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ORIGINAL ARTICLE

Glyphosate in agronomic performance and seed quality of soybean with *cp4-EPSPs* and *cry1Ac* genes

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Abstract

The "second generation" of glyphosate-tolerant soybean (GT2 soybean) was developed through a different technique of insertion of the glyphosate-insensitive EPSPs gene, in comparison with "first generation" of glyphosate-tolerant soybean. However, there is not enough information available about glyphosate selectivity in GT2 soybean and the effects on the quality of seeds produced. The aim of this study was to evaluate tolerance to glyphosate and seed quality of soybean cultivar NS 6700 IPRO (GT2) with *cp4-EPSPs* and *cry1Ac* genes, after application at post-emergence (V4). The experiment was conducted in a randomized block design with four replicates and seven treatments, or rates of glyphosate (0; 720; 1,440; 2,160; 2,880; 3,600; 4,320 g of acid equivalent – a.e. · ha^{-1}). Assessments were performed for crop injury, SPAD index and variables related to agronomic performance and seed quality. A complementary trial with the same cultivar and treatments in a greenhouse was conducted in a completely randomized design with four replications. Data analysis indicated no significant effect of glyphosate on V4 on agronomic performance and physiological quality of seeds, for two growing seasons. The soybean cultivar NS 6700 IPRO (GT2), with *cp4-EPSPs* and *cry1Ac* genes, was tolerant to glyphosate up to the maximum rate applied (4,320 g a.e. · ha^{-1}) at post-emergence (V4). The quality of soybean seeds was not affected by glyphosate up to the maximum rate applied (4,320 g a.e. · ha^{-1}) at post-emergence (V4).

Keywords: crop injury, *Glycine max*, glyphosate-tolerant soybean, herbicide, selectivity

Introduction

Soybean [*Glycine max* (L. Merrill)] crops are very important in the Brazilian economic scenario. There has been a remarkable growth of areas occupied by soybean crops in Brazil; in the 2017/18 harvest, this area reached 35.02 million ha (Companhia Nacional de Abastecimento – CONAB 2018). Considering soybean in the world scenario, there has been a large number of research studies aimed at increasing the quality and quantity of soybean production. This has required the constant reformulation and adaptation of technologies, e.g. a more adequate use of herbicides, such as glyphosate.

Glyphosate is a post-emergence herbicide, classified as non-selective [selective for glyphosate-tolerant (GT) crops only]. It has a broad spectrum of action and inhibits the activity of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) (Galli and Montezuma 2005).

The development of GT soybean (Roundup Ready™ soybean) was achieved by introducing the *cp4-EPSPs* gene, which encodes a glyphosate-insensitive EPSPs enzyme, from the bacterium *Agrobacterium tumefaciens* strain cp4 (Padgette *et al.* 1995; Dill 2009). The "second generation" of GT soybean (GT2 soybean) was developed through a different technique of insertion of the *cp4-EPSPs* gene (in addition to the *cry1Ac* gene, from the bacterium *Bacillus thuringiensis* (Bt), which makes it insect resistant), under the trademark Intacta™ Roundup Ready™ 2 Pro (Martinell *et al.* 2002; Zobiole *et al.* 2010a).

Some injuries caused by the use of glyphosate in GT soybean should be taken into account. Given the increased use of the product worldwide, injuries are found after application of the product (Zablotowicz and Reddy 2007). These injuries are characterized by yellow flashing of the upper leaves (Zobiole *et al.* 2011). Yellow flashing is a visual symptom of the negative effect of glyphosate on photosynthetic parameters and chlorophyll content (Zablotowicz and Reddy 2007). According to Zobiole *et al.* (2010b), higher rates of glyphosate and late applications (V6) may negatively affect photosynthetic parameters of GT2 soybean. However, in early applications (V2), damage is lower, probably because of the longer recovery time of soybean plants, after application of glyphosate.

