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Effect of herbicides on the activity of antioxidant enzymes and ALA-D in transgenic hybrid corn

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Abstract

A corn cultivar with resistance to 2,4-D, glyphosate, ammonium-glufosinate, and haloxyfop-R has been developed. However, it is known that the application of herbicides generally induces the accumulation of reactive oxygen species (ROS), which can cause oxidative damage to plants. In this study, we applied these herbicides in Enlist™ corn hybrids, in the doses recommended for weed control of herbicides alone and in mixtures, being 0, 670, 1080 400, and 60 g ha^{-1} a.e. of glyphosate, 2,4-D, ammonium-glufosinate, and haloxyfop-R, respectively. The experiment was conducted in a greenhouse at *Universidade Federal da Fronteira Sul* (UFFS), Erechim, RS, Brazil. The plants were grown in a soil and substrate mix (proportion of 1:1), in plastic vases (0.5 L). The design was completely randomized, with four replicates and two plants per replicate. The herbicides were sprayed 22 days after corn emergence (V2 stage). At seven days after the application, all plants were collected and macerated, then maintained in an ultra-freezer at -80 ºC until the biochemical analysis could be performed. The activity of antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), ALA-D and the lipid peroxidation levels were evaluated to know the effects of herbicide application in these plants. The herbicides, especially the mixtures, increased the activity of the antioxidant enzymes that decompose H_2O_2, such as CAT and APX, and also caused an inhibition of the activity of GPX and ALA-D while promoting lipid peroxidation. This may be associated with the increased generation of H_2O_2, which was added to the production of other ROS, causing an overload of the antioxidant defense system of transgenic corn, resulting in damage to plant lipids.

Keywords: *Zea mays* L, Enlist™, oxidative stress, antioxidant defense, ALA-D.

Introduction

In pursuit of a strategy to manage weed problems, Corteva Agrosciences™ developed a hybrid of transgenic corn resistant to 2,4-D, glyphosate, ammonium-glufosinate, and haloxyfop-R. Resistance was achieved by the insertion of bacterial genes into corn DNA (Wright et al., 2010; USDA-APHIS, 2013).

In order to exhibit resistance to 2,4-D herbicide, which is classified as a synthetic auxin, but at high concentrations hinders normal plant growth, which lead to lethal damages to the plant (Grossmann, 2010), by generating leaf epinasty, stem twisting and terminal bud thickening (Silva et al., 2007), the genetically modified plant had the transgene aryloxyalkanoate dioxygenase (aad-1) from the soil bacterium *Sphingobium herbicidorovans* inserted into its DNA. This bacterium gene allows the plant to expresses enzymes which can degrade the herbicide into 2,4-dichlorophenol (2,4-DCP), a compound that does not exhibit herbicidal activity (Wright et al., 2010). Besides, aad-1 provides resistance to herbicides of the aryloxyphenoxypropionate (AOPP) chemical group, more specifically haloxyfop-R and quizalofop-p-ethyl. These herbicides act by inhibiting the Acetyl-CoA carboxylase (ACCase) enzyme in grasses, which catalyzes the first step of fatty acid biosynthesis (Wenger and Niderman, 2007), thereby interrupting the formation of lipids and cell membranes (Vidal and Merotto Jr, 2001; Roman et al., 2007). Aad-1 can catalyze the degradation of these herbicides to their inactive phenol equivalents (Wright et al., 2010), thus conferring resistance to these herbicides. This event also contains the gene from *Agrobacterium tumefaciens* which encodes the CP4-5-enolpyruvyl-shikimate-3-phosphate synthase (CP4 EPSPs), an enzyme that confers resistance to glyphosate (Wright et al., 2010). This herbicide inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) enzyme of the shikimate pathway, which catalyzes the reaction of shikimate-3-

phosphate (S3P) and phosphoenolpyruvate to form 5-enolpyruvylshikimate-3-phosphate (EPSP) (Maeda and Dudareva, 2012). The inhibition of EPSPs specifically results in the accumulation of shikimate (Singh and Shaner, 1998), which ultimately inhibits the biosynthesis of aromatic amino acids (tyrosine, tryptophan, and phenylalanine) and is lethal to sensitive plants (Sammons and Gaines, 2014). The ammonium-glufosinate is a contact herbicide that translocates within the plant over a short distance (Carvalho, 2013). This technology also confers resistance to ammonium-glufosinate by inhibiting the action of glutamine synthesis, which assists in the production of glutamine and the detoxification of ammonia in animals and plants (Jewell and Buffin, 2001). This inhibition leads to the accumulation of ammonia, the inhibition of the synthesis of amino acid precursor proteins, the production of free radicals, and a disruption in photosynthesis, leading to plant death (Carvalho, 2013).

