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Metabolic changes, agronomic performance, and quality of seeds in soybean with the *pat* gene after application of glufosinate

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Research Article

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Abstract

The transgenic Liberty Link® (LL) soybean is tolerant to glufosinate, conferred by the enzyme phosphinothricin acetyltransferase (PAT), which is encoded by the *pat* gene from *Streptomyces viridochromogenes*. Because symptoms of injury can be observed in soybean [*Glycine max* (L.) Merr.] plants in some situations, this study evaluated the effects of rates of glufosinate on agronomic performance; quality of LL soybean seeds; and the ammonia, glufosinate, and *N*-acetyl-L-glufosinate concentration (NAG) in soybeans with and without the *pat* gene after application of increasing glufosinate rates. Field and greenhouse experiments were conducted; the first evaluated the selectivity of glufosinate in LL soybeans, and the second evaluated the metabolic changes in soybeans with (LL) and without (RR2) the *pat* gene, after application of glufosinate. For fieldwork, application of glufosinate at rates up to four times the maximum recommended caused initial injury symptoms (up to 38.5%) in LL soybean plants. However, no negative effect was found on seed quality and agronomic performance of LL plants, including yield. This shows the selectivity of glufosinate promoted by *pat* gene insertion for application in POST (V4), in LL soybean. For the greenhouse experiment, it was concluded that the LL soybean plants presented high glufosinate metabolism, lower ammonia concentration, and no reduction in dry matter, in comparison with RR2 soybean, after application of high rates of glufosinate.

Introduction

Glufosinate is a broad-spectrum, nonselective herbicide (selective only to crops that express the *pat* or *bar* gene). It has limited contact action and translocation; the first symptoms are the yellowing of leaves and other green tissues, followed by wilting and death of the plant. It acts by inhibiting the enzyme glutamine synthetase (GS), fundamental for nitrogen assimilation. After uptake, plants show rapid accumulation of ammonia accompanied by destruction of chloroplasts, reduced levels of photosynthesis, and decreased production of amino acids, thereby resulting in inhibition of photosynthesis and cell death (Brito et al. 2018; Brunharo et al. 2014; Sauer et al. 1987).

Transgenic soybean tolerant to glufosinate (events A2704-12 and A5547-127, Liberty Link® [LL]) was developed using recombinant DNA technology (ISAAA 2020). Tolerance is conferred by the enzyme phosphinothricin acetyltransferase (PAT), encoded by the *pat* gene from *Streptomyces viridochromogenes* (Herouet et al. 2005). This enzyme metabolizes glufosinate in transgenic plants to *N*-acetyl-L-glufosinate (NAG) (Müllner et al. 1993). This compound does not inhibit GS, which explains the selectivity of glufosinate for plants with the *pat* gene. Thus, NAG is a compound related to the degradation of the herbicide glufosinate. The greater the expression of the *pat* gene in plants, the higher the production of NAG, and the greater the tolerance to glufosinate (Krenchinski et al. 2018).

In Brazil, according to Technical Report No. 2273.2010, the insertion of the *pat* gene allows applications of glufosinate in LL soybean [*Glycine max* (L.) Merr.] at rates up to three times higher than label-recommended rate for weed control (CTNBio 2010). However, even with the tolerance to high rates of glufosinate for LL soybean, some reports of injury have been observed. Aulakh and Jhala (2015) observed injury symptoms of up to 15% for the application of glufosinate (740 + 593 g ai ha^{-1}) in LL soybean. However, Landry et al. (2016) observed no injury symptoms or reductions in yield of LL soybean in three field experiments for applications of glufosinate (700 + 600 g ai ha^{-1}, sequential application at V2/V4 follow by V6/V8).

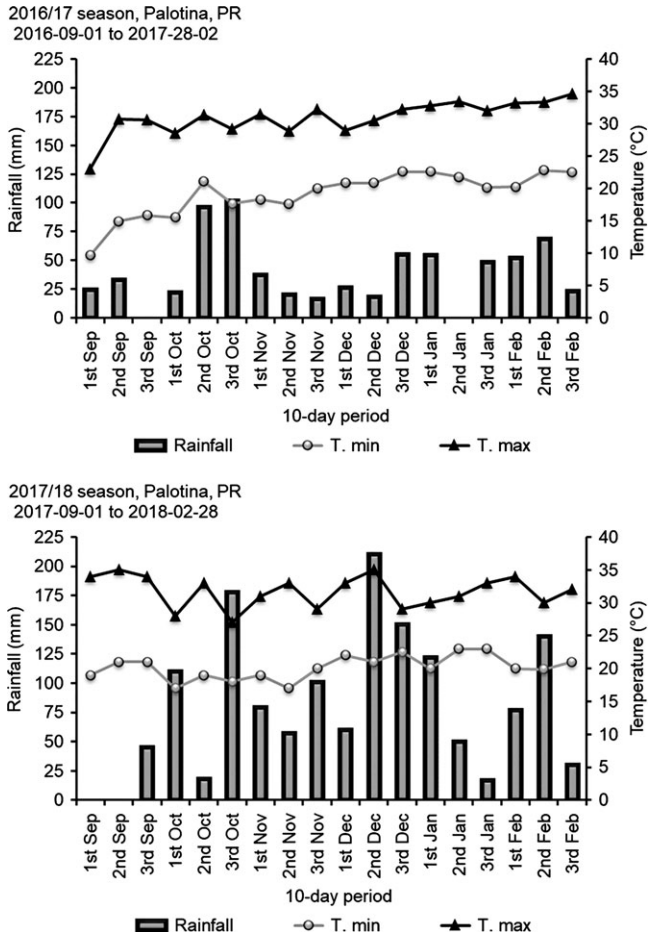

Figure 1. Representation of rainfall and average temperature over the course of the experiments for field experiments (2016 to 2017 and 2017 to 2018 seasons). Labels on the x-axes refer to the first, second, and third 10-day periods in each month.

The maximum recommended label rate in Brazil for the application of glufosinate in LL soybean is 700 g ai ha^{-1} (Rodrigues and Almeida 2018). As injury symptoms can be observed in some conditions in LL soybean plants, high rates can affect the selectivity of the herbicide (even with the introduction of the resistance gene) and the physiological responses of and quality of seeds produced by LL soybeans. Therefore, this study aimed to evaluate the effects of high rates of glufosinate on seed quality, physiological responses, and agronomic performance of LL soybean, as well as the effects of increasing rates of glufosinate on concentrations of ammonia, glufosinate, and NAG in soybean with and without the *pat* gene after application of glufosinate.

