Effect of glyphosate and glufosinate on nutritional content and agronomic performance of maize possessing *cp4epsps* and *pat* transgenes

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Abstract

The aim of this study was to check the tolerance level of maize possessing the transgenes *cp4epsps* and *pat*, exposed to high rates of glyphosate or glufosinate. Field experiments were carried out in 2016/17 at the first growing season; and the other in the second growing season, in Palotina, state of Paraná (PR), Brazil. The design was completely randomized block with 4 replications, which were set up with variations of rates of herbicides: glyphosate (0; 2,160; 4,320; 6,480 and 8,640 g ae ha^{-1}) (experiment 1) and glufosinate (0; 1,500; 3,000; 4,500 and 6,000 g ai ha^{-1}) (experiment 2). The application was performed at the V4 stage. Plants were evaluated for injuries, final plant height, stem diameter, 100-grain mass, yield and content of P, K, S, Fe, Cu and Zn. The results show that there was no reduction in yield and 100-grain mass, as well as changes in nutrient contents, even with the use of high rates of glyphosate and glufosinate. This is explained by the rapid recovery of maize hybrids under favorable developmental conditions. Despite causing injuries and decreased height, application of glyphosate (up to 8,640 g ae ha^{-1}) or glufosinate (up to 6,000 ai ha^{-1}) did neither reduce yield and 100-grain mass nor the nutrient contents of grains. This shows the high selectivity of these herbicides. Nonetheless, crop injury and reduction in height were observed in maize plants for herbicide application. This indicates the risks of using herbicides above the rates recommended in the package insert, despite the absence of deleterious effects on yield.

Keywords: chemical control; crop injury; herbicides; morphological response; herbicides selectivity; *Zea mays.*
Abbreviations: ae_ acid equivalent; ai_active ingredient; DAA_days after application; D_stem diameter; EPSPs_5-enolpyruvylshikimate-3-phosphate synthase; GS_glutamine synthase; H_height; LL_Liberty Link; M_100-grain mass; PAT_phosphinothricin N-acetyltransferase; RR_Roundup Ready; RR2_Roundup Ready 2; Y_yield.

Introduction

The natural competition between the crop of interest and weeds generates significant yield losses. Estimated, world grain production is affected by 13% (Stiegelmeier et al., 2016). Weed control consists of the adoption of practices that culminate in the reduction of infestation, the level of control depends, among other factors, on the weed species, crop and methods employed (Lorenzi, 2014).

In the past, several herbicides were used on weeds and crops to find out which one was selective, however, today a single herbicide may show selectivity for several plant species, due to the transfer of genes that confer tolerance to a particular molecule. As an example of this advance, Roundup Ready (RR) technology has been widely accepted by farmers because of the effective weed control using a previously non-selective product (Albrecht et al., 2014).

The "first generation" of glyphosate herbicide tolerant maize (RR) refers to the transgenic event GA21 (Roundup Ready and Agrisure GT). Glyphosate tolerance is conferred by the *mepsps* gene (modified *epsps* gene from *Zea mays*), which

encodes an enzyme EPSPs insensitive to this herbicide (Sidhu et al., 2000). The "second generation" of glyphosate tolerant maize (RR2) is represented by events NK603 (Roundup Ready 2 maize) and MON87427 (Roundup Ready maize). Tolerance is conferred by the *cp4epsps* gene from *Agrobacterium tumefaciens* strain CP4 that encodes a glyphosate-insensitive EPSPs enzyme (Ridley et al., 2002).

Event T25 confers tolerance to the herbicide glufosinate in maize (Liberty Link™ - LL). Tolerance is conferred by the *pat* gene from the bacterium *Streptomyces viridochromogenes* (Matsuoka et al. 2001). In addition, insect-resistant maize - Bt11 and TC1507 events - also has tolerance to glufosinate since the *pat* gene was used as a marker in its selection process (Silva et al., 2017; Albrecht et al., 2021).