Plants are continually exposed to various stress factors, which affect their production. These environmental stresses generally induce the accumulation of reactive oxygen species (ROS), which can cause severe oxidative damage to plants, such as the inhibition of growth, development, and decreased grain yield (Bailey-Serres and Mittler, 2006). Among these factors, salinity, dry matter, metals, high temperatures, nutrient deficiency, pollution and especially herbicides (Gill and Tutteja, 2010) can lead to several effects on plant physiology, promoting changes in the antioxidant system in organisms and sensitive plants (Alves et al., 2018).

ROS are produced naturally through cellular metabolism in various metabolic pathways located in chloroplasts, mitochondria, and peroxisomes. About 1–2% of the O_2 consumed by plants is diverted to the formation of ROS (Gill and Tutteja, 2010). However, when in excess, they end up triggering damage to proteins and nucleic acids, as well as the peroxidation of membrane lipids, which can lead to the death of the cells (Miller et al., 2010). Among the ROS are singlet oxygen ($^1O_2$), superoxide radical ($^{\bullet}O2^-$), hydroxyl ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) (Sharma et al., 2012). To eliminate the damage caused by ROS, plants have developed protection mechanisms: the enzymatic and the non-enzymatic (low molecular weight antioxidants) antioxidant systems (Mittler, 2002).

The enzymatic system consists of antioxidant enzymes, among which are superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and guaiacol peroxidase (GPX, EC 1.11.1.7) (Deuner et al., 2008; Barbosa et al., 2014). APX and CAT have the role of converting H_2O_2 into H_2O and O_2 (Silva et al., 2016). The GPX also acts on the removal of H_2O_2 (Moller et al., 2007).

In addition to the antioxidant enzyme system, there is an enzyme that is considered a biomarker for stress generation: delta-aminolevulinate dehydratase x (δ-ALA-D EC4.2.1.24); this does not act in the antioxidant defense system, but is affected in situations of oxidative stress (Folmer et al., 2003). It is very important due to its participation in several main processes in the plant, such as photosynthesis and respiration (Tanaka and Tanaka, 2007). This enzyme catalyzes the asymmetric condensation of two δ-aminolevulinic acid molecules to porphobilinogen, (Pereira et al., 2006), which promotes the formation of compounds such as porphyrins, heme, and chlorophylls (Jaffe et al., 2000). Some studies have already investigated its effects on photosynthesis and the generation of oxidative stress in plants exposed to the herbicides employed in this study (Moldes et al., 2008; Ahn, 2008; Gomes and Juneau, 2016; Gomes et al., 2016; Yeo, et al., 2018). The objective of this study was to evaluate whether the herbicide application negatively affects the activity of antioxidant enzymes and ALA-D in corn hybrid resistant to the herbicides glyphosate, 2,4-D, ammonium-glufosinate, and haloxyfop-R.

Results

Effects on lipid peroxidation after spraying the herbicides

In the present work, an increase in lipid peroxidation levels was observed in the Enlist™ corn seven days after the application of herbicide treatments. The use of glyphosate alone created around 41.37% of lipid peroxidation, and the mixtures glyphosate + 2,4-D (50.61%), 2,4-D + ammonium-glufosinate (45.83%), glyphosate + 2,4-D + ammonium-glufosinate (51.61%), glyphosate + 2-glyphosate + 4-D + haloxyfop-R (36.46%), ammonium-glufosinate + haloxyfop-(46.68%), and glyphosate + ammonium-glufosinate + haloxyfop-R (48.60%) also increased lipid peroxidation compared to the control, showing increased oxidative stress (Figure 2).

Effects on enzymes activities after spraying the herbicides

The activity of the catalase enzyme (Figure 3) was increased by 157.80% and 173.39% compared to the control when the plant was exposed to haloxyfop-R alone and to the ammonium-glufosinate + haloxyfop-R mixture, respectively. The were no significant increases in the other treatments when compared to the control.