Material and Methods

Field Experiment

Site and Experimental Design

The experiments were conducted in Palotina, state of Paraná (PR), Brazil (24.34°S, 53.85°W, 346 m altitude), in the 2016 to 2017 and 2017 to 2018 seasons, in clayey soil (sand: 16.3%; silt: 15%; clay: 68.7%), with CEC of 13 $cmol_c$ dm^{-3}, and pH (CaCl) of 4.7. Data related to rainfall and temperatures during the study period are shown in Figure 1. The experiments were grown in a no-till system, with previous cultivation of wheat (*Triticum aestivum* L.). Sowing was carried out in October in both experiments, with a row spacing of 0.45 m. Plots had six rows of 5 m in length; only the central 2 m of the four center rows were used for the evaluations, with the two outside rows and the first and last 1.5 m of the central rows discarded to avoid plot border effects. The experimental design was a randomized complete block design with four replications.

In the 2016 to 2017 season, the treatments consisted of five rates of glufosinate (200 g ai L^{-1}; Liberty®, Bayer S.A., São Paulo, SP, Brazil): 0, 350, 700, 1,050, and 1,400 g ai ha^{-1}, and the cultivar used was 'BS 0043 LL' (Bayer S.A.). In the 2017 to 2018 season, the treatments consisted of rates of 0, 700, 1,400, 2,100, and 2,800 g ha^{-1}, and the cultivar used was 'BS 1590 LL' (Bayer S.A.). The cultivar was changed according to commercial availability; however, the two cultivars are from the same introgressed event. The treatments were applied when the soybean was at the V4 phenological stage, using a CO_2 pressurized backpack sprayer at a constant pressure of 150 kPa. The sprayer was equipped with a six fan-nozzle bar AD-IA 110.015 (MagnoJet®, Ibaiti, PR, Brazil), spaced at 0.5 m, with an application speed of 1 m s^{-1} at a spray volume of 150 L ha^{-1}. Aureo® (Bayer S.A.) adjuvant (0.25% v/v) was always added to the application mixtures.

Assessments and Statistical Analysis

Crop injury was assessed at 7, 14, 21, 28, and 35 d after application (DAA) by means of visual assessments in each plot (0% for no injuries and 100% for plant death) considering visible symptoms (Velini et al. 1995). To determine plant height, 10 plants chosen at random from the plot area used were measured with a millimeter ruler at 28 DAA. The number of pods per plant was evaluated at full maturity (R8), counting all pods present in 10 plants per plot.

For the evaluation of the 100-seed weight, eight subsamples were weighed for each field repetition. For yield, the useful area was harvested, and the total amount of seeds was weighed. The seed weight of each plot was extrapolated to kilograms per hectare (kg ha^{-1}), with seed moisture corrected to 13%.

To analyze the quality of the seeds produced, assessments were carried out in accordance with the seed analysis rules for soybeans (MAPA 2009). Analyses were performed for germination, vigor, seedling length (shoot), and electrical conductivity.

The germination test was conducted with four subsamples of 50 seeds per field plot of each treatment. The germination of each field plot corresponded to the average of these four subsamples. Seeds were placed between three sheets of filter paper that had been moistened with demineralized water until they measured three times the weight of the dry paper. Following guidelines from the Brazilian Ministry of Agriculute (MAPA 2009), the germination filter papers were rolled up and stored in plastic bags to preserve the moisture required for germination and placed in a regulated growth chamber maintained at a constant temperature of 25 C. The first count (indicative of percentage vigor) was carried out at 5 d, with all germinated seeds, regardless of whether they were normal or abnormal, removed from the roll; the remaining seeds were returned to the growth chamber until the second count, which occurred 8 d after installation (germination was determined by adding the two counts together). The germination test was used to determine the average seedling length; during the first count, 10 seedlings were randomly selected from each roll of each field plot and shoots and roots were measured.

For the determination of electrical conductivity, two subsamples of 50 seeds each for each field plot were weighed and placed in plastic cups, and 75 ml of deionized water was added. The cups were kept at 25 C for 24 h in a growth chamber with a 12-h

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photoperiod, then the electrical conductivity was measured with a conductivity meter. The value obtained was divided by the weight of the seeds to obtain the value in μS cm^{-1} g^{-1} (Loeffler et al. 1988). The current metabolic condition of seeds is indirectly assessed through the electrical conductivity test; the larger the amount of exudates (electrolytes) released, the higher the electrical conductivity of the solution (Marcos Filho 2015).

Regarding statistical analysis, data were analyzed according to Pimentel-Gomes and Garcia (2002). Regression analysis was applied ($P \leq 0.05$). The analyses were run in the Sisvar v. 5.6 software (UFLA, Lavras, MG, Brazil), according to Ferreira (2011).

Greenhouse Experiments

Experimental Design

Two main experiments were installed in a greenhouse in Botucatu, state of São Paulo (SP), Brazil, (22.84°S, 48.42°W), with a temperature of 27 C (±2 C). Each experiment was conducted in duplicate in a completely randomized design with 10 replications.

For the first experiment, the treatments were arranged in a 2 by 5 factorial arrangement (event by rate). For the event, we used: Intacta™ Roundup Ready™ 2 Pro (RR2; without the *pat* gene) and Liberty Link® (LL; with the *pat* gene). Rates of 0, 350, 700, 1,400, and 2,800 g ai ha^{-1} glufosinate (200 g ai L^{-1}; Finale®, Bayer S.A.) were used, and the cultivars 'M 6410 IPRO' (Monsanto) without the *pat* gene, and BS 0043 LL (Bayer S.A.), with the *pat* gene.

In the second experiment, a 2 by 7 factorial arrangement (cultivar by rate) was used. Two cultivars with the *pat* gene, BS 0043 LL and BS 1590 LL (Bayer S.A.) were used, the same as those used in field experiments. Rates of 0, 87.5, 175, 350, 700, 1,400, and 2,800 g ha^{-1} glufosinate were used.