Glyphosate inhibits EPSPs (5-enolpyruvylshikimate-3-phosphate synthase) not allowing the transformation of shikimate to chorismate, resulting in lack of aromatic amino acids (phenylalanine, tyrosine and tryptophan), dysregulation of plant carbon flux and accumulation of toxic

intermediate compounds (shikimate-3-phosphate and shikimate). It has a broad spectrum of action, its action is systemic, being absorbed by leaves and green tissues, and preferentially redistributed by phloem to the meristematic tissues of the plant (Oliveira Júnior, 2011).

Glufosinate is a non-selective contact herbicide that binds to GS (glutamine synthase) which is an important enzyme in the metabolic pathway of nitrogen uptake. As a result, there is accumulation of ammonia inside the cell, decreased photosynthesis, lack of amino acids and accumulation of substances such as glyoxylate (which is a potent inhibitor of rubisco) and toxic oxygen radicals that lead the plant to death (Oliveira Júnior, 2011). A more recent study indicates that glufosinate is mainly toxic to plants due to a light-dependent generation of reactive oxygen species, rather than ammonia accumulation 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 effects of glyphosate on nutritional content and/or yield in tolerant transgenic crops have been studied for soybean (transformed *cp4epsps*) (Zobiole et al., 2012; Albrecht et al., 2018). However, these studies are less consolidated for glyphosate tolerant maize. More recently, Reddy et al. (2018) observed no deleterious effects for two glyphosate applications (870 g acid equivalent - ae - ha^{-1}) on the nutritional content and yield of maize (transformed *cp4epsps*). For glufosinate tolerant maize (transformed *pat*), there are also few studies on the effects of this herbicide on nutritional content and agronomic performance. Costa et al. (2018) reported no negative effects of glufosinate (300 + 300 g active ingredient - ai - ha^{-1}) on nutritional content and yield of maize (transformed *pat*).

The maximum recommended single application rate of glyphosate for maize (transformed *cp4epsps*) is 1,080 g ae ha^{-1}. For glufosinate, 600 g ai ha^{-1} in maize (transformed *pat*) (Rodrigues & Almeida, 2018). High rates of herbicides (out of the range recommended in the package insert) are known to aggravate resistance problems (due to selection pressure), increase control costs per area, cause damage to cultivated plants, and cause environmental damage, but they comprise a practice still performed by some farmers.

In this sense, it is believed that rates above the recommended in package inserts of glyphosate and glufosinate may affect the nutritional content and agronomic performance of transgenic maize with *cp4epsps* and *pat* genes. Thus, the aim of this study was to evaluate the nutritional content and agronomic performance of maize with the transgenes *cp4epsps* and *pat*, subjected to the application of glyphosate or glufosinate.

Results

Regression analysis
The regression analysis indicated a significant effect ($P \leq$ 0.05) of glyphosate rates on the variables: crop injury, at 7, 14 and 21 DAA and plant height, for the 1st growing season. For the 2nd growing season, on crop injury at 7, 14 and 21. The regression analysis indicate no significant effect of glyphosate rates on the other variables ($P > 0.05$) (Supplementary Table).

For glufosinate, it was observed a significant effect ($P \leq 0.05$) of the rates on the following variables: crop injury at 7, 14, 21 and 28 DAA and plant height for both growing seasons. Analysis indicated no significant effect ($P > 0.05$) of glufosinate rates on the other variables for both growing seasons (Supplementary Table).

Glyphosate rates effect
When significant effect was detected, increasing glyphosate rates increased the percentage of crop injury and reduced plant height (Figures 1 and 2), with linear model adjustment. The crop injury was 7.25% for the rate 8,640 g ae ha^{-1} at 21 DAA, in the 1st growing season (Figure 1C). For the 2nd growing season, at 21 DAA, an injury of 2.75% was also found for the highest rate (8,640 g ae ha^{-1}) (Figure 2C). It is noteworthy that despite the crop injury, no deleterious effects of glyphosate rates on yield and 100-grain mass were observed for both harvests. The same was verified for the contents of nutrients, evaluated in the 2nd growing season.

Glufosinate rates effect
With increasing rates of glufosinate, an increase in crop injury was observed for all evaluations and a reduction in maize plant height for both growing seasons (Figures 3 and 4), with linear model adjustment. For the highest rate (6,000 g ai ha^{-1}), injury was observed at 27.0% (1st growing season) and 20.3% (2nd growing season) at 28 DAA (Figures 3D and 4D). Similar to glyphosate rates, there were no deleterious effects of glufosinate rates on yield and 100-grain mass for both harvests. Likewise, no significant effect on the contents of nutrients was observed.