As for seed quality and physiology of the plant itself, when damage relative to glyphosate application is detected, it is likely to have been caused by compounds from the degradation of glyphosate rather than from the herbicide itself (Albrecht and Ávila 2010). The application of glyphosate (1,440 to 2,880 g a.e. · ha^{-1}) may adversely affect the physiological quality of GT soybean seeds at the V6 and R2 stages (Albrecht *et al.* 2012). These negative effects on seed physiological quality are probably related to potential damage, or deleterious action of glyphosate. This herbicide can cause stress even on GT soybean plants for which it should be selective (Zablotowicz and Reddy 2007; Zobiole *et al.* 2011; Albrecht *et al.* 2018).

Selectivity of glyphosate in GT soybean, and the effects on the quality of seeds produced, have been studied by other researchers who evaluated glyphosate effects on GT2 soybean (Zobiole *et al.* 2010b; Krenchinski *et al.* 2017). However, studies on these aspects are less consolidated in GT2 soybean. As previously mentioned, the glyphosate-insensitive *EPSPs* gene was inserted by using another technique in GT2 soybean. The GT and GT2 soybean are different transgenic products, GTS-40-3-2 (Roundup Ready™ soybean) and MON87701 × MON89788 (Intacta™ Roundup Ready™ 2 Pro soybean), respectively (Conselho de Informações sobre Biotecnologia – CIB 2018).

Given the above, the aim of this study was to evaluate tolerance to glyphosate and seed quality of the soybean cultivar NS 6700 IPRO (GT2), with *cp4-EPSPs* and *cry1Ac* genes, after application at post-emergence (V4).

Materials and Methods

Field and greenhouse experiments

The experiment was conducted in the field during the 2013/14 and 2014/15 seasons (experiments I and II) and in a greenhouse in 2014/15 (experiment III) in Piracicaba, São Paulo, Brazil, altitude: 536 m. Table 1 shows the soil chemical analysis of the experimental area.

Fertilization practices, crop installation and phytosanitary management were carried out in accordance with recommendations of Embrapa (2013). All the parcels and plots were kept free of weed interference by means of manual weeding. The climate of the region is Cwa by the Köppen climate classification. Figure 1 shows data on rainfall and temperature distribution over the experimental periods in the two growing seasons. For 2013/14 rainfall values were close to the normative average of the region, with slightly lower values in January and February. For 2014/15 rainfall was slightly higher than the annual average. For both seasons the temperature was in the range of the typical average for the period in the region.

The experiment was conducted in a randomized block design for experiments I and II and in a completely randomized design for experiment III, with four replications and seven treatments. The treatments consisted of seven rates of the herbicide glyphosate (Roundup Ready™) (0; 720; 1,440; 2,160; 2,880; 3,600; 4,320 g a.e. · ha^{-1}). The maximum recommended rate of glyphosate for GT soybean is 1,440 g a.e. · ha^{-1} (Rodrigues and Almeida 2018).

Table 1. Results of soil chemical analysis, depth 0 to 20 cm, Piracicaba – SP

pH ($CaCl_2$)	Al	H + Al	P (resin)	K	Ca	Mg	SB	CEC	V	Clay	Silt	Sand
5.3	< 1.0	25.0	10.0	2.8	26.0	13	41.8	66.8	63	41.0	5.0	54.0

SB – sum of bases; CEC – cation exchange capacity
Units: Al, H + Al, K, Ca, Mg, SB, CEC [$mmol_c \cdot dm^{-3}$]; P (resin) [$mg \cdot dm^{-3}$]; V, clay, silt, sand [%]