Soybean plants were grown in pots (capacity of 0.0017 m^3) filled with substrate composed of sphagnum peat, vermiculite, and roasted rice husk, with pH 5.7 (± 0.5). Eight seeds were sown per pot, and at 7 d after emergence, pots were thinned to four plants per pot. The pots were kept under full sunlight in the greenhouse, with irrigation as needed.

Glufosinate was applied when the plants reached the V4 stage, via a stationary sprayer, in a closed room, with a spray bar with four nozzles XR 110015 (TeeJet®, São Paulo, SP, Brazil), spaced at 0.5 m and positioned at 0.5 m above plants, with spray volume corresponding to 150 L ha^{-1}, at a constant pressure of 150 kPa, pressurized by compressed air.

Evaluations

Leaves were collected from two plants per replication at 2 DAA, washed with distilled water, ground in liquid nitrogen with a mortar and pestle, and stored in an ultra-freezer (−80 C). To determine glufosinate and NAG, the samples were packed in plastic tubes and lyophilized for 60 h. A 0.1-g aliquot of each lyophilized sample was weighed and placed in a 15-ml tube to which 10 ml of methanol: water extraction solution (80:20 v/v) was added. The tubes were placed in an ultrasonic bath for 0.5 h and centrifuged at 4,000 × *g* for 5 min. The samples were then filtered through a 0.45-μm membrane and transferred to 2-ml amber flasks for liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis (Carbonari et al. 2016; Krenchinski et al. 2018).

For quantification of the glufosinate and NAG levels, an LC/MS/MS system consisting of a Prominence UFLC (Shimadzu do Brasil, Barueri, SP, Brazil) high-performance liquid chromatograph equipped with two LC20AD pumps, a SIL-20AC autoinjector, a DGU-20A5 degasser, a CBM20A controller system (allows fully automated operation), and a CTO-20AC column oven (for column temperature control) was used. A 4500 hybrid triple quadrupole mass spectrometer (Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was coupled to the high-performance liquid chromatograph. Chromatographic analysis was conducted with a C18 column (Phenomemex Gemini 5μ C18 110A, Phenomenex Inc., São Paulo, SP, Brazil), with 5 mM ammonium acetate in water (phase A) and 5 mM ammonium acetate in methanol (phase B). The gradient used was as follows: 0 to 1 min = 60% phase B and 40% phase A, 1 to 3 min = 95% phase B and 5% phase A, 5 min = 95% phase B and 5% phase A, and 5 to 7 min = 60% phase B and 40% phase A, with a flow rate of 0.800 ml min^{-1} and a total reading time of 10 min. The retention time in the chromatographic column of each compound was as follows: glufosinate, 3.15 min; and NAG, 3.12 min. The mass spectrometer was operated in the positive electrospray ionization mode. The glufosinate and NAG were analyzed using the multiple reaction monitoring mode, optimized to 182.02 and 221.92, respectively, and confirmation was achieved through ion transition (135.8, 118.9, and 164.8 for glufosinate; 136.0, 133.9, and 134.0 for NAG). A standard curve of serial dilutions of glufosinate and NAG analytical standard with a certified purity level of 99.9% (Sigma-Aldrich, Gillingham, Dorset, UK) was used for quantification. The levels of glufosinate and NAG were expressed in relation to the dry mass of the sample (μg g^{-1} dry mass).

Ammonia was extracted immediately after collection from fresh leaf tissue (5 g) from two other plants per replication at 2 DAA (Carbonari et al. 2016; Krenchinski et al. 2018). The samples were placed in beakers containing 300 ml of water acidified with hydrochloric acid (pH 3.5) and placed in an ultrasonic bath for 30 min. The ammonia concentration of the solution was determined by spectrophotometry according to published methods (Dayan et al. 2015; Wendler et al. 1990) using a spectrophotometer (Cintra 40, GBC Scientific Equipment, Hampshire, IL, USA).

To evaluate initial growth, plant shoots were collected at 15 DAA and dried in a forced ventilation oven at 60 C to constant weight. The dry mass of the plants was expressed as a percentage relative to the dry mass observed for nontreated plants.

Statistical Analysis

Data were tested by ANOVA ($P \leq 0.05$) according to Pimentel-Gomes and Garcia (2002); for events or cultivars, the means were compared using the *t*-test ($P \leq 0.05$). A combined analysis of the data was also performed in duplicate for both greenhouse experiments.

For rates, correlation analysis was performed using glufosinate, NAG, and ammonia concentration. As there was a significant correlation, the adapted nonlinear regression model of Mitscherlich (1909) was fit:

$$y = a\left[1 - 10^{(-c(x+b))}\right] \quad [1]$$

where *a* is the maximum asymptote of the model, the lateral displacement of the curve corresponds to parameter *b*, and the concavity of the curve to parameter *c*.

For dry matter only, the correlation with glufosinate rates was not performed by fitting the nonlinear regression model, so the means were compared using the *t*-test ($P \leq 0.05$). The analyses were run with the aid of SAS v. 9.1.3 (Statistical Analysis

