. 1976; Pihakaski and Pihakaski 1980; Nilsson 1985) or immobilization of Mg and Mn required for chlorophyll formation and photosynthesis. Since glyphosate is a glyphosate a strong metal chelator (Jaworski 1972; Kabachnik et al. 1974; Coutinho and Mazo 2005), it may immobilize essential micronutrients required as components, co-factors or regula-

tors of physiological functions, such them Fe (Bellaloui et al. 2009), Mn (Johal and Huber 2009). According to Cakmak (Cakmak et al. 2009), the period for the observed 'yellow flashing' is most likely dependent on the ability of the plants to recover through adequate root uptake of the specific elements that are immobilized by glyphosate in plant tissues.

Similar results reported by Zobiole et al. (2010a), were also noticed in this study, which *A, gs* and *E* were significantly decreased with increased glyphosate rates and late applications (Fig. 1b and c). Other studies have shown a high correlation between leaf conductance and *A* within diverse vegetation types distributed world-wide (Körner 1995). As stomatal closure is an important factor contributing to depressed CO_2 assimilation (Zlatev and Yordanov 2004) and stomata also respond to CO_2 as stomatal conductance decreases as CO_2 concentration increases (Centritto et al. 1999), this is in accordance with the results found for *Ci*, with significant increased as glyphosate increased (Fig. 1d). Zobiole et al. (2010b) also reported significant increased *Ci* after glyphosate application and at R1 growth stage.

Light energy used to drive photosynthesis can be dissipated as heat or re-emitted as light at a longer wavelength, the latter process is known as fluorescence (Maxwell and Johnson 2000). According to Horton et al. (1996) decreases in photosynthesis rate are associated with decreases in *Fv′/Fm′* with consequently increased excitation energy quenching the PS2 antennae and are generally considered indicative of "down regulation" of electron transport. These results were also noticed in this study, which decreased *A* (Fig. 1a), reflecting decreases in Fv′/Fm′ (Fig. 2d).

Effective PS2 quantum yield represents the plant's capacity to convert photon energy into chemical energy once steady-state electron transport has been achieved (Genty et al. 1989). Thus, considering that glyphosate affected *Fo', Fm′ and Fs*, consequently *Fv′/Fm', PhiPS2, PhiCO2, ETR, qP* and *qN* the Fv′/Fm′ were also affected by glyphosate (Fig. 2). However, there was a different behavior observed between time and rate of glyphosate application, in which higher and later applications resulted in greater reduction in fluorescence (Fig. 2). In addition, RR2 was more sensitive to glyphosate applications than RR1.

As stated previously, chlorophyll fluorescence indicates the extent to which PS2 is using the energy absorbed by chlorophyll and the extent to which it is vulnerable to damage by excess light (Maxwell and Johnson 2000). Decreases in *Fv′/Fm′* may indicate photoinhibition of PS2 (Martínez-Ferri et al. 2004). The flow of electrons through PS2 indicates the overall rate of photosynthesis. It is known that PS2 is the most vulnerable component of the photosynthetic apparatus to light-induced damage. Damage to PS2 will often be the first manifestation of stress in a leaf (Maxwell and Johnson 2000). *PhiPS2* measures the proportion of the light absorbed by chlorophyll associated with PS2 that is used in photochemistry. As such, it can give a measure of the rate of linear electron transport and so an indication of overall photosynthesis (Genty et al. 1989; Maxwell and Johnson 2000). Therefore, glyphosate probably affected PS2 in plants applied with herbicide (Fig. 2).

Genty et al. (1989) found a linear relationship between *PhiCO2*, *qP* and *Fv′/Fm′* by open PS2 centers, and has been used as a calibration curve to estimate the rate of non-cyclic electron transport associated with Rubisco activity (Cheng et al. 2001). It is known that thermal dissipation of absorbed light helps protect the photosynthetic apparatus from damage, particularly by controlling the rate of damage to the D1 protein of PS2 (Long et al., 1994). Because non-photochemical quenching of chlorophyll fluorescence (*qN*), indicated as the heat of dissipation, protects the photosynthetic process against deleterious effects of excess light (Jiang et al. 2006), the present study suggests that glyphosate might exert negative effects on PS2, which were more pronounced at higher and later applications. The inhibition of photosynthesis with glyphosate has been reported to occur in the Hill reaction in isolated chloroplast as well as in intact plant tissues (Campbell et al. 1976).