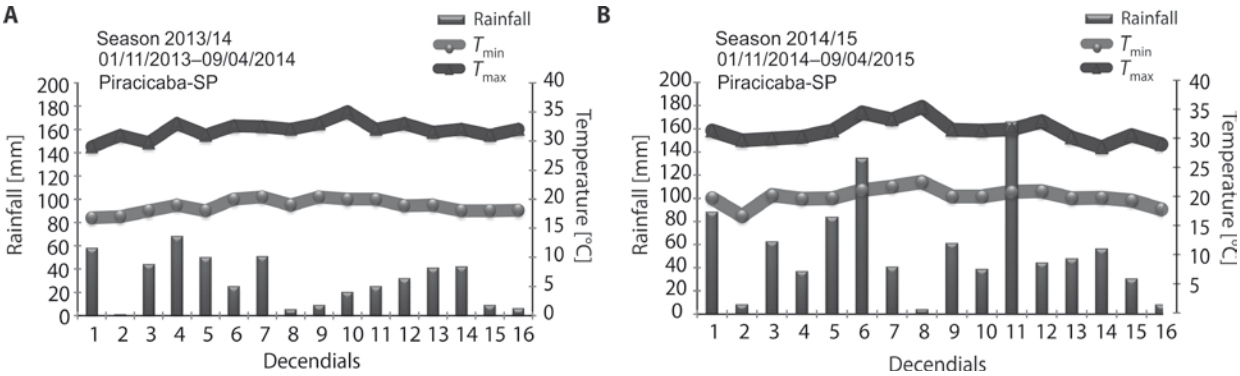

Fig. 1. Representation of rainfall, average minimum and average maximum temperatures (T_{min}, T_{max}) for periods relative to soybean crop seasons 2013/14 (A), and 2014/15 (B). Piracicaba, São Paulo, Brazil. Source: LEB – USP/ESALQ

Soybean cultivar NS 6700 IPRO (GT2), which has a cycle of 120–150 days was used. Seeding was carried out in the first week of December, with harvest in the second week of April, for both seasons. For experiments I and II, the experimental units were composed of plots 5 m in length with six rows of soybean plants. The four central rows were used but the first and last meters of the plot were discarded. For experiment III, 7 L pots were filled with medium-textured soil. Six seeds were sown per pot and after emergence, thinned to three plants per pot. Experiment III began in the first week of October 2014 and was completed in the second week of November of the same year.

Treatments were applied at growth stage V4 – four nodes on the main stem beginning with the unifoliate node (Fehr *et al.* 1971) of soybean plants. The best time for application of glyphosate (Roundup Ready™) for post-emergence weed control is 20 to 30 days after emergence of the crop (Rodrigues and Almeida 2018), at approximately the V3–V6 stages of soybean.

The treatments were applied via a CO_2 pressurized backpack sprayer with a bar fitted with four spray nozzles, at a constant pressure of 2 bar, flow rate of 0.65 l · min^{-1}, working at a height of 50 cm and at a speed of 1 m · s^{-1}, reaching an applied band of 50 cm wide per spray nozzle, and providing a volume flow of 200 l · min^{-1}.

Data collection

Crop injury was assessed through visual evaluations, in which percentage scores were assigned to each experimental unit (0 for no injuries, up to 100% for plant death) (Velini *et al.* 1995). This evaluation was performed 7, 14, 21 and 28 days after application (DAA) for experiments I and II and at 7, 14 and 21 DAA for experiment III.

The Soil and Plant Analyzer Development (SPAD) index was evaluated at 14 and 28 DAA for experiments I and II, and at 7, 14 and 21 DAA for experiment III. For this evaluation, a portable meter was used (SPAD-502, Konica Minolta, Inc., Japan). This instrument quantitatively evaluates the intensity of leaf greenness through the measurement of light transmissions; the equipment calculates a SPAD index which is usually highly correlated with the chlorophyll content of leaves (Markwell *et al.* 1995; Uddling *et al.* 2007). Ten plants were evaluated in experiments I and II, randomly chosen in the used area of the plots, while the three plants of each pot were evaluated for experiment III.

For experiments I and II, evaluations were also made of the variables related to agronomic performance (plant height, total number of pods per plant, number of pods with two seeds per plant, number of pods with three seeds per plant, yield and 1000-seed mass). For determination of plant height, 10 plants were randomly chosen and measured with a wooden millimeter ruler, and the results were expressed in centimeters. The number of pods per plant was evaluated at full maturity (R8) (Fehr *et al.* 1971) by manually counting the number of pods in 10 plants randomly chosen in the used area of each plot. The plants were harvested manually at the R8 stage. Pods were threshed in a thresher for experiments, cleaned with sieves and packed in paper bags for further analysis and evaluation.