The ammonium-glufosinate and haloxyfop-R herbicides activated the enzyme in relation to the control, by 183.92% and 127.33%, respectively (Figure 4). Among the herbicide combinations, mixtures of glyphosate + 2,4-D (173.52%), glyphosate + haloxyfop-R (111.18%), 2,4-D + ammonium-glufosinate (317.62%), ammonium-glufosinate + haloxyfop-R (189.98%), and glyphosate + ammonium-glufosinate + haloxyfop-R (145.18%) (Figure 4) showed increased enzyme activity when compared to the control.

Another enzyme that was affected by the treatments was guaiacol peroxidase (GPX). This enzyme had its activity inhibited in relation to the control only when some mixtures were applied and it was not impaired when herbicides were sprayed alone. The glyphosate + ammonium-glufosinate and 2,4-D + haloxyfop-R mixtures reduced about 48.57% and 50.86% of its activity compared to the control, respectively. The lowest GPX activity occurred when mixing glyphosate + 2,4-D + ammonium-glufosinate (75.38%), glyphosate + 2,4-D + haloxyfop-R, (92.51%), glyphosate + ammonium-glufosinate + haloxyfop-R (75.59%), 2,4-D + ammonium-glufosinate + haloxyfop-R (87.93%) and glyphosate + 2,4-D + ammonium-glufosinate + haloxyfop-R (91. 92%) compared to the control (Figure 5). This indicates that mixtures involving the association of a greater number of mechanisms of action were more damaging than herbicides applied alone.

The activity of ALA-D was reduced by the herbicide treatments when compared to the control with no herbicide spraying. Treatments with glyphosate, 2,4-D, ammonium-glufosinate, and haloxyfop-R inhibited the enzyme in comparison to the control by about 52.7%, 59.82%, 67.63%,

and 66.52%, respectively. A similar inhibition occurred for the herbicide mixtures compared to the control: glyphosate + 2,4-D (67.91%), glyphosate + ammonium-glufosinate (60.66%), glyphosate + haloxyfop-R (64.84%), 2,4-D + haloxyfop-R (50.06%), ammonio-glufosinate + haloxyfop-R (66.33%), and glyphosate + 2,4-D + ammonium-glufosinate (64.56%).

Discussion

In normal conditions, the production of ROS is limited, and these are efficiently removed by the plant's antioxidant defense system, preventing oxidative damage to cellular compounds (Gill and Tutteja, 2010). However, it is known that when ROS production increases in a stress situation, such as herbicide application, in addition to acting as a signal in the activation of stress response pathways and defense mechanisms, they can pose a threat to the plant (Mittler, 2002), and may have several consequences such as damages to plant membranes.

In the present study, among the herbicides that were applied alone, glyphosate was the only one that promoted a significant increase in lipid peroxidation (Figure 2). Indeed, 2,4-D, ammonium-glufosinate, and haloxyfop-R did not induce any increase in peroxidation levels when applied individually (Figure 2). Oxidative stress caused by the increase in ROS contents after the applications of these three herbicides was not sufficient to cause the degradation of cell membranes, or this was due to the efficiency of the antioxidant system of the plants, thereby preventing damage to the cells (Ekmekci and Terzioglu, 2005).

The treatments with mixtures and membrane damage were the associations of 2,4-D + glyphosate, 2,4-D + ammonium-glufosinate, 2,4-D + haloxyfop-R, ammonium-glufosinate + haloxyfop-R, and glyphosate + 2,4-D + ammonium-glufosinate, glyphosate + 2,4-D + haloxyfop-R and glyphosate + ammonium-glufosinate + haloxyfop-R, which caused the highest peroxidation values (Figure 2).

Overall, the results demonstrated that exposure to combinations of these herbicides directly affected the antioxidant defense system, leading to lipid peroxidation. The increase in lipid peroxidation in plants after treatments with these herbicides is found in some studies in which the products were applied in isolation. In the study by Gomes et al. (2016), the authors observed an increase in lipid peroxidation and the greater accumulation of H_2O_2 after plants were treated with glyphosate. This also was observed after the application of 2,4-D in wheat (Agostinetto et al., 2016). Also, the ammonium-glufosinate induced a significant increase in lipid peroxidation (Yeo et al., 2018), which was also observed after the treatment of *Acanthospermum hispidum* with the herbicide fluazifop-P-buthyl, from the same group as haloxyfop –R (Luo et al., 2004).