Discussion

Glyphosate application was selective for maize (transformed *cp4espsps*) at rates 720, 1,440, 2,160 and 2,880 g ae ha^{-1}, with no negative effects on height and no crop injury, according to research by Albrecht et al. (2016). Similarly, Krenchinski et al. (2018a) did not observe reductions in chlorophyll indices, height, diameter and yield of maize for glyphosate application (1,080 g ae ha^{-1}). Other studies also report glyphosate selectivity for maize (transformed *cp4espsps*) at rates 1,260 g ae ha^{-1} (Ganie et al., 2017), 1,080 g ae ha^{-1} (Giovanelli et al., 2018).

The results verified in the present study agree with the previously mentioned studies. Nevertheless, higher rates of glyphosate (up to 8,640 g ae ha^{-1}) were applied in the present study, and despite the crop injury, no reductions in grain yield and nutrient content were detected. It is noteworthy that the maximum recommended rate of glyphosate for application in tolerant maize is 1,080 g ae ha^{-1} (Rodrigues & Almeida, 2018). Thus, high rates are not recommended for weed management, despite being used in some situations by some farmers.

Soltani et al. (2018) observed crop injury, and in some situations even reductions in maize yield (transformed *cp4epsps*) for glyphosate application (1,800 g ae ha^{-1}) in association with 2,4-D. This result may be related to a higher absorption of 2,4-D by maize plants, due to the adjuvants present in the glyphosate formulation, and/or the glyphosate rate used. It is believed that the application of glyphosate at the rates used in the present study, in case of combination with other herbicides could reduce maize yield. This reinforces the recommendation to use glyphosate only up to the maximum recommended rate in the package insert as a precautionary principle. There are descriptions of different side effects generated by herbicides, effects that may be considered latent in the plant of interest. These include changes in nitrogen metabolism, hormone levels and secondary plant metabolism (Rizzardi et al., 2003). When
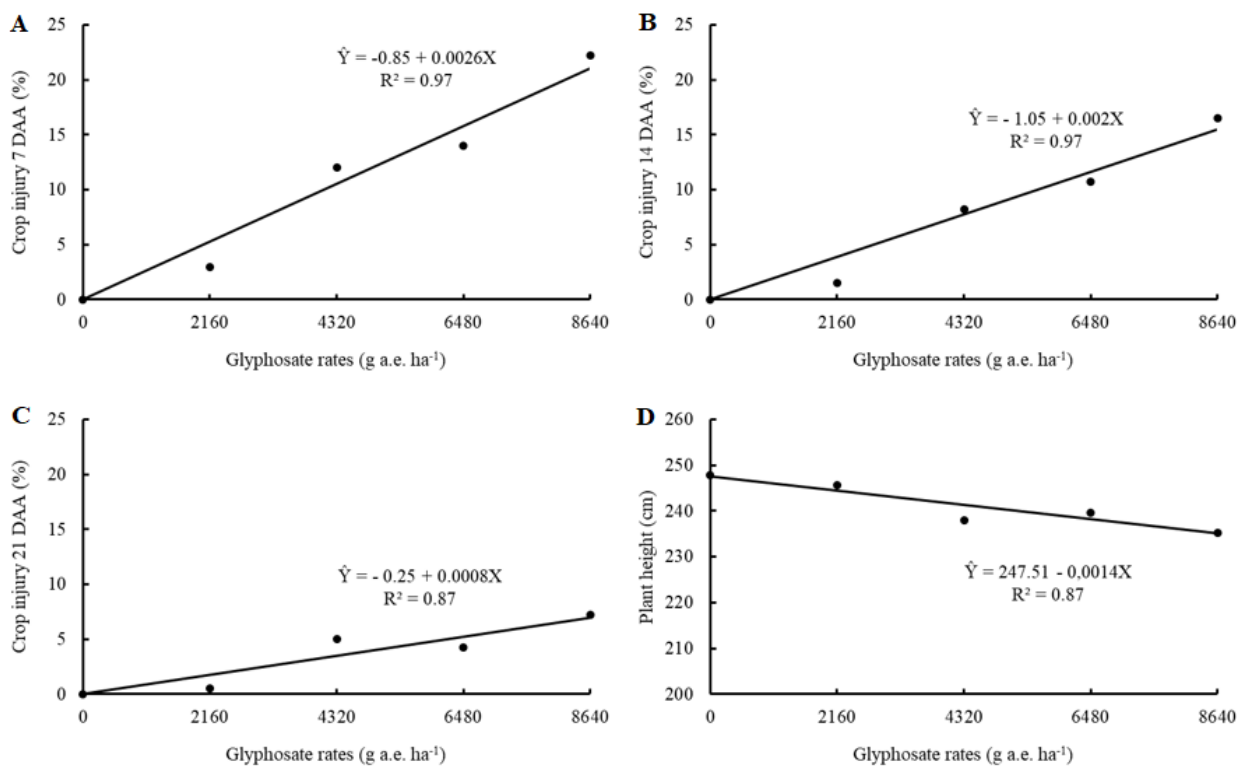
Figure 1. Crop injury at 7 (A), 14 (B) and 21 (C) DAA and plant height (D) of maize plants, under glyphosate rates application at post-emergence. Palotina, PR, Brazil, 2016/17 (Experiment 1, 1st growing season).
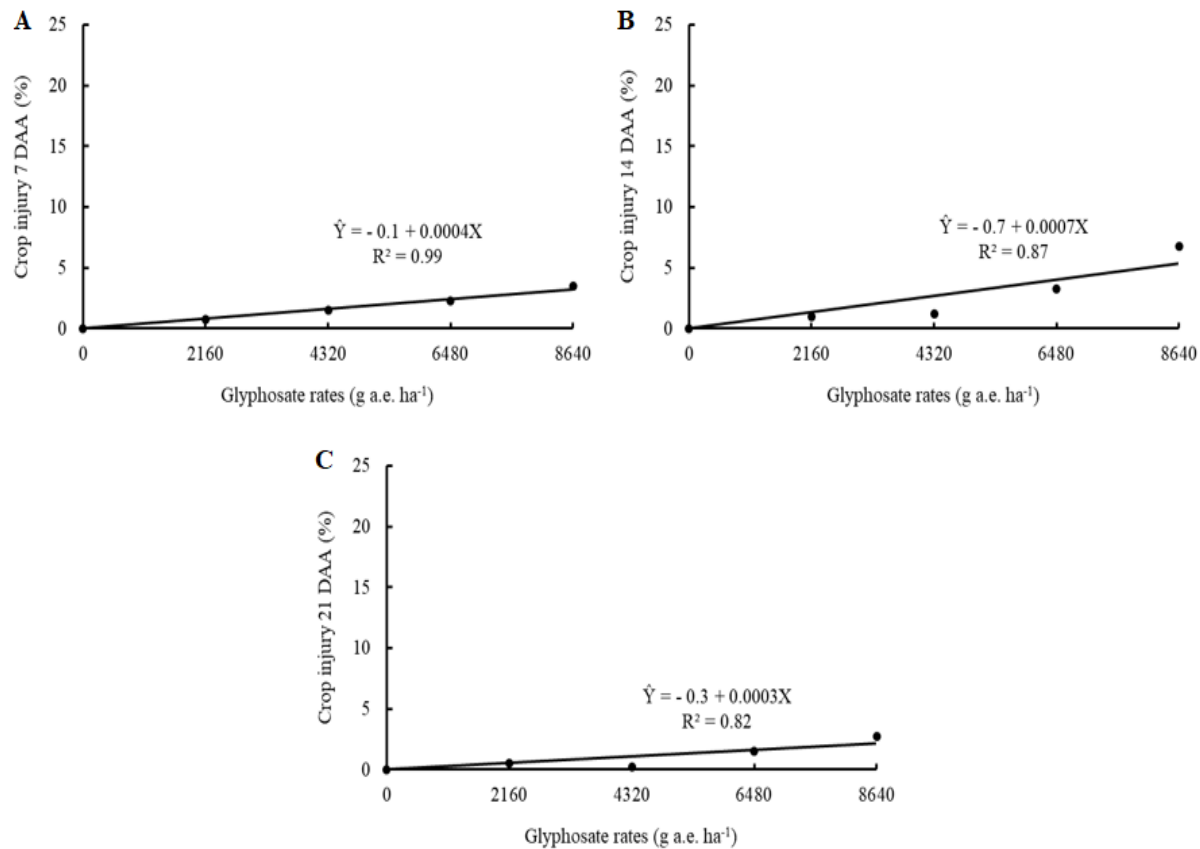