The physiological quality of seeds was analyzed in experiments I and II by means of the first count of germination test (indicative of vigor), second count (germination), percentage of abnormal seedlings and dead seeds, according to Brasil (2009). For experiment III, the height of soybean plants at 7, 14 and 21 DAA was also evaluated. Three plants of each pot were measured with a ruler and the results were expressed in centimeters. When most of the plants reached the R2 stage, the shoots and the root system of the plants of each pot were collected to determine the fresh and dry matter mass of shoots and of the root system. For drying, the material was placed in a forced ventilation oven at 65°C for 72 h and an analytical balance, accurate to three decimal places, was used to determine the mass.

Statistical analyses

The data were analyzed according to the methods of Pimentel-Gomes and Garcia (2002). Once the basic assumptions for the variance analysis were met, the treatment averages were subjected to regression analysis ($p \leq 0.05$).

Results

Crop injury and SPAD index

Data analysis indicated no significant effect of glyphosate at post-emergence (V4) on crop injury (Table 2). With increased glyphosate rates, there was a linear increase in crop injury percentage of GT2 soybean plants. For experiment I (Fig. 2), percentages did not exceed 11.25; this value was found for the rate 3,600 g a.e. · ha^{-1} at 14 DAA. At 28 DAA, higher values (8.75%) were found for rates 3,600 and 4,320 g a.e. · ha^{-1}.