The enzyme CAT goes into action to disrupt H_2O_2 generated by SOD in H_2O and O_2 to decrease its cytotoxic effects in the plant (Shehab et al., 2010), acting without the need for an electron donor (Apel and Hirt, 2004), making it one of the most effective antioxidant enzymes (Akcay et al., 2010).

The present study demonstrated that catalase activity increased in comparison to control when corn was exposed to the herbicide haloxyfop-R and also the ammonium-glufosinate (ammonium-glufosinate + haloxyfop-R) mixture (Figure 3). The herbicides of the chemical group of aryloxyphenoxypropionates, such as haloxyfop-R, in addition to their mechanism of action, have the power to generate ROS, which can lead susceptible plants to develop oxidative stress (Luo et al., 2004). The results obtained in this work show that haloxyfop-R could affect the studied corn cultivar, generating the accumulation of reactive species, even though it was resistant. The same happened with the mixture of haloxyfop-R with ammonium-glufosinate, which shows that the association of these two herbicides with different mechanisms of action intensified the generation of ROS, and reached critical levels since CAT has a greater capacity for the removal of H_2O_2 compared to PODs such as APX (Gill and Tuteja, 2010; Sharma et al., 2012). Although transgenic plants contain the genes that detoxify these herbicides, the above result implies that they induce phytotoxicity in Enlist™ corn and demonstrate that CAT was stress-induced and participated in the antioxidant mechanism and plant cell protection.

The induction of phytotoxicity in transgenic cultivars by herbicides was also observed by Ahn (2008) in her study with an ammonium-glufosinate-resistant rice cultivar (*Oryza sativa*), where the herbicide triggered an accumulation of H_2O_2, showing that even when the plant was resistant there was a situation of toxicity caused by the herbicide. The herbicides ammonium-glufosinate and haloxyfop-R when applied alone and in combination, increased APX activity (Figure 3), the enzyme that is responsible for the conversion of H_2O_2 to water and O_2, using ascorbate as an electron donor. APX and CAT act together to combat the high accumulation of H_2O_2, the two enzymes have different affinities over H_2O_2: APX performs fine regulation, while CAT removes any excess from that ROS (Mittler, 2002; Gill and Tuteja, 2010).

Regarding the mixtures of haloxyfop-R + ammonium-glufosinate + glyphosate and haloxyfop-R + glyphosate and 2,4-D + glyphosate, there was an increase in APX (Figure 4). The increase in this activity seems to be related to the increased tolerance to oxidative stress and shows that the association of these herbicides with glyphosate, which already has potential in the generation of reactive species, has helped in the accumulation of ROS. Glyphosate has been reported in several studies to induce the accumulation of ROS, although this herbicide does not have oxidative stress as its main action (Moldes et al., 2008; Gomes et al., 2016). Besides, AMPA (aminomethylphosphonic acid), the main metabolite of glyphosate degradation, as well as the inert ingredients of the commercial product, are at risk of being even more toxic than when alone (Giesy et al., 2000; Tsui and Chu, 2003; Gomes et al., 2016). In some plant species, glyphosate is metabolized, while in others it is detected as an intact molecule. These differences are due to several reasons since it depends on several factors such as the rate of absorption, genotypes, and environmental conditions.

It can be seen that the mixture of glyphosate with 2,4-D also promoted an APX increase, proving that the herbicide 2,4-D also has the power to promote the accumulation of H_2O_2 (Figure 4), showing that the two herbicides together intensified the generation of reactive species.

Some studies show that ROS can participate in cell wall changes, allowing cellular elongation, which is necessary for 2,4-D, demonstrating that reactive oxygen species play a central role in the toxicity of 2,4-D in susceptible plants (Pazmiño, 2011). In the present study, it can be observed that even without exerting its herbicidal role, the 2,4-D associated with other products can cause an increase of ROS in the plants. One hypothesis would be based on the

Table I: Treatments and doses of herbicides applied on Enlist ™ corn hybrid (13K288 PWE). UFFS, Erechim/RS, 2018.