The SPAD values significantly decreased as glyphosate rates increased. The SPAD meter measures absorption at 650 and 940 nm wavelength to estimate chlorophyll level (Richardson et al. 2002), therefore chlorophyll content did not recover after glyphosate treatment, as evidenced by chlorotic symptoms persisting at the R1 growth stage. Indeed, the chlorophyll content decreased proportionally as glyphosate rates increased (Fig. 3a). This decrease could be due to direct damage of the chloroplast (Campbell et al. 1976; Pihakaski and Pihakaski 1980; Nilsson 1985) in the presence of glyphosate. Zobiole et al. (2010a) also noted that RR soybean from different maturity groups exposed to a single or

sequential application of glyphosate frequently had chlorophyll concentrations lower than plants that were not exposed to the herbicide. Glyphosate may also prevent chlorophyll synthesis by inhibiting the formation of the porphyrin precursor d-aminolevulinic acid (ALA) (Cole 1985; Zaidi et al. 2005).

Thompson et al. (1996) observed strong correlations of chlorophyll content with SPAD readings and leaf area in soybean and reported that SPAD meter readings could be used to distinguish high and low leaf area genotypes in experimental lines selected to differ in this trait. Contrary to their findings, regression analyses of similar data by Fritschi and Ray (2007) indicated that neither SPAD meter readings nor chlorophyll content were good predictors for leaf area among 833 lines examined. In the present study, SPAD correlated not only with leaf area but also with shoot biomass production (Fig. 3a–c), which significantly decreased with higher and later glyphosate applications.

The extent of injury in glyphosate-treated RR soybean is correlated with levels of AMPA formed within the plant (Zablotowicz and Reddy 2007). This primary phytotoxic metabolite is also toxic to RR soybean as evidenced by the reduction in chlorophyll and shoot fresh weight (Reddy et al. 2004). Other authors also reported reduced shoot and root dry weight in RR soybean, using glyphosate at 1,680 g a.e. ha^{-1} (Reddy et al. 2000) and 6,300 g a.e. ha^{-1} (King et al. 2001). Similarly, Bott et al. (2008) also noted that the recommended label rate of glyphosate applied to a RR soybean cultivar significantly inhibited root biomass, and root elongation.

Zobiole et al. (2010b) previously reported that as glyphosate rates increase, both root and shoot biomass are affected, probably by additive detrimental effects on photosynthesis. In the current study, the damage caused by glyphosate was more intense on shoot than root (Fig. 3c, d). Comparing shoot and root dry weight on different timing of glyphosate applications (V2, V4 and V6), shoot dry weight decreased more with late than with early applications. In contrast, roots exhibited an opposite trend, suggesting that with early applications (V2), plants probably have more time to recover from glyphosate effects. According to Shibles and Weber (1965) the total biomass production by soybean fundamentally depends on energy supplied by photosynthesis, which is based on solar energy to synthesize carbon compounds. Thus with adequate leaf area, carbon production is optimized with this input of energy (Taiz and Zeiger 1998). Therefore, the reductions in photosynthesis (Figs. 1 and 2), leaf area, and consequently shoot biomass observed at the R1 stage (Fig. 2) long after herbicide application, suggests that either glyphosate or its metabolites may exert long term effects on physiology of the plant. In either case, glyphosate molecules can remain in plants until complete physiological maturity (Duke et al. 2003; Arregui et al. 2004).

Even though RR2 demonstrated higher photosynthetic and transpiration rates (Fig. 1a), it produced less leaf area, shoot and root biomass than RR1 (Fig. 3b–d). Thus, RR1 possesses higher physiological activity (photosynthesis and respiration) and functional chlorophyll than RR2. However both cultivars were affected by glyphosate or its metabolites. Recent findings showing that glyphosate decreased water use efficiency by RR soybeans (Zobiole et al. 2010b) further suggests that RR soybean plants are more sensitive to drought and less efficient in converting water into biomass under glyphosate application compared to non-treated RR plants.

Conclusion

Glyphosate caused undesirable effects on photosynthesis and biomass production in both first and second generation RR soybean. Results suggest that management strategies are needed to minimize these effects in the field, which could include using lower glyphosate rates as possible and early applications, with consideration of weed populations and the critical period of weed control, to assure optimum crop growth.

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