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Does the application of herbicides with distinct mechanisms of action change enzymatic activity and grain yield of Clearfield® canola?

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Abstract

Herbicides have a high potential for phytotoxicity and generate oxidative stress, even on tolerant crops. The canola hybrid Hyola 571 CL obtained through Clearfield® technology shows tolerance to imidazolinones. Given the importance of this hybrid, this work assessed the effect of using herbicides with different mechanisms of action on the activity of antioxidant enzymes, ALA-D, gas exchange, and grain yield of canola hybrid tolerant to imidazolinones. Treatments applied to Hyola 571 CL canola hybrid were: control without herbicides, diclosulam, imazaquim, sulfentrazone, sulfentrazone + diuron pendimenthalin, flumioxazin, imazethapyr + imazapic, imazapic + imazapyr, imazethapyr, imazamox, nicosulfuron, chlorimuron, netsulfuron-methyl, iodosulfuron, and pyroxsulam. Seven, 14, 21, and 28 days after application we assessed herbicide phytotoxicity to canola. Antioxidant enzyme systems, variables related to plant physiology, and grain yield of Hyola 571 CL were also determined. It was observed that the hybrid, even tolerant, showed sensitivity to herbicides. Pendimethalin, flumioxazin, chlorimuron-ethyl, metsulfuron-methyl, and iodosulfuron were the most harmful to Hyola 571 CL. Commercial mixtures by [imazethapyr + imazapic] and [imazapic + imazapyr], as well as imazamox, were the most selective ones. Catalase was inhibited by most treatments. Sulfentrazone promoted the greatest negative interference along with its mixture with diuron and nicosulfuron, chlorimuron, and metsulfuron-methyl. The hybrid showed sensitivity to herbicides and the differences in gas exchange rates and other analyzes for the types of treatment do not result in productivity.

Keywords: ALS; *Brassica napus* var. *oleifera*; Chemical control; Clearfield®; Oxidative stress.
Abbreviations: Al^{3+}_aluminum ion, ALA-D_δ-aminolaminated dehydratase, Al_aluminum, ALS_Acetolactate synthase APX_ascorbate peroxidase, Ca^{2+}_calcium ion, CAT_ catalase, $CEC_{(t)}$ _effective cation exchange capacity, $CEC_{(T)}$ _total cation exchange capacity, CO_2_carbon dioxide, DAT_days after treatment, H_2O_2_ hydrogen peroxide , H_hydrogen, K_potassium, MDA_ malondialdehyde , Mg^{2+}_magnesium ion, O_2_oxygen, O^{2-}_superoxide ion , OH^-_hydroxyl ion , P_phosphorus, PQ_plastoquinone, PROTOX_protoporphyrinogen IX oxidase, ROS_reactive oxygen species, SB_sum of bases, SOD_superoxide dismutase, SOM_soil organic matter, V_base saturation.

Introduction

The introduction of canola hybrids (*Brassica napus* L.) tolerant to imidazolinones obtained through Clearfield® technology, such as Hyola 571 CL, enables its cultivation in areas infested with weeds, enabling selective control in post-emergence. Canola is also important for collaborating with the rotation of winter crops and also mechanisms of action, mitigating the selection of resistant weed biotypes (Durigon et al., 2018). The canola hybrid Hyola 571CL was developed from a mutant, called PM1, obtained by microspore-induced mutagenesis. This PM1 mutant has the substitution of serine for asparagine in codon 653, providing tolerance to only one group of ALS-inhibiting herbicides, that of imidazolinones (Krato et al., 2012). However, nowadays with the emergence of weeds resistant to many herbicides, it is necessary to evaluate the possibility of applying products of the most varied mechanisms of action to canola, and spread of weed resistance ALS-inhibiting herbicides. Herbicides that inhibit the enzyme acetolactate synthase (ALS) interrupt the biosynthesis of branched-chain amino acids such as valine, leucine, and isoleucine, which can lead susceptible plants to death (Yu and Powles, 2014). The main effects resulting from the decrease in the synthesis of these amino acids is the reduction of the synthesis of proteins and DNA, and the reduction of the transport of photoassimilates in the leaves, thus having as a consequence, the decrease in plant growth, elongation and leaf chlorosis (Tan et al., 2006). The herbicides of this mechanism of action involve advantages such as low toxicity to mammals and the ability to use low doses due to its effectiveness (Endo et al., 2013). Imidazolinones are among the chemical families of ALS-

inhibiting herbicides, this mechanism is still formed by the groups of sulfonylurea, triazolopyrimidines, pyrimidinylthio-benzoates, and sulfonylamino-carbonyltriazolinones (Mallory and Retzinger, 2003).