Figure 2. Crop injury at 7 (A), 14 (B) and 21 (C) DAA in maize plants, under glyphosate rates application at post-emergence. Palotina, PR, Brazil, 2016/17 (Experiment 1, 2nd growing season).
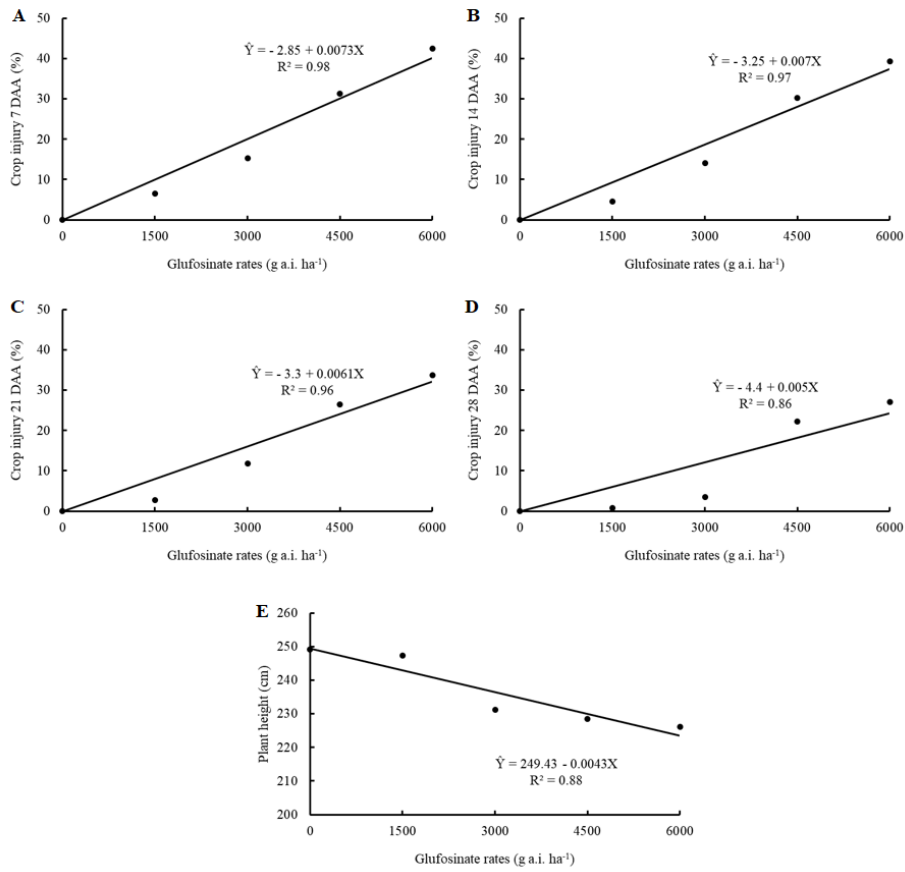
Figure 3. Crop injury at 7 (A), 14 (B), 21 (C) and 28 (D) DAA and plant height (E) of maize plants, under glufosinate rates application at post-emergence. Palotina, PR, Brazil, 2016/17 (Experiment 2, 1st growing season).
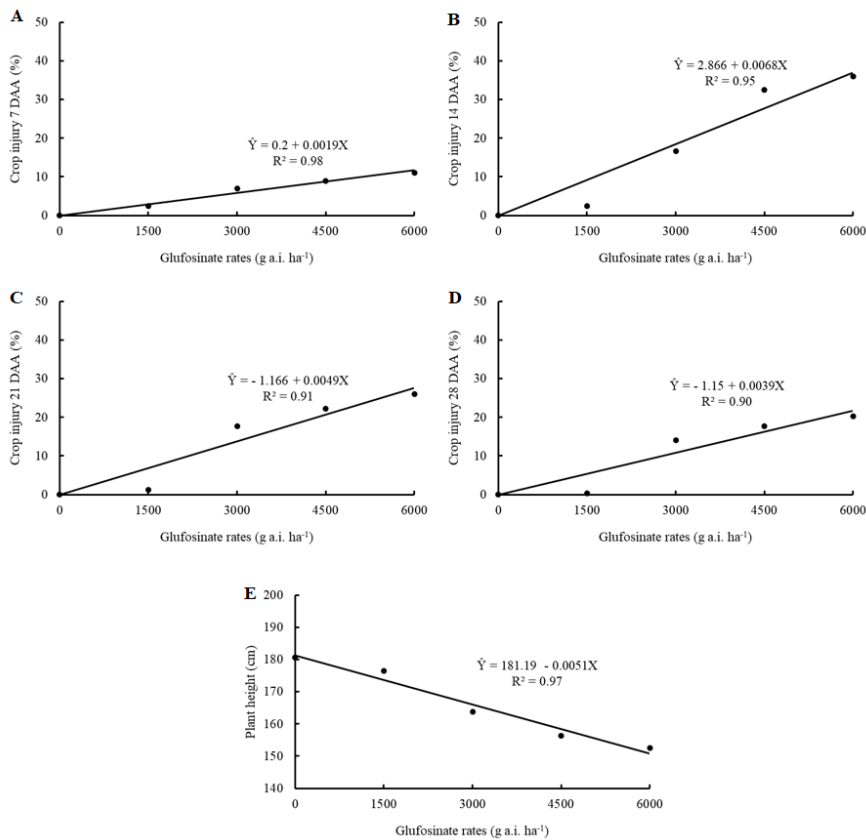