Treatments	Dose (g ha^{-1}) of (a.i. or a.e.)*	Commercial Name	Commercial Dose (L ha^{-1})*
Control without herbicide	---	---	---
Glyphosate	1080	Roundup®Original	3,0
2,4-D	670	DMA® 806 BR	1,0
Ammonium-glufosinate	400	Finale®	2,0
Haloxyfop-R	60	Verdict®	0,5
Glyphosate + 2,4-D	1080+670	Roundup® Original+ DMA® 806 BR	3,0+1,0
Glyphosate+ammonium-glufosinate	1080+400	Roundup®Original+ Finale®	3,0+2,0
Glyphosate + haloxyfop-R	1080+60	Roundup®Original+ Verdict®	3,0+0,5
2,4-D + ammonium – glufosinate	670+400	DMA® 806 BR +Finale®	1,0+2,0
2,4-D +haloxyfop-R	670+60	DMA® 806 BR +Verdict®	1,0+0,5
ammonium -glufosinate+ haloxyfop-R	400+60	Finale® +Verdict®	2,0+0,5
Glyphosate+2,4-D+ ammonium -glufosinate	1080+670+ 400	Roundup®Original+ DMA® 806 BR +Finale®	3,0+1,0+2,0
Glyphosate+2,4-D+ haloxyfop-R	1080+670 +60	Roundup®Original+ DMA® 806 BR + Verdict®	3,0+1,0+0,5
Glyphosate + ammonium -glufosinate + haloxyfop-R	1080+400 +60	Roundup®Original +Finale® +Verdict®	3,0+2,0+0,5
2,4-D + ammonium - glufosinate +haloxyfop-R	670+400 +60	DMA® 806 BR +Finale® + Verdict®	1,0+2,0+0,5
Glyphosate+2,4-D+ ammonium -glufosinate +haloxyfop-R	1080+670 +400+60	Roundup®Original + DMA® 806 BR+ Finale® + Verdict®	3,0+1,0+2,0+ 0,5

* Recommended dose by the manufacturer for weed control.

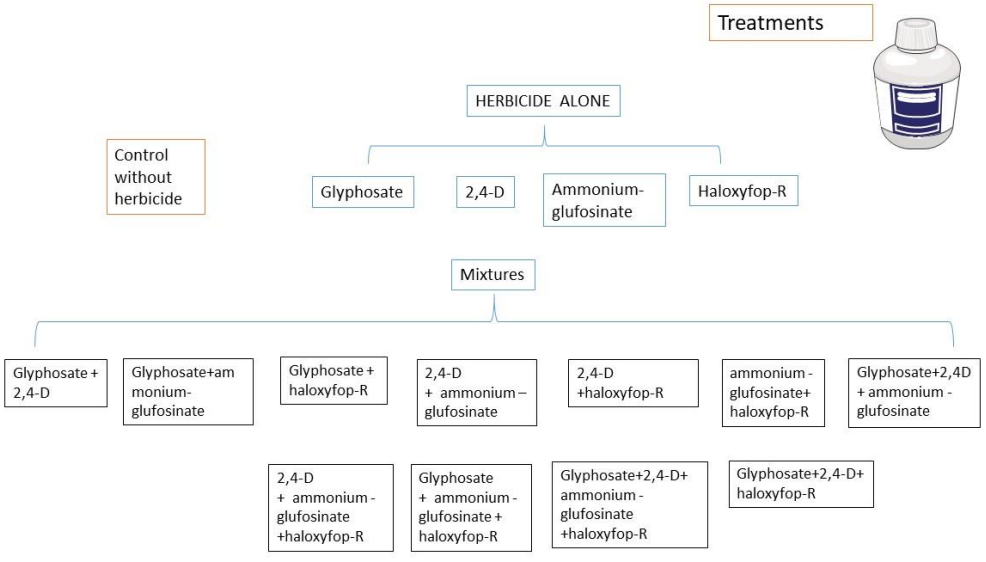

Figure 1. Treatments of herbicides sprayed on the Enlist ™ corn hybrid (13K288 PWE). UFFS, Erechim/RS, 2018.