No studies are showing which herbicides belonging to this mechanism of action can be used in the Hyola 571 CL hybrid without causing injury (Bandeira et al., 2013), mainly biochemical changes. Thus, new products must be tested for greater flexibility in controlling weeds in canola without causing phytotoxicity (Alves et al., 2018a). These herbicides have the potential to inhibit the enzyme protoporphyrinogen IX oxidase (PROTOX), necessary for the heme and chlorophyll biosynthesis in the plant (Dayan and Dayan, 2011). The inhibition of PROTOX results in the accumulation of protoporphyrin IX, which in the presence of light, generates large amounts of singlet oxygen ($1O_2$), an oxidizing agent. Peroxidation of unsaturated fatty acid bonds in the plant's cell membranes, causing loss of membrane integrity, leaf necrosis, and death (Zagar et al., 2019).

Another herbicide that can be used is the commercial mixture of sulfentrazone + diuron. Diuron, in turn, inhibits the photosynthetic reactions of plants as a mechanism of action. It competes with plastoquinone (PQ), binding at the site of the complex D1 protein in photosystem II (FSII). Thus, it inhibits electron transport between photosystems II and I. This process, inhibits the formation of NADPH and ATP, promotes the production of reactive oxygen species (ROS) that causes lipid peroxidation, leading to plant death. Pendimethalin is also interesting to be tested as this herbicide is a microtubule inhibitor. Microtubule inhibitors bind to the α-tubulin subunit reversibly and competitively, modifying the organization of microtubules (Mitra and Sept, 2006), which are important structural polymers, mediators of several processes that occur in cells through dynamic reorganization (Mitra and Sept, 2006).

When the plant is subjected to stresses such as herbicides, there may be an overproduction of ROS in several metabolic pathways, especially in chloroplasts, mitochondria, and peroxisomes (Gill and Tuteja, 2010). The antioxidant defense mechanism has the role of protecting plants against damage caused by oxidative stress. Among the main enzymes superoxide dismutase (SOD) which, together with other enzymes such as catalase (CAT), and ascorbate peroxidase (APX), promotes the alteration of ROS (Barbosa et al., 2014). The balance of these enzymes activity is essential in eliminating damaging levels of ROS in plants (Barbosa et al., 2014).

When there is an imbalance between the production and removal of ROS, there is damage to proteins and nucleic acids, in addition to the peroxidation of membrane lipids, causing cell death or resulting in damage at several magnitudes (Miller et al., 2010). Besides, other enzymatic processes act as plant defense responses to combat the damaging effects (Marchezan et al., 2017), such as a δ-aminolaminated dehydratase (ALA-D, EC4.2.1.24) which is a key enzyme in plants (Alla and Hassan, 2014; Tanaka and Tanaka, 2007), by catalyzing the condensation of two molecules of delta-aminolevulinic acid (δ-ALA), forming the monopyrrole compound porphobilinogen (PBG) (Breinig et al., 2003). Therefore, understanding how plants react in response to exposure to adverse stresses is one of the primary steps for the development of resistant crops (Marchezan et al., 2017).

Since ALA-D is essential in the process of chlorophyll synthesis, when its activity is altered drawbacks to the photosynthetic rate may be observed. Due to the importance of photosynthesis, studying stomatal functions generates a better understanding of the different regulatory mechanisms at the local and systemic levels that promote changes and modulate gas exchange (Ehonen et al., 2020).

In this way, this work aimed to evaluate the effect of herbicides with different mechanisms of action on the activity of antioxidant enzymes, ALA-D activity, gas exchange, and grain yield of canola hybrid resistance to imidazolinones.

Results

Herbicide phytotoxicity on canola

There was a significant treatment effect on the phytotoxicity of canola plants 7, 14, 21, and 28 days after treatment (DAT). Pendimethalin flumioxazin, chlorimuron-ethyl, metsulfuron-methyl, and iodosulfuron were the most harmful ones to canola, from 7 to 28 DAT (Supplementary Table 4). The commercially available mixtures of [imazethapyr+imazapic], [imazapic+imazapyr] as well as imazamox, otherwise, were the most selective ones to Hyola 571 CL.

Of all tested herbicides applied on the hybrid Hyola 571 CL, the only imazamox is officially registered for the Clearfield (CL) technology (Agrofit, 2021). However, our results report that both mixtures, [imazethapyr+imazapic] and [imazapic+imazapyr], were also selective to Hyola 571 CL. The other tested herbicides presented intermediary phytotoxicity. Herbicides with other mechanisms of action, however, could serve as important tools to manage resistance in areas of Clearfield canola and avoid further spread of weed resistance to ALS inhibitors.