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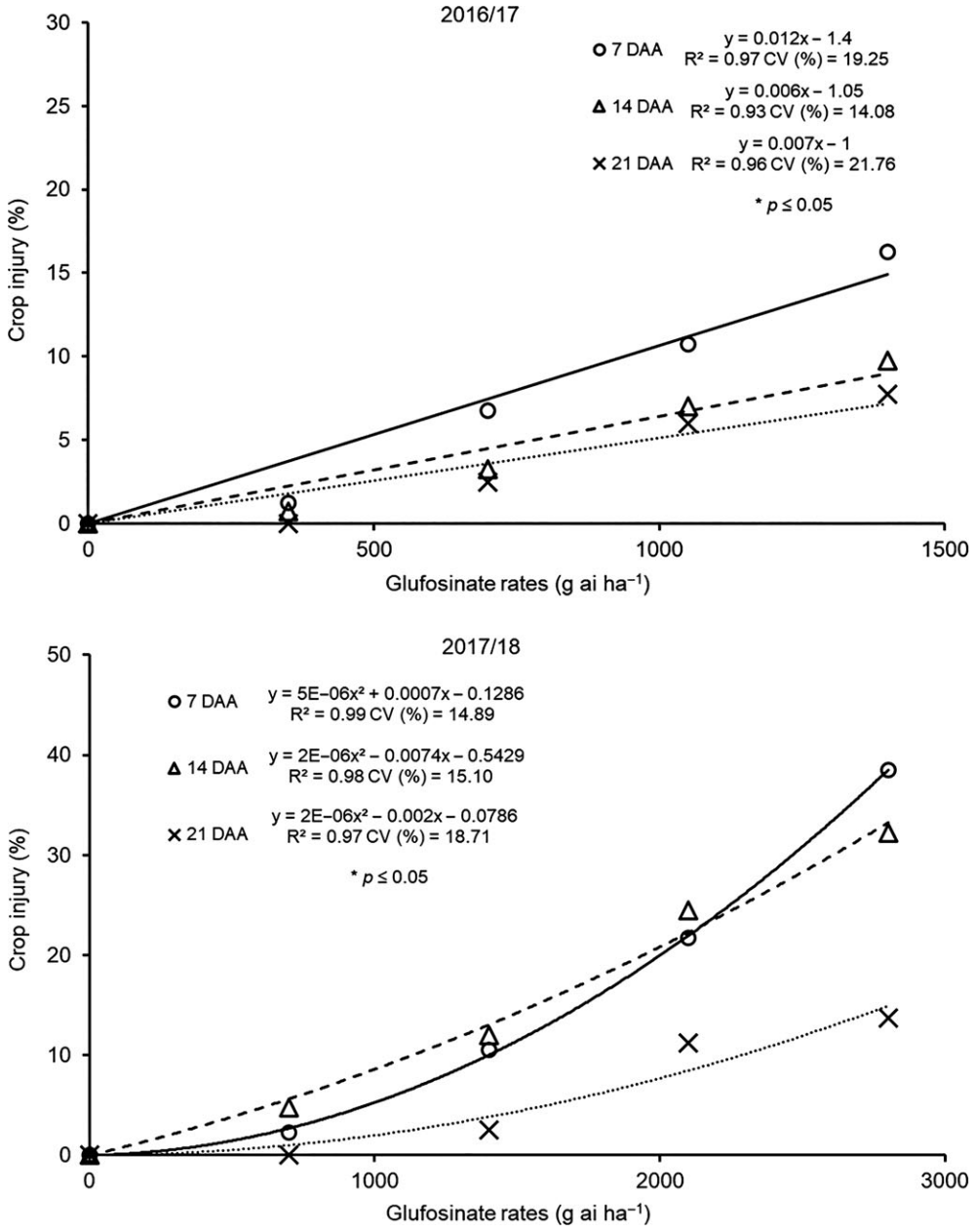

Figure 2. Crop injury of Liberty Link® soybean (with *pat* gene) plants after glufosinate application in the field experiments.

System, SAS Institute, Cary, NC, USA), and the graphics were constructed in SigmaPlot v. 12.0 (Systat Software, San Jose, CA, USA).

Results and Discussion

Field Experiment

There was a significant effect of glufosinate rate on symptoms of injury to soybean plants (up to 21 DAA). With increasing linear fit as rates increased incrementally, an increase in symptoms was observed for the first harvest (Figure 2). Higher absolute values were found at 7 DAA (16.25%), for a higher rate of glufosinate applied (1,400 g ha^{-1}). After 28 DAA, no symptoms were verified.

For the second harvest, there was also a significant effect of rate for the symptoms of injury to soybean plants. Polynomial fit was possible, with scores of up to 38.5% at 7 DAA for the highest rate (2,800 g ha^{-1}); however at 28 and 35 DAA, the symptoms were low and did not allow fit (data not plotted). It is important to note that higher rates were applied in the second growing season precisely because of the mild symptoms observed in the first harvest.

For the agronomic variables of soybean plants, there was no significant effect ($P > 0.05$) of glufosinate rate on any variable in either experiment (Table 1), and for seed quality variables, there was no effect of glufosinate rate ($P > 0.05$) on any variable in either experiment (Table 2). It is must be noted that in view of the results for the first harvest, in which injury symptoms were observed but glufosinate rate had no effect on the agronomic and seed quality variables, higher rates were used in the second harvest. Even so, no effects were detected up to the highest application rate of 2,800 g ha^{-1} glufosinate, only more substantial visual symptoms of injury were seen at the highest rates.

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Table 1. Results of regression analysis (*F*-value) for agronomic performance for glufosinate application rates[a] on Liberty Link® soybean(with *pat* gene) soybean plants in the two field experiment seasons.

	Plant height	Pods plant^{-1}	100-seed weight	Yield
Season 2016 to 2017	cm		g	kg ha^{-1}
Mean	64.1	58.8	16.4	3,598
F	0.5^{ns}	0.6^{ns}	1.0^{ns}	0.1^{ns}
CV (%)	6.4	15.2	3.5	9.8
Season 2017 to 2018				
Mean	68.8	30.7	15.4	4,112
F	1.8^{ns}	0.9^{ns}	2.5^{ns}	0.8^{ns}
CV (%)	4.6	7.0	3.5	5.5

[a] Glufosinate rates: 2016/2017: 0, 350, 700, 1,050, and 1,400 g ai ha^{-1}; 2017/2018: 0, 700, 1,400, 2,100, and 2,800 g ha^{-1}.
[ns] Nonsignificant ($P > 0.05$).

Table 2. Results of regression analysis (*F*-value) for seed quality for glufosinate application rates[a] on Liberty Link® soybean(with *pat* gene) soybean plants in the two field experiment seasons.

	Germination	Vigor	Electrical conductivity	Seedling length
Season 2016 to 2017	%	%	µS cm^{-1}g^{-1}	cm
Mean	91.7	80.5	104.5	14.0
F	0.2^{ns}	0.1^{ns}	0.2^{ns}	0.4^{ns}
CV (%)	5.5	9.0	10.3	7.2
Season 2017 to 2018				
Mean	93.2	88.0	100.4	13.83
F	4.3^{ns}	3.4^{ns}	0.3^{ns}	2.8^{ns}
CV (%)	2.2	2.3	5.8	3.5

[a] Glufosinate rates: 2016/2017: 0, 350, 700, 1,050, and 1,400 g ha^{-1}; 2017/2018: 0, 700, 1,400, 2,100, and 2,800 g ha^{-1}.
[ns] Nonsignificant ($P > 0.05$).