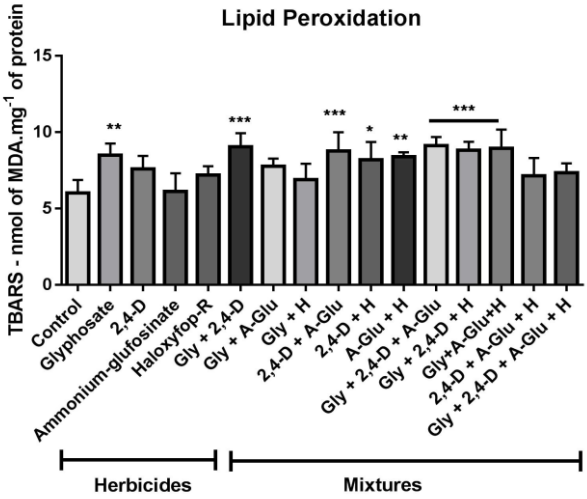

Figure 2. Effect of herbicides after seven days of application on lipid peroxidation of Enlist™ corn hybrid. * Gly: glyphosate; A-Glu: ammonium-glufosinate; H: haloxyfop-R. Data are expressed as nmol of MDA mg^{-1} of protein. Asterisks indicate statistical significance when treatments differ by Dunnet test compared to the control without herbicide: p <0.05 (*); p <0.005 (**); p <0.001 (***) and p <0.0001 (****). The bars represent the standard error of the means of four replicates.

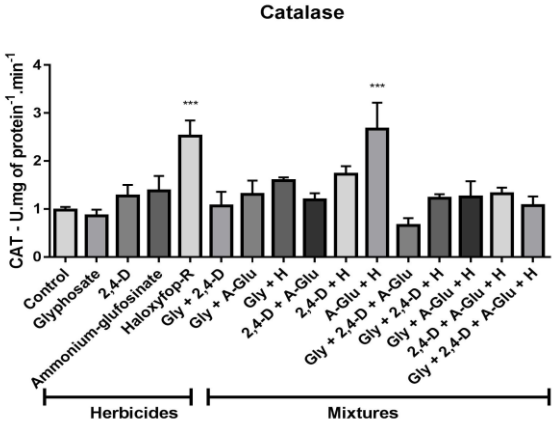

Figure 3. Effect of herbicides after seven days of application on catalase of Enlist™ corn. Gly: glyphosate; A-Glu: ammonium-glufosinate; H: haloxyfop-R. Data are expressed as U.mg of $protein^{-1}.min^{-1}$. Asterisks indicate statistical significance when treatments differ by the Dunnet test compared to the control without herbicide: p <0.05 (*); p <0.005 (**); p <0.001 (***) and p <0.0001 (****). The bars represent the standard error of the means of four replicates.

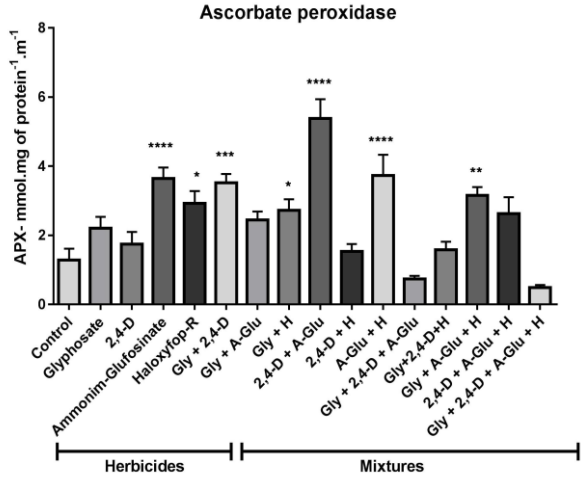

Figure 4- Effect of herbicides after seven days of application on ascorbate peroxidase of Enlist™ corn hybrid . Gly: glyphosate; A-Glu: ammonium-glufosinate; H: haloxyfop-R. Data are expressed as nmol mg of $protein^{-1}$ min^{-1}. Asterisks indicate statistical significance when treatments differ by the Dunnet test compared to the control without herbicide: p <0.05 (*); p <0.005 (**); p <0.001 (***) and p <0.0001 (****). The bars represent the standard error of the means of four replicates.