Figure 4. Crop injury at 7 (A), 14 (B), 21 (C) and 28 (D) DAA and plant height (E) of maize plants, under glufosinate rates application at post-emergence. Palotina, PR, Brazil, 2016/17 (Experiment 2, 2nd growing season).

thoroughly analyzed, the relationship that glyphosate could have with these side effects, it is noticeable that in unmodified plants the herbicide leads to the reduction of aromatic amino acid biosynthesis among them tryptophan (Moreira et al., 2007). Tryptophan is a known precursor of indolylacetic acid, a plant growth promoting auxin (Taiz & Zeiger, 2010). By analyzing the mechanism of action of glyphosate and how it acts, without taking into account herbicide insensitivity, it can be assumed that the injury caused would lead to reduced growth, as observed for the first growing season for plant height, however, the same result was not observed in the second harvest.

Regarding the nutrient content of the grains for glyphosate application, no changes were verified, which corroborate Reddy et al. (2018) for the application of glyphosate at the rate 870 g ae ha^{-1}. Correia & Santos (2013) also observed no effects of glyphosate (1,200 g ae ha^{-1}) on leaf contents of N, P, K, Ca, Mg, S, B, Mn and Zn maize plants. Again, in these studies, the rates used of glyphosate are lower than those used in the present study, showing the high tolerance of transgenic genotypes to this herbicide.

Ganie & Jhala (2017) observed crop injury between 2 and 12% for glufosinate application (590 g ae ha^{-1}) in maize (transformed *pat*) in different chemical management programs. However, they did not observe reductions in maize yield, as verified in the present study. As well as Krenchinski et al. (2018a) did not register reductions in chlorophyll indices, height, diameter and yield of maize for glufosinate application (500 g ai ha^{-1}). Glufosinate selectivity was also found for maize (transformed *pat*) at rate 500 g ai ha^{-1} (Silva et al., 2017). Costa et al. (2018) found no negative effects of glufosinate (300 + 300 g a.i. ha^{-1}) on N, P, K, Ca, Mg, S, Mn, Fe, Cu, and Zn contents and maize yield (transformed *pat*). Such information corroborates the results of the present study.