Effect of herbicides application on lipid peroxidation rate

There was an increase in the levels of MDA (lipid peroxidation) compared to the control when the canola was subjected to sulfentrazone (D) (29.54%) PROTOX inhibitor and nicosulfuron (L) (47.52%), chlorimuron (M) (39.08%) and metsulfuron-methyl (N) (60.55%) from the sulfonylurea class (ALS). On the other hand, some herbicides showed lower peroxidation levels than the control, being they diclosulam (B) (5.04%), imazaquin (C) (30.59%), sulfentrazone + diuron (E) (26.68 %), pendimethalin (F) (37.09), imazamox (K) (48.61%) and iodosulfuron (O) (62.35%) which indicates that they did not promote damage to the plant membrane (Supplementary Figure 1a).

Effect of herbicides application on catalase (CAT) activity

The CAT enzyme had its activity inhibited in most treatments (Supplementary Figure 1b). Among them, we have diclosulam (B) with a decrease of about 60.53%, sulfentrazone + diuron (E) with 57.83%, pendimethalin (F) 63.65%, flumioxazin (G) 46.22%, imazethapyr + imazapic (H) 69.56%, imazapic + imazapyr (I) 93.20%, chlorimuron (M)71.90%, metsulfuron-methyl (N) 79.09%, iodosulfuron (O) 57.14% . In contrast, pyroxylin (P) was the only herbicide that increased the activity of the enzyme (45.45%). Among the treatments studied, those involving imazethapyr + imazapic (H) imazapic + imazapyr (I), chlorimuron (M), and metsulfuron-methyl (N), which, are ALS inhibitors, were the ones that showed the greatest effects of inhibition on the enzyme.

Effect of herbicides application on ascorbate peroxidase (APX) activity

The only treatments in which the APX enzyme activity was significantly increased were sulfentrazone (D) (118.69%) and

imazapic + imazapyr (I) (92.99%), respectively of the PROTOX mechanism and ALS (imidazolinones). A result different from that found in the other treatments, which did not show an increase in enzyme activity compared to the control, as can be seen in Supplementary Figure 1c.

Effect of herbicides application on δ aminolevulinate dehydratase (ALA-D) activity

Some treatments promoted inhibition of the ALA-D enzyme, among them are the herbicides: imazaquin (C) (49.84%), sulfentrazone (D) (42.90%) pendimethalin (F) (41.32%) imazethapyr + imazapic (H) (41.32%) and imazamox (K) (36.58%). As noted, the mechanisms involved in reducing the activity of this important enzyme were those of the class of imidazolinones (ALS), PROTOX, and microtubules (Supplementary Figure 1d).

Effect of herbicides application on gas exchange

Regarding the determination of gas exchange rates, the results showed that the carboxylation coefficient (EC) and the internal concentration of CO_2 (Ci) did not change significantly concerning the control (Supplementary Figures 2b e 2e). Photosynthetic activity (A) was reduced only in treatment with nicosulfuron (52.29%) (Supplementary Figure 2a). Another herbicide, pyroxulan (30.39%) caused an increase in water use efficiency (WUE), the opposite result to that found for the herbicide iodosulfuron, which caused a decrease of about 27.97% (Supplementary Figure 2d). That same herbicide promoted an increase of 25.73% in transpiration (E). The rate that E was decreased when the plant was exposed to Flumioxazin (51.77%) and chlorimuron (31.14%) (Supplementary Figure 2f). Stomatal conductance (Gs) had significantly increased values after exposure to sulfentrazone + diuron (118.69%) and nicosulfuron (162.46%) (Supplementary Figure 2c).

Effect of herbicides application on canola productivity rate

No treatment negatively affected crop grain yields of canola CL compared to the control without herbicide. The results also showed a significant increase in the values of grain production after exposure to imazapic + imazapyr (I) (63.42%), imazethapyr (J) (78.73%), and imazamox (K) (71.79 %), both herbicides of the mechanism of action to which the plant is tolerant (imidazolinones) (Supplementary Figure 3).

Discussion

This study demonstrates the effect of exposure of canola genetically modified to tolerate ALS-inhibiting herbicides, more specifically those of the chemical group of imidazolinones, Hyola 571 CL. These crop-selective herbicides were tested, some of the other groups of this mechanism of action, such as sulfonylureas and triazolopyrimidines, and still other products with different mechanisms of action: PROTOX inhibitors, FSII inhibitors, and microtubule formation inhibitors (Supplementary Table 2). It was observed that the presence of herbicides presented variable behavior depending on the mechanism of action and the chemical group of each one.