Table 3. Parameter estimation of the adjusted Mitscherlich (1909) model[a] for glufosinate application rates[b] on soybean plants with and without the *pat* gene in the greenhouse experiments (average of the two experiments).

	Glufosinate concentration				
	a	*b*	*c*	R^2	*F*
RR2[c]	950.10	−1.3795	0.000423	0.9978	445.3
LL[d]	1069.10	10.8951	0.000176	0.9989	947.8
	$F_{Events} = 66.8^*$	$F_{Rates} = 251.2^*$	$F_{(Events \times Rates)} = 5.6^*$	CV (%) = 14.3	
			N-acetyl-L-glufosinate		
	a	*b*	*c*	R^2	*F*
RR2	–	–	–	–	–
LL	23396.9	0	0.00000167	0.9997	6223.7
		$F = 53.1^*$		CV (%) = 41.3	
			Ammonia concentration		
	a	*b*	*c*	R^2	*F*
RR2	6695.10	40.7531	0.000167	0.9969	323.4
LL	358.00	23.0826	0.000230	0.9831	58.1
	$F_{Events} = 309.0^*$	$F_{Rates} = 57.9^*$	$F_{(Events \times Rates)} = 45.1^*$	CV (%) = 31.6	

[a] $y = a\left[1 - 10^{(-c(x+b))}\right]$, where *a* is the maximum asymptote of the model, the lateral displacement of the *b*, and the concavity of the curve to parameter *c*.
[b] Glufosinate rates: 0, 350, 700, 1,400, and 2,800 g ha^{-1}.
[c] RR2, Intacta™ Roundup Ready™ 2 Pro soybean (without *pat* gene).
[d] LL, Liberty Link® soybean (with *pat* gene).
*$P \leq 0.05$.

Greenhouse Experiment

Glufosinate Rates in Soybeans with and without the pat Gene

According to the ANOVA for glufosinate concentration, differences and the interaction between the factors evaluated were verified (Table 3). For all rates, higher levels of glufosinate were observed in RR2 soybean plants compared with LL plants, and the nonlinear regression model (Equation 1) was fit for each event as a function of the applied rates (Figure 3) with a high degree of correlation.

Soybean plants without the *pat* gene do not have the ability to metabolize glufosinate to NAG; this characteristic is present only in plants with the *bar* and/or *pat* genes. Thus, in RR2 soybean plants, NAG concentration was not identified, and only the results for LL plants are presented. The concentration of NAG quantified in LL soybean plants was higher with the application of the highest rate, with an increase directly proportional to the rate (Table 3). The obtained levels allowed Equation 1 (Figure 3) to be fit with a high degree of correlation, using the parameter estimates listed in Table 3.

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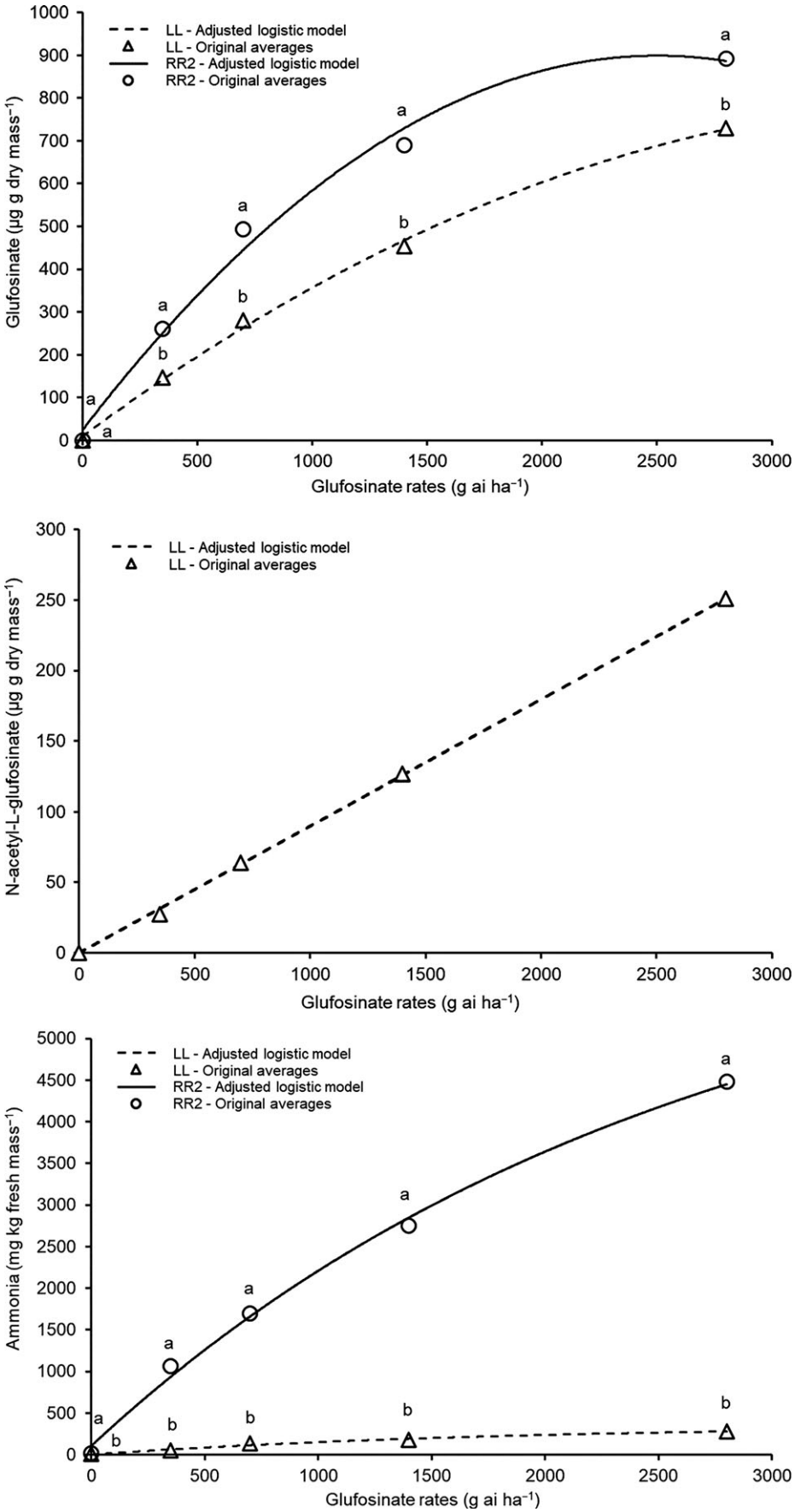

Figure 3. Mitscherlich (1909) model adjusted for glufosinate effects in Intacta™ Roundup Ready™ 2 Pro soybean (without *pat* gene) (RR2) and Liberty Link® soybean (with *pat* gene) (LL) plants in the greenhouse experiments. For events, means followed by the same letter, do not differ by the *t*-test ($P \leq 0.05$).