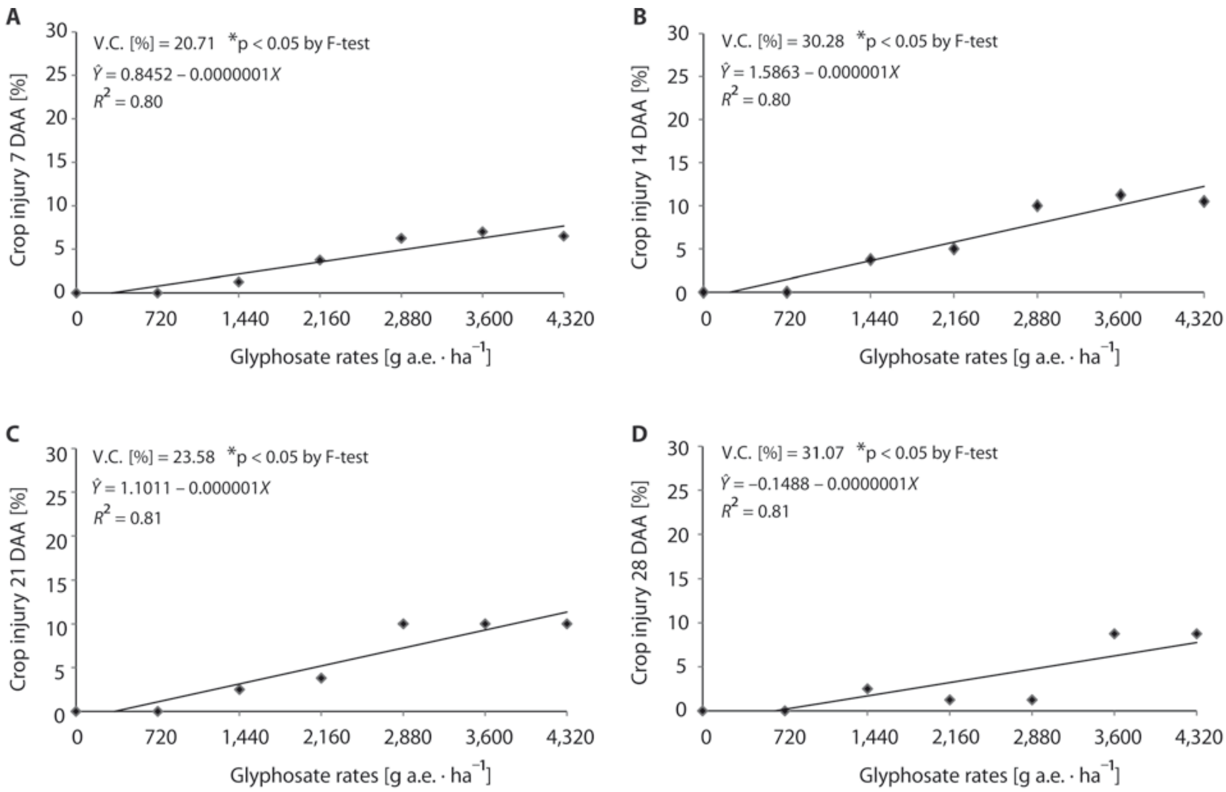

Fig. 2. Crop injury evaluation at 7 (A), 14 (B), 21 (C) and 28 (D) days after application (DAA), from GT2 soybean plants after application of glyphosate rates in post-emergence (V4). Season 2013/14 (experiment I), Piracicaba, São Paulo, Brazil. V.C. [%] – variation coefficient

Table 2. Results of the analysis of variance for crop injury of GT2 soybean plants after application of glyphosate rates in post-emergence (V4)

Source	Experiment I DAA				Experiment II DAA		Experiment III DAA	
	7	14	21	28	7	14	7	14
				F				
Rates	15.434*	8.870*	20.721*	12.120*	19.364*	13.231*	15.920*	10.496*
				P > F				
	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
V.C. [%]	20.71	30.28	23.58	31.07	13.95	16.46	18.02	20.21

DAA – days after application; V.C. [%] – variation coefficient
*p < 0.05 significant

For experiment II (Fig. 3), even lower values were found, with the highest percentage of 4.0, for the rate 4,320 g a.e. · ha^{-1} at 14 DAA. At 21 and 28 DAA, there were no symptoms of injury in soybean plants (data not shown). For experiment III (Fig. 4) percentages did not exceed 5.0; this value was found for the rate 3,600 g a.e. · ha^{-1} at 7 DAA. At 21 DAA, there were no symptoms of injury in soybean plants (data not shown).

The results of the analysis of variance were conclusive for the SPAD index (Table 3). There was no influence of glyphosate doses in this variable on GT2 soybean.

Growth, development and agronomic performance of GT2 soybean plants

For experiment III, there were no significant effects of glyphosate rates applied at post-emergence (V4) for the following variables: plant height at 7, 14 and 21 DAA, and dry mass of shoots and roots. Therefore, the results of the analysis of variance were conclusive (Table 4).

Moreover, data analysis indicated no significant effect of glyphosate at post-emergence (V4) on variables

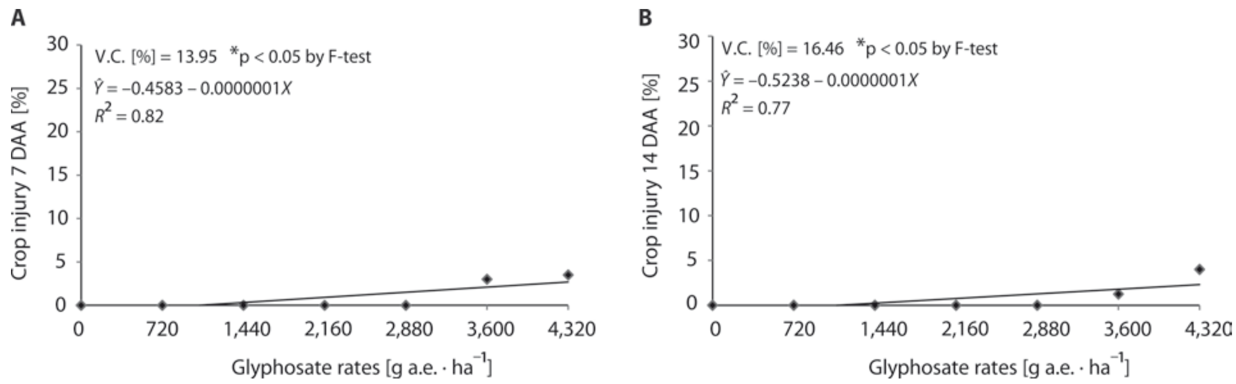

Fig. 3. Crop injury evaluation at 7 (A), and 14 (B) days after application (DAA) from GT2 soybean plants after application of glyphosate rates in post-emergence (V4). Season 2014/15 (experiment II), Piracicaba, São Paulo, Brazil