Commercial mixtures of [imazapyr+imazapic] and [imazethapyr+imazapic] present low toxicity rates on CL canola, with injuries below 2%; only imazapyr resulted in higher toxicity levels (Galon et al., (2015). And imazapyr causes higher phytotoxicity even on Clearfield plants, and this phytotoxicity is also reported for soybeans (Merotto Jr. et al., (2000).

Sulfentrazone, alachlor, metribuzin and pendimethalin applied to soybean result in remarkable carryover effect with canola planting in succession; thus, these herbicides are unsuitable for weed control in canola (Vargas et al., 2011). Spader et al., (2014) studied the effects of ALS-inhibiting herbicides on canola hybrid Hyola 571 CL, and reported that plants were not affected 10 DAT, but remarkable phytotoxicity was observed 20 DAT. Young plants are usually most susceptible to herbicides compared to older ones.

Lipid peroxidation generates malondialdehyde (MDA), which is a product of the breakdown of membrane fatty acids, as it one of the first damages as a stress-induced response on plants (Amri and Shahsavar, 2010). This biomarker is an excellent parameter in the evaluation of the structural integrity of membranes, indicating the level of damage caused to lipids by oxidation reactions (Ekmekci and Terzioglu, 2005). In our study herbicides that caused significant increases in lipid peroxidation values in canola CL, were nicosulfuron, chlorimuron, and metsulfurom-methyl (Supplementary Figure 1a), belonging to the group of sulfonylureas and sulfentrazone, a PROTOX inhibitor.

This may have occurred because of the tolerance Hyola 571 CL, happen especially to herbicides in the group of imidazolinones, because the plant has an S653N mutation that gives high selectivity to imidazolinones, but not to sulfonylureas (McCourt et al., 2006) even though they have the same mechanism of action. Accumulation of MDA was observed in wheat when exposed to metsulfuron-methyl, favoring the idea that this chemical group may cause some toxicity to plants. In the same way, sulfentrazone also showed a potential for oxidative stress (Agostinetto et al. (2016). According to Alves et al., (2018b) the increase in sulfentrazone concentration induced an increase in ROS levels and the promotion of lipid peroxidation in *R. sativus* and *L. albus*.

This result indicates that there was an induction of a state of stress, related to membrane damage, which probably occurred due to the generation of H_2O_2, and other ROS (Gill and Tuteja, 2010). This usually occurs when ROSs are generated in high concentrations, which ends up overloading the antioxidant system.

In the other treatments (Supplementary Figure 1a), it is possible that the oxidative stress caused by the increase in the levels of H_2O_2 or other ROS during the period in which the plants were exposed to the herbicides was not sufficient to cause degradation of the cell membranes, or due to the efficiency of the antioxidant system of plants, both enzymatic and non-enzymatic, which can prevent damage to cell membranes.

So, to try to reduce these effects of oxidative stress, the cells and organelles (chloroplasts, mitochondria, and peroxisomes) of the plants developed defense mechanisms, both enzymatic and non-enzymatic, that play an excellent role acting against the accumulation of ROS (Mittler, 2002). The enzyme antioxidant system is formed by antioxidant enzymes, such as catalase, ascorbate peroxidase, and guaiacol peroxidase, specialized in the removal of H_2O_2 (Barbosa et al., 2014), among other enzymes.

There was an inhibition of CAT enzyme activity in almost all herbicides tested: diclosulam, sulfentrazone, sulfentrazone + diuron, pendimethalin, flumioxazin, imazethapyr + imazapic, imazapic + imazethapyr, imazethapyr, nicosulfuron, chlorimuron, chlorimuron, chlorimuron, chlorimuron methyl, and iodosulfuron when compared to the control without herbicide. This suggests that they did not activate the defense system or that the time studied is not enough to

obtain this response (Langaro et al., 2016). Qian et al. (2011) also noted that CAT activity in *Arabidopsis thaliana* decreased by about 33.9% of the control after exposure to the herbicide imazethapyr, an ALS inhibitor. The decrease in enzyme activity is believed to be probably due to sensitivity to the concentration of the herbicide. Therefore, it is notable that CAT was very sensitive to the increase in the dose of isoproturon. An adjustment in the production of ROS that modulates the activity of antioxidant enzymes is prone to affect the redox state, causing oxidative stress. Results show that the only herbicide that increased catalase activity was pyroxulam.