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Table 4. Dry matter for glufosinate application rates at soybean plants with and without the *pat* gene in the greenhouse experiments (average of two experiments).[a]

	RR2[b]	LL[c]
Rate	Dry matter	
g ai ha^{-1}	% of 0 g ha^{-1}	
0	100 Aa	100 Aa
350	42.0 Bb	105.8 Aa
700	41.2 Bb	83.0 Aa
1,400	38.1 Bb	82.9 Aa
2,800	36.7 Bb	82.9 Aa
F_{Events}	62.806*	
F_{Rates}	10.364*	
$F_{(Events \times Rates)}$	5.441*	
CV (%)	19.68	

[a] Means followed by the same letter, uppercase on the column (rates) and lowercase on the row (events), do not differ by the t-test, at the level of 5% probability.
[b] RR2, Intacta™ Roundup Ready™ 2 Pro soybean (without *pat* gene).
[c] LL, Liberty Link® soybean (with *pat* gene).
*P ≤ 0.05.

For the accumulation of ammonia, a difference was detected between the factors and the interaction between them. Ammonia accumulation increased with increasing rate, with higher concentration in RR2 soybean plants. It was possible to adjust Equation 1 with a high level of correlation (Table 3). For both events, the highest rate provided the largest accumulation of ammonia; however, there was no linearity in the correlation between applied rate and ammonia concentration in the tissues, as demonstrated by the adjusted model (Figure 3).

For dry matter, a difference was detected between the factors analyzed and the interaction between them. The application of glufosinate, even at the lowest rate (350 g ai ha^{-1}), was sufficient for the maximum reduction in the dry matter of RR soybean plants. In contrast, for the LL soybean, even the highest rate (2,800 g ai ha^{-1}) caused insufficient injury to cause reduction of dry matter (Table 4).

Glufosinate Rates in Two LL Soybean Cultivars (with the pat Gene)

For glufosinate concentration, no significant effect was found for cultivars, only for rates. For the highest applied rate, the highest levels of the herbicide were quantified. The Equation 1 fit for each cultivar showed a similar behavior between the cultivars and for the increase in the herbicide concentration remaining in the plant tissues at 2 DAA (Table 5; Figure 4).

The NAG values did not differ between cultivars. The fit of the nonlinear regression model (Equation 1) demonstrates the increase in relation to the applied rate (Table 5; Figure 4), with the highest rates responsible for the highest NAG concentrations.

The ANOVA showed that there was a significant effect for cultivar, glufosinate rates, and an interaction of cultivar by rate in regard to ammonia accumulation. Ammonia accumulation was greatest at the highest rates (Table 5; Figure 4). The higher levels of ammonia for the BS 1590 cultivar suggest greater sensitivity at the two highest rates tested. Glufosinate-sensitive plants, when sprayed with this herbicide, show an increase in ammonia levels, as does a maize (*Zea mays* L.) glufosinate-sensitive hybrid, due to the permanent binding of glufosinate to GS (Dayan et al. 2015; Sellers et al. 2004).

The rates of glufosinate, despite causing initial injury, did not show differences in relation to zero rate, with no reduction in dry matter and no differences between the LL cultivars (Table 6).

The results of the field experiment demonstrated the tolerance of LL soybean plants to glufosinate, even after the application of rates up to 2,800 g ha^{-1}. The maximum recommended label rate for application of glufosinate in LL soybean in Brazil is 700 g ha^{-1} (Rodrigues and Almeida 2018), that is, four times less than the maximum rate applied in one of the experiments. Despite injury symptoms, glufosinate rates did not affect the agronomic performance of soybean plants, and there were no reductions in productivity.

Aulakh and Jhala (2015) also observed symptoms of injury up to 15% for the application of glufosinate (740 g ha^{-1}) at the V2 stage, with sequential application at the V6 with a rate of 593 g ha^{-1}. Beyers et al. (2002) observed symptoms of injury up to 21% for the application of glufosinate in mixtures, but without reduction in LL soybean yield. On the other hand, other studies do not even report injury symptoms for the application of glufosinate (Barnes et al. 2017; Chahal and Jhala 2015; Jhala et al. 2017; Landry et al. 2016).

Reddy et al. (2011) observed changes in the composition of LL soybean seeds under two applications of glufosinate (450 and 450 g ha^{-1} at the V3 and V6 stages, respectively). Glufosinate increased the protein concentration and decreased the oil concentration compared with the treatment without application. However, the authors did not evaluate the effects of glufosinate on the germination or vigor of soybean seeds.

For the application of this herbicide as a preharvest desiccant in soybean (not tolerant to glufosinate), negative effects on the quality of the seeds produced may or may not be observed, with variations due to the stage of application and rate (Pereira et al. 2015; Zuffo et al. 2019). Studies evaluating the quality of LL soybean seeds following application of glufosinate are scarce. However, in LL rice (*Oryza sativa* L. with the *bar* gene), there was no reduction in germination and vigor of seeds produced following application of glufosinate (840 g ai ha^{-1}) at the vegetative stage of plants (Webster et al. 2003), similar to observed in the present study.

Regarding metabolic changes, the application of the highest rates of glufosinate were responsible for the recovery and quantification of the highest levels of the herbicide, whether in LL or RR2 soybean. Nevertheless, lower levels were found for the LL soybean, an effect that is related to the transgenics of the plants.

Genetically modified crops tolerant to glufosinate were developed from the insertion of an exogenous gene capable of detoxifying the herbicide to a residue nontoxic to the plants. This gene can be isolated from two species of bacteria, *Streptomyces hygroscopicus* (*bar*) and *S. viridochromogenes* (*pat*), that express the PAT enzyme, which rapidly metabolizes glufosinate into a stable compound, NAG, that is nontoxic to plants (Mullner et al. 1993). Thus, NAG is a compound causally related to the degradation of glufosinate, and its quantification demonstrates the expression of the PAT enzyme; a greater production of NAG can be related to greater plant tolerance to glufosinate.