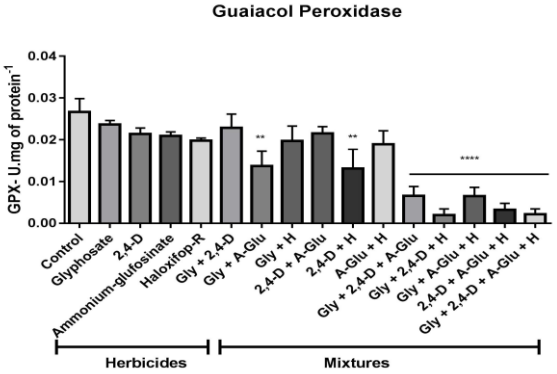

Figure 5. Effect of herbicides after seven days of application on guaiacol peroxidase of Enlist^{TM} corn hybrid. Gly: glyphosate; A-Glu: ammonium-glufosinate; H: haloxyfop-R. Data are expressed as U mg of protein^{-1}. Asterisks indicate statistical significance when treatments differ by the Dunnet test compared to the control without herbicide: p <0.05 (*); p <0.005 (**); p <0.001 (***) and p <0.0001 (****). The bars represent the standard error of the means of four replicates

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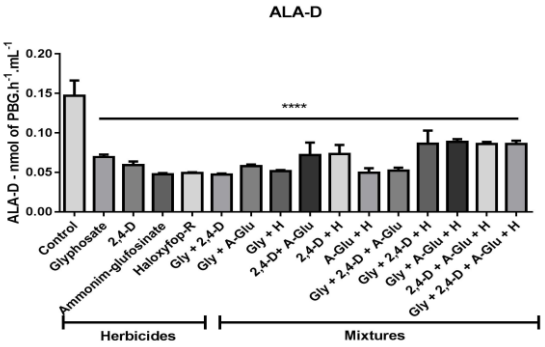

Figure 6. Effect of herbicides after seven days of application on δ acid aminolevulinate dehydratase of Enlist^{TM} corn hybrid. Gly: glyphosate; A-Glu: ammonium-glufosinate; H: haloxyfop-R. Data are expressed as nmol of PBG $h^{-1}mL^{-1}$. Asterisks indicate statistical significance when treatments differ by Dunnet test compared to control without herbicide: p <0.05 (*); p <0.005 (**); p <0.001 (***) and p <0.0001 (****). The bars represent the standard error of the means of four replicates.

performance of their respective metabolites in the plant as 2,4-DCP.

Among the antioxidant enzymes already seen in the study, the enzyme GPX is one containing the heme group that also acts in the detoxification of excess H_2O_2 (Das and Roychoudhury, 2014). Its activity in this work, there was a general decrease when comparing to the control when some mixtures were applied. Among those that caused the greatest inhibitions are those that involved the mixtures of glyphosate + 2,4-D + ammonium-glufosinate + haloxyfop-R and glyphosate + 2,4-D + ammonium-glufosinate; glyphosate + 2,4-D + haloxyfop-R and 2,4-D + ammonium-glufosinate + haloxyfop-R). In addition to those mentioned, 2,4-D + haloxyfop-R and glyphosate + ammonium-glufosinate also caused an inhibition of the enzyme, but it was less significant in comparison to the control (Figure 4). This result shows that when the activity of these enzymes decreases, as in the exposure to the mixtures, there may be some accumulation of ROS.

Inhibition of GPX in these treatments may impair cell wall synthesis in the plant and may cause a reduction of the same after the application of these treatments.

Oxidative stress may negatively modulate δ-ALA-D enzyme activity (Salgueiro et al., 2016), which is a hypothesis for the results of the present study that demonstrated that all treatments with herbicides, both alone or in mixtures, caused an inhibition of the enzyme when compared to the control (Figure 5). Besides, the inhibition of ALA-D may further exacerbate ROS production by increasing the levels of its substrate, δ-ALA, which has pro-oxidant effects and contributes to a decrease in antioxidant defenses of the plant, since its reducing activity can harm the synthesis of tetrapyrrolic compounds and end up decreasing enzyme synthesis, such as CAT and peroxidases, which have these components (heme) in their structure (Jaffe et al., 1995; Neal et al., 1997). Perhaps the decrease in GPX enzyme activity may be related to this cause, as it is also formed by the heme group.

With the inhibition of ALA-D, it may be suggested that there is some effect on the synthesis of tetrapyrrolic compounds such as bilians, chlorophylls, and heme (Tanaka and Tanaka, 2007). As it is a key enzyme in the process (Tanaka and Tanaka, 2007) the present study suggests that such inhibitioncould promote a decrease in plant chlorophyll synthesis.