In the present study, crop injury of up to 27.0% and reductions in height were observed, however, without reductions in yield for glufosinate application. It is noteworthy that the rates (6,000 g ai ha^{-1}) were higher than those used in the abovementioned studies, and above the maximum recommended rate in the package insert (600 g ai ha^{-1}) (Rodrigues & Almeida, 2018). As for glyphosate, also for glufosinate, rates above the recommended should not be used; however, it is practical that occurs in some situations. The results of the present study indicate some undesirable effects when high rates are applied, characterizing the potential for risk.

The *pat* gene encodes the phosphinothricin N-acetyltransferase (PAT) enzyme; this enzyme inactivates glufosinate through acetylation reactions (Murakami et al., 1986), so that the herbicide does not reach its site of action, forming non-toxic compounds, metabolizable by the plant. As the rate used is much higher than usual, it is assumed that the reaction of transformation of glufosinate into non-toxic by-products is not so rapid as to avoid all symptoms, aggravated by its elevation, thus explaining the injury observed even with the presence of the enzyme.

Insect-resistant maize (Bt11 and TC1507 events) also has tolerance to glufosinate since the *pat* gene was used as a marker in its selection process. For example, Agrisure® Duracade™ 5222, Agrisure® Viptera™ 2100 (Viptera™ 2), Agrisure® Viptera™ 3100, Agrisure® Viptera™ 3110 (Viptera™ 3), Agrisure® Viptera™ 3111 (Viptera™ 4), Agrisure™ CB/LL (TL), SmartStax™, Herculex™ I, Leptra®,

Optimum™ Intrasect, Power Core™, among other combinations of these insect-resistant transgenic technologies are tolerant to glufosinate because of the *pat* gene in the selection process (Silva et al., 2017; Krenchinski et al., 2018b).

According to Krenchinski et al. (2018b), *pat* gene expression is proportional to the level of tolerance to glufosinate. *Pat* gene expression was as follows Leptra > Power Core > Optimum Intrasect >> Herculex I > Agrisure TL = Viptera 3. Therefore, there may be differences in selectivity for application of glufosinate in maize (transformed *pat*).

As already mentioned, glufosinate influences the metabolic pathway of nitrogen absorption; this nutrient in turn is constituent of amino acids, coenzymes, alkaloids, nucleotides, chlorophyll and others. Its availability is almost always a limiting factor, directly influencing plant growth more than any other nutrient (Bredemeier & Mundstock, 2000). This helps to explain, for example, the decrease in height of maize plants with increasing rates of glufosinate, which may have transiently affected the N balance in the plant, and thus its growth.

Importantly, any stress can have a negative effect on the normal growth and development of the plant (Taiz & Zeiger, 2010). However, according to Morô & Damião Filho (1999), even presenting strong crop injury due to the use of an herbicide, production can still be higher because of weed control. The response of the plant to herbicide application may or may not result in injury, which in turn is characteristic of the species and of the product in question. The effect is a result of complex interactions between the plant, herbicide and the environment.

The results show that there was no reduction in yield and 100-grain mass, as well as changes in nutrient contents, even with the use of high rates of glyphosate and glufosinate. This is explained by the rapid recovery of maize hybrids under favorable developmental conditions. The plants were able to recover from stress even at lower height and develop reproductive structure normally. Although not having impacts on yield, it is noteworthy that the use of high rates (out of the range recommended) of chemical pesticides can generate both economic and environmental losses.

Materials and methods

Design and experimental conditions

The experiments were carried out in the field, in Palotina, State of Paraná (PR), Brazil. In experimental area with very clayey soil, whose chemical and physical analysis showed; P: 10.00 mg dm^{-3}; C: 14.11 g dm^{-3}; K: 0.22 cmol$_c$ dm^{-3}; Mg: 1.04 cmolc dm^{-3}; H + Al: cmol$_c$ dm^{-3}; cation exchange capacity (CEC): 10.98 cmol$_c$ dm^{-3}; Al: 0.10 cmol$_c$ dm^{-3}; sum of bases (SB): 5.22 cmol$_c$ dm^{-3}; Zn: 5.13 mg dm^{-3}; Fe: 24.78 mg dm^{-3}; Mn: 22.98 mg dm^{-3}; Cu: 9.25 mg dm^{-3}; pH (CaCl$_2$): 4.64; V: 47.54%; sand: 16.25%, silt: 15.00%; clay: 68.75%. The climate of the region is Cfa according to the Köppen classification. Rainfall and temperature data during the experimental period are illustrated in Supplementary Figure. Experiment 1 consisted of the application of glyphosate rates (0; 2,160; 4,320; 6,480 and 8,640 g ae ha^{-1}) (Roundup Ready®, 480 g ae L^{-1}, Monsanto do Brasil Ltda, Brazil). Experiment 2 consisted of the application of the rates (0; 1,500; 3,000; 4,500 and 6,000 g ai ha^{-1}) of glufosinate (Finale®, 200 g ai L^{-1}, Bayer S.A., Brazil). The experimental