Plants without the *pat* or *bar* gene do not have the ability to metabolize glufosinate into NAG (Carbonari et al. 2016; Krenchinski et al. 2018). A high level of expression of the *bar* gene in glufosinate-resistant LL cotton (*Gossypium hirsutum* L.; 'IMACD 6001LL') was compatible with the very high level of PAT activity in an in vitro test, and the total amount of glufosinate was acetylated in the first 60 min of incubation (Carbonari et al. 2016). The same authors also observed that in the cotton cultivar ('FM 975WS') with the *pat* gene (inserted as a selection marker), low levels of expression and little activity of the PAT enzyme occurred in the enzymatic assay, however giving tolerance to the herbicide glufosinate.

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Table 5. Parameter estimation of the adjusted Mitscherlich (1909) model[a] for glufosinate application rates[b] on Liberty Link® soybean(with *pat* gene) soybean cultivars (BS 0043 and BS 1590) in the greenhouse experiments (average of the two experiments).

	Glufosinate concentration				
Cultivars	*a*	*b*	*c*	R^2	*F*
BS 0043	1044.40	−19.0592	0.000186	0.9979	933.8
BS 1590	908.70	−20.0940	0.000242	0.9976	832.3
	$F_{Cultivars} = 0.6^{ns}$	$F_{Rates} = 359.2^{\star}$	$F_{(Cultivars \times Rates)} = 0.3^{ns}$	CV (%) = 16.4	
			N-acetyl-L-glufosinate		
Cultivars	*a*	*b*	*c*	R^2	*F*
BS 0043	2935.80	−43.9791	0.000016	0.9992	2521.2
BS 1590	1108.10	−55.5605	0.000053	0.9985	1367.42
	$F_{Cultivars} = 3.0^{ns}$	$F_{Rates} = 154.7^{\star}$	$F_{(Cultivars \times Rates)} = 0.7^{ns}$	CV (%) = 30.2	
			Ammonia concentration		
Cultivars	*a*	*b*	*c*	R^2	*F*
BS 0043	352.60	−1.5572	0.000241	0.9867	148.8
BS 1590	473.70	−28.0827	0.000219	0.9839	122.6
	$F_{Cultivars} = 8.3^{\star}$	$F_{Rates} = 147.3^{\star}$	$F_{(Cultivars \times Rates)} = 3.4^{\star}$	CV (%) = 25.2	

[a] $y = a\left[1 - 10^{(-c(x+b))}\right]$, where *a* is the maximum asymptote of the model, the lateral displacement of the curve corresponds to parameter *b*, and the concavity of the curve to parameter *c*.
[b] Glufosinate rates: 0, 87.5, 175, 350, 700, 1,400, and 2,800 g ha^{-1}.
$^{\star}P \leq 0.05$.
^{ns}Nonsignificant ($P > 0.05$).

The metabolism of glufosinate to NAG in different maize cultivars with the *pat* gene continued to increase between 2 and 4 DAA (Krenchinski et al. 2018). This may explain the high levels of tolerance to the herbicide observed in this study in soybean even without the total metabolization of glufosinate at 2 DAA.

The concentration of NAG quantified in LL soybean plants was higher for higher rates of glufosinate, with an increase directly proportional to the rate. When comparing quantification of NAG for cultivars with and without the *pat* gene, the relationship between glufosinate metabolization and glufosinate tolerance is evident, indicating this is the mechanism used by the plants, as the compound was not identified in this study in RR2 soybean plants. As in the comparison of LL cultivars, no differences were detected for NAG concentration. Higher levels of glufosinate were also found in RR2 soybean compared with LL soybean, for the same reason that NAG was not identified in RR2 soybean plants. Another point is that there were no differences between the levels of glufosinate in the comparison between the LL cultivars (BS 0043 and BS 1590).

All rates caused an increase of ammonia in leaf tissues between events with the highest content expressed at the highest rate (2,800 g ha^{-1}) of approximately 4,500 mg ammonia kg^{-1} fresh matter found for RR2 soybean, and 280 mg ammonia kg^{-1} fresh matter for LL soybean. Again, the higher concentration in RR2 soybean is related to the absence of the *pat* gene. The inhibition of GS activity by glufosinate leads to the rapid accumulation of high levels of ammonia, due to the lack of nitrogen metabolism, as well as the depletion of the amino acid glutamine (Avila-Garcia and Mallory-Smith 2011; Avila-Garcia et al. 2012; Salas-Perez et al. 2018).

Plant cells prevent ammonium toxicity by rapidly converting ammonium generated from the assimilation of nitrate or photorespiration into amino acids (Lacuesta et al. 1992), and this assimilation is interrupted by the action of the herbicide. These effects are followed by chlorosis, wilting, and necrosis, causing plants to die within 2 wk (Brunharo et al. 2014; Silva et al. 2016). Meanwhile, a more recent study indicates that glufosinate is mainly toxic to plants due to the light-dependent generation of reactive oxygen species instead of accumulation of ammonia or inhibition of carbon assimilation. The generation of reactive oxygen species causes lipid peroxidation of cell membranes and rapid cell death (Takano et al. 2019).

The difference for ammonia concentration between the LL cultivars occurred only at the two highest rates tested (1,400 and 2,800 g ha^{-1}), with greater accumulation for the BS 1590 variety. However, it is important to note that the levels presented may not be the total produced by the analyzed plants, but what was present in the leaves at the time of collection. Manderscheid et al. (2005) analyzed four weed species, namely common lambsquarters (*Chenopodium album* L.), barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.], black nightshade (*Solanum nigrum* L.) and scentless false mayweed [*Tripleurospermum perforatum* (Mérat) M. Lainz], and reported volatilization of ammonia to the atmosphere after the application of 1,400 g ha^{-1} glufosinate. The levels were considered low in all species, but there were also differences between them, with the concentration volatilized by *C. album* plants being about one-third of the total recorded for *S. nigrum* plants. That being said, the equivalent tolerance of LL cultivars to glufosinate application is evident, as the values for both cultivars were equivalent for all other measured components and for dry matter.