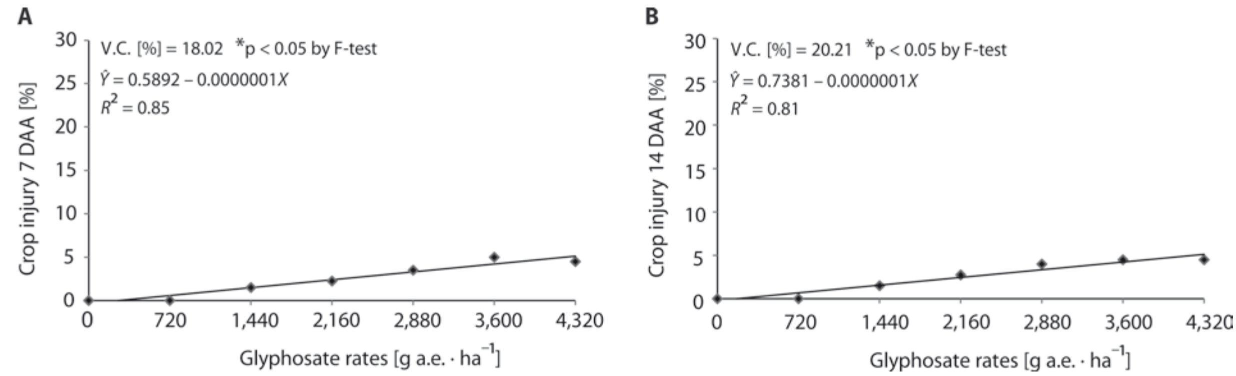

Fig. 4. Crop injury evaluation at 7 (A), 14 (B) days after application (DAA) from GT2 soybean plants after application of glyphosate rates in post-emergence (V4). Greenhouse, 2014 (experiment III), Piracicaba, São Paulo, Brazil

Table 3. Results of the analysis of variance for Soil and Plant Analyzer Development (SPAD) index of GT2 soybean plants after application of glyphosate rates in post-emergence (V4)

Source	Experiment I DAA		Experiment II DAA		Experiment III DAA		
	14	28	14	28	7	14	21
				F			
Rates	1.927 ns	0.689 ns	0.638 ns	0.861 ns	0.814 ns	1.998 ns	2.512 ns
				P > F			
	0.1313	0.6610	0.6987	0.5414	0.5709	0.1130	0.0644
V.C. [%]	3.70	3.38	7.42	9.39	11.08	9.97	6.39

SPAD – Soil and Plant Analyzer Development; DAA – days after application; V.C. [%] – variation coefficient
ns – non-significant ($p < 0.05$)

Table 4. Results of the analysis of variance for plant height, dry mass of shoots and roots of GT2 soybean plants after application of glyphosate in experiment III

Source	Plant height			DMS	DMR
	7 DAA	14 DAA	21 DAA		
			F		
Rates	0.768 ns	0.241 ns	0.354 ns	0.471 ns	0.966 ns
			P > F		
	0.6037	0.9576	0.8995	0.8223	0.4719
V.C. [%]	9.15	11.77	13.85	23.80	19.21

DMS – dry mass of shoots; DMR – dry mass of roots; DAA – days after application; V.C. [%] – variation coefficient
ns – non-significant ($p < 0.05$)

related to agronomic performance for experiments I and II (Table 5). Thus, there was no significant fit of the linear regression model, according to the criteria observed (biological explanation, significant regression, non-significant regression deviations, coefficient of determination and residual analysis).