Moreover, the decrease in chlorophyll as an effect of the herbicides studied in this work is addressed in some studies showing that the effects of glyphosate occur in photosynthesis, such as the inhibition of carotenoid and chlorophyll biosynthesis (Fedtke and Duke, 2005). It is thus perceived that there is an indirect effect caused by them in plants. Furthermore, since the present study was carried out with a plant that is able to metabolize these herbicides or

decrease their affinity, as in the case of glyphosate, the presented effects cannot be secondary to the inhibition of their mechanisms of action.

However, the analyses carried out in this study showed that, despite the mediation of the removal of ROS by the CAT and APX enzymes after the application of treatments, there was a decrease in GPX activity, which shows that it was not sufficient to remove H_2O_2. In general, the enzymes failed to reverse the stress situation in glyphosate treatments and mixtures, which in turn caused an increase in lipid peroxidation mediated by ROS, which are generated when then plant is under herbicide toxicity. Besides, inhibition of the ALA-D enzyme shows that the herbicides negatively affected its activity. This also happened with GPX, which was also inhibited and, because it is involved in the synthesis of lignin, its inhibition may cause a decrease in the formation of cell walls in the plant. These results reinforce that the corn plant, even when resistant to herbicide treatments, will show toxicity symptoms after application.

Material and Methods

Description of experimental site and plant materials

The experiment was conducted in a greenhouse at the *Universidade Federal da Fronteira Sul* (UFFS), Erechim, RS, Brazil, from June to July 2018. Seed of corn hybrid Enlist™ 13K288 (PWE)with resistance to herbicides glyphosate, ammonium-glufosinate, 2,4-D, and haloxyfop-R were grown in a soil plus substrate mix (1:1) in plastic vases with a volume of 0.5 L. Soil characteristics were: pH (water)=5.1; organic matter=3.0%; clay=>60%, P=5.2 $mg.dm^{-3}$, K=118 $mgdm^{-3}$, Ca^{+2}=5.5 $cmol_c\ dm^{-3}$, Mg^{+2}=3.0 $cmol_c\ dm^{-3}$, Al^{+3}=0.3 $cmol_c\ dm^{-3}$, H+Al=7.7 $cmol_c\ dm^{-3}$; CEC=16.6 $cmol_c\ dm^{-3}$.

Experimental design

The design was completely randomized, with four replicates and two plants per replicate. The treatments were composed of a control treatment and herbicides and their respective recommended rates for weed control; these are presented in Figure 1 and Table 1.

The herbicides were sprayed 22 days after corn emergence (DAE), in the V2 stage (two completely developed true leaves). Seven days after herbicide application, all plants were collected and macerated using liquid nitrogen, then maintained in an ultra-freezer at -80 ºC until the biochemical analysis was performed.

Extract Preparation

The enzymatic extract for determination of the activities of catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) was obtained using a protocol previous established by Alexieva et al. (2001). For ALA-D activity determination, extract preparation follows the method described by Morsch et al. (2002). In all enzymatic determinations, protein quantification was determined by the method of Bradford (1976).

Enzymatic activity

CAT activity was determined using the method of Havir and McHale (1987) with modifications. APX activity was assessed following the method of Nakano and Asada (1981). The activity of the GPX enzyme was determined according to Zeraik et al. (2008). The activity of ALA-D was determined according to Sassa (1982) and Morsch et al. (2002). Malondialdehyde (MDA) content was measured according to the method proposed by Hodges et al. (1999).

Statistical analysis

The data were submitted to analysis of variance (ANOVA) using the GraphPad Prism 7.0 software, and, when significant, compared to the control treatment using Dunnett test ($p < 0.05$).

Conclusion

Herbicides, especially mixtures, cause the activation of catalase and ascorbate peroxidase enzymes, which are responsible for the decomposition of H_2O_2 in the corn hybrid. Furthermore, as evidenced by increasing levels of lipid peroxidation, there was damage to plant lipids. This may have been caused not only by H_2O_2 but also by other reactive oxygen species, causing an overload in the antioxidant defense system of corn plants.

Additionally, the ALA-D enzyme was inhibited in all treatments with herbicide application, indicating their possible toxicity, which could also be linked to a reduction of GPX activity. So, we demonstrated that the deleterious effects of these herbicides on the antioxidant defense of the corn plants are extremely relevant, even for the transgenic plants.

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