Studies have shown that internal ammonia concentration is related to intoxication of plants by glufosinate (Brito et al. 2017; Carbonari et al. 2016; Krenchinski et al. 2019; Sellers et al. 2004; Tsai et al. 2006). Thus, the accumulation of ammonia after application of glufosinate can be used as a biochemical marker of GS inhibition and, consequently, as an indicator of the action of the herbicide (Petersen and Hurle 2001) and the level of sensitivity of the plants. This can also be observed in the present study, in which the largest accumulations of ammonia and major injury for the same cultivars in the field (with values of up to 38.5%) were observed for the highest glufosinate rates (≥1,400 g ha^{-1}).

Glufosinate application POST (V4) on LL soybeans with rates up to four times the recommended maximum caused initial injury symptoms (up to 38.5%) in LL soybean plants. However, there was no negative effect on seed quality and agronomic performance of plants, including yield.

LL soybean plants presented high glufosinate metabolism, lower ammonia concentration, and no reduction in dry matter for application of high rates of glufosinate in comparison with

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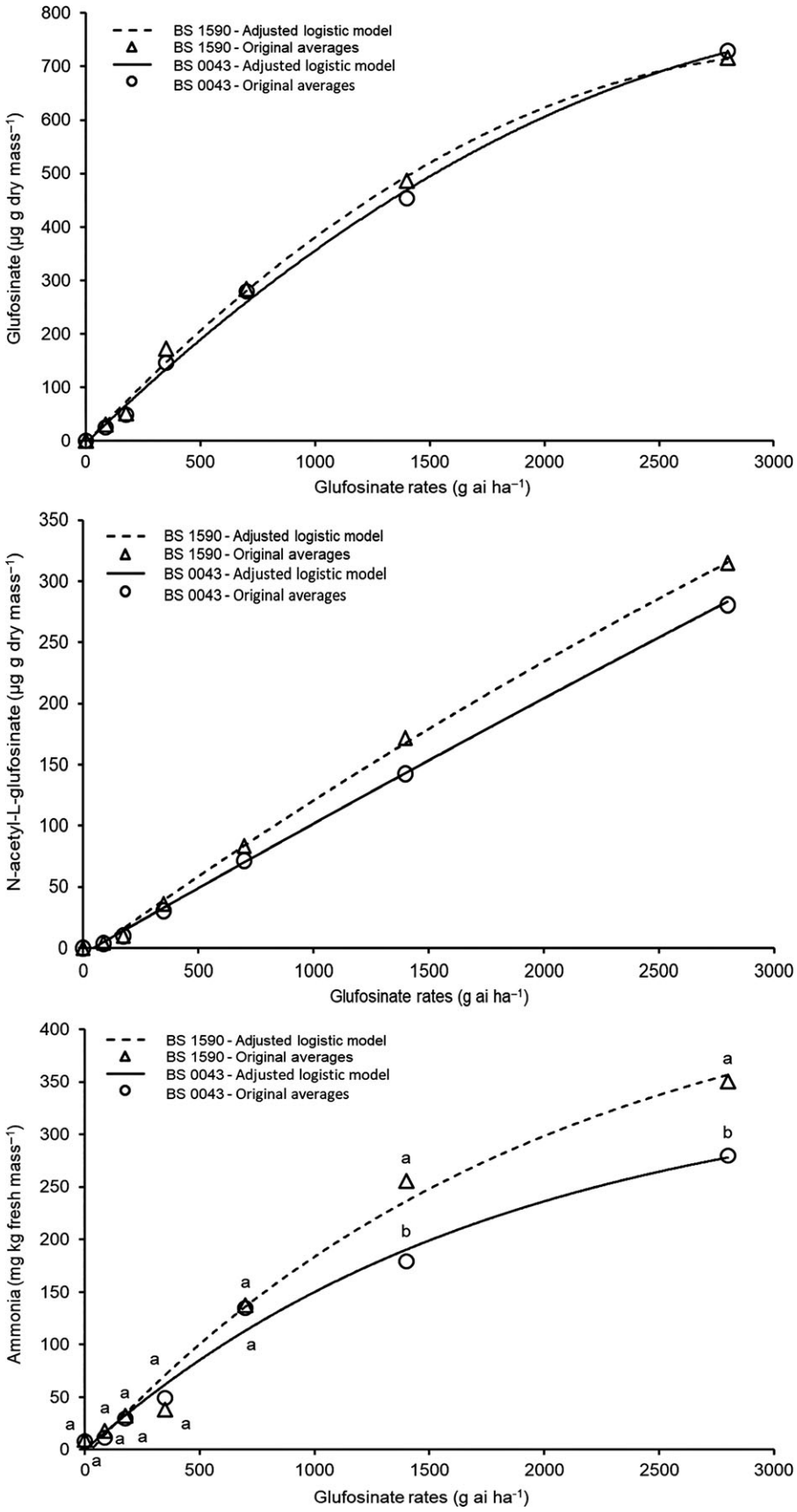

Figure 4. Mitscherlich (1909) model adjusted for glufosinate effects in Liberty Link® soybean (with *pat* gene) cultivars in the greenhouse experiments. For ammonia concentration, at cultivars, means followed by the same letter do not differ by the *t*-test ($P \leq 0.05$). For other variables, at cultivar, means do not differ by the *F*-test ($P > 0.05$).

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Table 6. Dry matter for glufosinate application rates on Liberty Link® soybean(with *pat* gene) soybean cultivars (BS 0043 and BS 1590) in the greenhouse experiments (average of two experiments).

	BS 0043	BS 1590
Rate	Dry matter	
g ai ha^{-1}	% of 0 g ha^{-1}	
0	100	100
87.5	83.3	82.3
175	86.9	81.6
350	105.8	80.3
700	83.0	69.6
1400	82.9	67.8
2800	82.9	60.4
$F_{Cultivars}$	1.8^{ns}	
F_{Rates}	1.4^{ns}	
$F_{(Cultivars \times Rates)}$	0.3^{ns}	
CV (%)	29.61	

^{ns} Nonsignificant ($P > 0.05$).

RR2 soybean. The effects were similar between the cultivars BS 0043 LL and BS 1590 LL, which indicates equivalent expression of the *pat* gene, and consequent equivalence in tolerance to the application of glufosinate.

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