The total number of pods per plant was not affected by the increase in glyphosate rates (Table 4) for both

Table 5. Results of the analysis of variance for variables related to agronomic performance of GT2 soybean plants after application of glyphosate rates in post-emergence (V4)

	Experiment I					
Source	PH	NP	NP2	NP3	Y	SM
			F			
Rates	1.334 ns	0.435 ns	5.609*	1.010*	1.180 ns	1.493 ns
			P > F			
	0.2930	0.8462	0.0020	0.0095	0.3600	0.2364
V.C. [%]	4.96	13.74	15.66	19.61	10.41	4.44
	Experiment II					
			F			
Rates	0.877 ns	1.379 ns	1.450 ns	0.967 ns	1.316 ns	1.466 ns
			P > F			
	0.5308	0.2759	0.2506	0.4745	0.3002	0.2451
V.C. [%]	4.80	10.64	14.35	10.25	7.57	5.53

PH – plant height; NP – total number of pods per plant; NP2 – number of pods with 2 seeds per plant; NP3 – number of pods with 3 seeds per plant; Y – yield; SM – 1000-seed mass; V.C. [%] – variation coefficient
*$p < 0.05$ significant, ns – non-significant ($p < 0.05$)

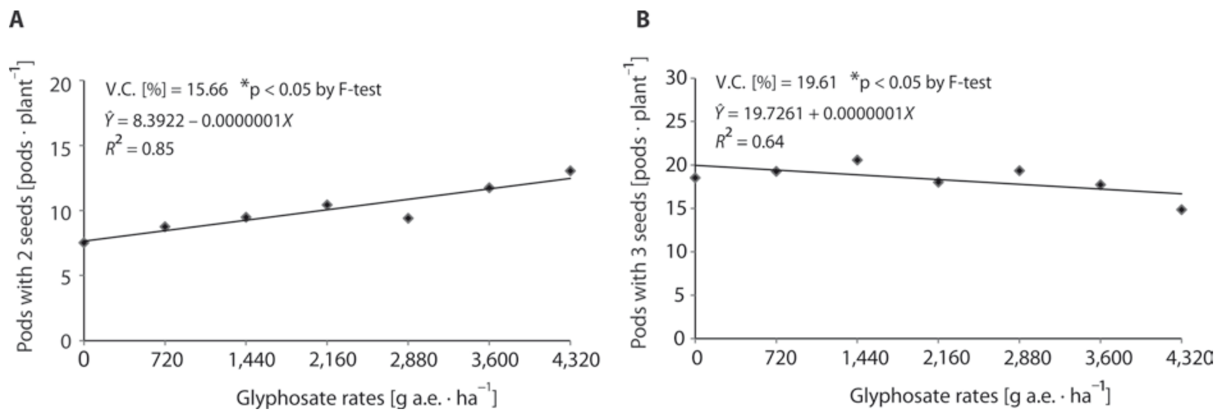

Fig. 5. Pods with 2 seeds (pods · plant^{-1}) (A), and pods with 3 seeds (pods · plant^{-1}) (B) from GT2 soybean plants after application of glyphosate rates in post-emergence (V4). Season 2013/14 (experiment I), Piracicaba, São Paulo, Brazil

Table 6. Results of the analysis of variance for variables related the physiological quality of seeds of GT2 soybean plants after application of glyphosate rates in post-emergence (V4)

Source	Experiment I				Experiment II			
	vigor	G	AS	DS	vigor	G	AS	DS
				F				
Rates	1.399 ns	1.605 ns	0.606 ns	2.549 ns	0.767 ns	0.369 ns	0.668 ns	1.062 ns
				P > F				
	0.2686	0.2029	0.7226	0.0577	0.6051	0.8832	0.6767	0.4201
V.C. [%]	3.55	3.01	40.56	51.54	5.07	4.58	51.16	51.81

G – germination; AS – abnormal seedlings; DS – dead seeds; V.C. [%] – variation coefficient
ns – non-significant ($p < 0.05$)

seasons. However, with increasing rates of glyphosate, there was a reduction in the number of pods with three seeds (Fig. 5A) and an increase in the number of pods with two seeds (Fig. 5B) for experiment I.

Physiological quality of soybean seeds

For variables related to the physiological quality of seeds, for both growing seasons, there were no significant effects of glyphosate applied at post-emergence (V4) with the rates used. Thus, the results of the analysis of variance were conclusive (Table 6).