design used was randomized blocks with four replications. Both were conducted in the first growing season (24º344'539''S 53º858'537''W) and the second growing season (24º346'748''S 53º861'839''W) of 2016/17. In the first growing season, it was used the hybrid 30F53 VYHR (Leptra® RR2 technology), sown on October 24, 2016 and harvested on March 09, 2017. For the second growing season, the 2B10 PW (Power Core™ technology) hybrid was sown on February 02, 2017 and harvested on July 02, 2017. Both hybrids have tolerance to glyphosate and glufosinate, conferred by the *cp4epsps* and *pat* genes, respectively.

Herbicide applications were performed post-emergence, at V4 stage of maize plants, via a CO_2 constant pressure backpack sprayer, equipped with a bar with four XR 110.015 fan-type nozzles and pressure of 2.5 kgf cm^{-2}, with a volume corresponding to 150 L ha^{-1}. For the first growing season, the applications of both experiments occurred on November 18, 2016, under the conditions of T: 26.3ºC; RH: 54.0% and wind speed: 5.5 km h^{-1}. While for the 2nd crop, on March 11, 2017, under T: 30.0ºC; RH: 59.9% and wind speed: 2.8 km h^{-1}.

Fertilization was carried out with 300 kg ha^{-1} of the formulated N-P-K (10-15-15), and the plots were kept free from weed interference by hand weeding. 0.45 m row spacing was used, with 3 plants m^{-1}. The experimental units consisted of plots of six rows and 4 m in length.

Evaluations and data collection

For all evaluations, the four central rows were considered, discarding the first and last meters, which was considered the useful area. The crop injury was evaluated at 7, 14, 21 and 28 days after application (DAA), through a visual scale from 0 to 100%, where 0 represented the absence of symptoms and 100% the plant death (Velini et al., 1995).

Variables related to agronomic performance were evaluated: plant height (height from the ground to the last leaf just below the tassel), stem diameter (2nd internode above the ground level, smallest diameter section), yield and 100-grain mass. For height and stem diameter, 10 plants were evaluated per plot.

The plants of the useful area of each plot were threshed in an experimental grain thresher. Afterwards the yield was determined, with the values extrapolated to kg ha^{-1}, and 100-grain mass. For both variables, the grain moisture was corrected to 13%, and for 100-grain mass, two repetitions were measured per plot.

For the second growing season, it was also evaluated the content of the nutrients P, K, S, Fe, Cu and Zn for harvested grains, using an X-ray fluorescence equipment; samples were sieved through a 100-mesh sieve and analyzed in triplicate under vacuum.

Statistical analysis

Data were analyzed according to Pimentel-Gomes and Garcia (2002). They were subjected to regression analysis ($P < 0.05$), with the aid of the software Sisvar 5.6 (Ferreira, 2011). In the regression analysis, the best fit model was selected according to the criteria: biological explanation, significant regression, non-significant regression deviations and coefficient of determination.

Conclusions

The application of glyphosate (up to 8,640 g ae ha^{-1}) or glufosinate (up to 6,000 ai ha^{-1}) did not reduce yield and 100-grain mass, nor did it influence the nutrient content in the grain of maize bearing *cp4epsps* and *pat* genes. This shows the high selectivity of these herbicides.

Nonetheless, crop injury and reduction in height were observed in maize plants for herbicide application. This indicates the risks of using herbicides above the rates recommended in the package insert, despite the absence of deleterious effects on yield.

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