Discussion

Crop injury and SPAD index

Glyphosate-tolerant (GT) soybean showed injury at 2.5% for application of glyphosate (1,200 g a.e. · ha^{-1}) (Correia *et al.* 2008). Silva *et al.* (2016) did not find visual symptoms of crop injury and reduction of the SPAD index for application of glyphosate (960 g a.e. · ha^{-1}) in GT soybean, while Albrecht *et al.* (2018) found symptoms of injury and reductions in chlorophyll content in GT soybean with increased doses of glyphosate. In the present study, injury was observed in GT2 soybean plants, however at doses higher than those used by Albrecht *et al.* (2018). As in the cited studies, no reduction in SPAD index values was observed for glyphosate application.

Although Krenchinski *et al.* (2017) found injuries in GT2 soybean with the application of glyphosate the GT2 soybeans recovered from visual injuries after glyphosate application. Alonso *et al.* (2013) reported crop injury up to 40% in GT soybean plants for application of glyphosate (1,200 + 1,200 g a.e. · ha^{-1}) at the post-emergence period (V1 to V2 and V3 to V4), but these injuries did not reduce soybean yield, as occurred in the present study.

Growth, development and agronomic performance of soybean plants

Albrecht *et al.* (2011), for application of glyphosate (0,1440 and 2,880 g a.e. · ha^{-1}) at post-emergence, reported no reduction in the height of GT soybean plants up to the rate of 2,880 g a.e. · ha^{-1}. For 100 seed-mass, they found a reduction for the rate 2,880 g a.e. · ha^{-1}; and a reduction in yield in the order of 0.40661 kg · ha^{-1} each g a.e. glyphosate. In contrast, our study found that there were no negative effects of glyphosate on variables related to agronomic performance.

However, with increasing rates of glyphosate, there was a reduction in the number of pods with three seeds for experiment I. The reduced number of pods may be related to a decrease in pod retention because of nutritional problems or problems in photosynthesis and water use (Zobiole *et al.* 2010a). Albrecht *et al.* (2011) found a reduction in the total number of pods per GT soybean plant with the application of glyphosate (2,880 g a.e. · ha^{-1}) at the V6 stage and mainly at the R2 stage.

Physiological quality of soybean seeds

For variables related to the physiological quality of seeds, for both growing seasons, there were no significant effects of glyphosate applied at post-emergence (V4) with the rates in use. This is contrary to the results of Albrecht *et al.* (2012), in which the application of glyphosate (1,440 to 2,880 g a.e. · ha^{-1}) negatively affected the seed physiological quality of GT soybean at the V6 and R2 stages.

Conclusions

It should be noted that the maximum recommended rate of glyphosate for GT soybean is 1,440 g a.e. · ha^{-1} (Rodrigues and Almeida 2018). In the present study, in general, no deleterious effects were found up to the rate 4,320 g a.e. · ha^{-1}, thus showing the high tolerance of the soybean cultivar NS 6700 IPRO (GT2). Similar results were found by Scursoni and Satorre (2010), Bohm *et al.* (2014), and Hungria *et al.* (2014). However, injuries, even yield reduction, resulting from the use of high rates of glyphosate have been reported (Melhorança-Filho *et al.* 2010; Zobiole *et al.* 2010c; Albrecht *et al.* 2011, 2014).

As previously mentioned, the glyphosate-insensitive EPSPs gene was inserted by using another technique in GT2 soybean. It should be noted that although there is information available about the selectivity of glyphosate in GT soybean, there is little information on the selectivity of this herbicide in GT2 soybean. Thus, the results of Krenchinski *et al.* (2017) as well as the results found in the present work are noteworthy and important in the use of glyphosate in GT2 soybean.

The soybean cultivar NS 6700 IPRO (GT2), with *cp4-EPSPs* and *cry1Ac* genes, was tolerant to glyphosate up to the maximum rate applied (4,320 g a.e. · ha^{-1}) at post-emergence (V4).

The soybean seed quality was not affected by glyphosate up to the maximum rate applied (4,320 g a.e. · ha^{-1}) at post-emergence (V4).

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