The opposite happened with APX, where a greater activity of the enzyme in plants treated with sulfentrazone and with the combination of imazapic+imazapyr was verified (Supplementary Figure 1c). This result may be associated with a greater accumulation of H_2O_2, due to the application of these herbicides. APX is also involved in the elimination of H_2O_2 using ascorbate as an electron donor. Furthermore, APX has a greater affinity for H_2O_2 when compared to CAT, therefore, even in smaller quantities, H_2O_2 can activate APX to convert this radical to H_2O stress (Gill and Tuteja, 2010). This may be related to the fact that the APX enzyme has been activated in these treatments and CAT has suffered inhibition of its activity.

As mentioned, the sulfentrazone herbicide that is part of the PROTOX inhibitory mechanism of action caused an increase in APX in CL canola. Another study also found an increase in the levels of ROSs as the concentration of fomesafen and sulfentrazone increased in plants used as green manure (Alves et al., 2018b). In the case of the formulation imazapic + imazapyr, increased activities of this enzyme after exposure to imazethapyr + imazapic and imazapyr + imazapic (Marchezan et al., (2017).

Some herbicides of the class of imidazolinones and sulfonylureas (ALS) and PROTOX inhibited the enzyme ALA-D (Supplementary Figure 1 d) such as imazaquin, sulfentrazone, and nicosulfuron. We can see until now that sulfentrazone promoted negative changes in most analyzes. We can see that even herbicides from the ALS inhibitor group affected the activity of the ALA-D enzyme. The damage to plants caused by herbicides in this group can be assessed by their indirect influence on the variables associated with photosynthesis (Taiz and Zeiger, 2013), and in this case even on tolerant hybrids. The decline of chlorophyll compounds can affect photosynthetic activity, impairing plant growth (Marchezan et al., 2017).

But, as observed by the productivity analysis (Supplementary Figure 3) despite the inhibition of the ALA-D enzyme in most treatments, there was no immediate negative effect on the productivity of the canola Hyola 571 CL after the treatments. Perhaps a possible change in this variable could occur in the long run. It was also evidenced that the herbicides imazapyc+imazapyr, imazhethapyr, and imazamox to which the plant is tolerant, presented satisfactory results in the increase in grain production.

Photosynthesis is divided in two processes. First some light reactions that form NADPH and ATP and the second that includes carbon addition responses on Calvin-Benson Cycle. To the second process to occur, is necessary the entry of diffuse CO_2 of the atmosphere into the leaf through the stomata plays an extremely important role. On gas exchange parameters, according to the analyzes, the photosynthetic rate was that just the nicosulfuron decreased the photosynthetic potential of the plant. When taking the other treatments concerning the photosynthetic rate, both did not promote any significant decrease. It shows that a plant has managed to detoxify the effect of the herbicides, without further damage.

Sulfonylurea group, mainly the active ingredient nicosulfuron, inhibits ALS enzymes which reflect on the photosynthetic rate on Hyola 571CL hybrid. These herbicides caused an increase in energy dissipation utilizing chlorophyll fluorescence, and i.a. nicosulfuron caused a decrease in maximum carbon assimilation in about a day after its application.

Plants under stress induce stomatal conductance and transpiration and increase water use efficiency, so photosynthesis is also reduced. In this experiment, the carboxylation coefficient (EC) and internal CO_2 concentration (Ci) parameters remained close to the control. Ci does not show increases. When an internal CO_2 concentration is higher, there is a chance that plants are not able to assimilate the CO_2 that is available for conversion to more energetic molecules.

The evapotranspiration water production for dry mass, and stand out for the relationship that happens between CO_2 correction and H_2O loss. Each opening of the stomata to assimilate CO_2, water evaporates from the leaf tissues (Bramley et al., 2013). Thus, more efficient crops in the use of disciplinary water plus dry matter per gram of transpired water (Torres et al., 2012). The water use efficiency is parameters to assess the plant's ability to adapt to adversities (Rosa *et al*., 2017). The herbicide iodosulfuron, caused a decrease in this rate.

Iodosulfuron increased the transpiration coefficient (E). When sweating is controlled, stomata also influence leaf temperature, no flow of metabolites, and painted signs over long distances (Morais et al., 2017). This rate was decreased when a plant was exposed to flumioxazin and chlorimuron. The reduction in transpiration by these herbicides, may be related to the damage of the cuticle and stomata of the plant and the leaf blade, and may directly affect the gas conductance index (Gs) in plants, reflecting a lower loss of water vapor (Curvêlo et al., 2013). Here, it was observed that although flumioxazin and chlorimuron decreased respiration, both values were close to the control with stomatal conductance, causing no damage. This increase promotes greater dissemination of CO_2 in the leaves, which may increase the rate of photosynthesis (Taiz and Zeiger, 2013).

Herbicides had different effects on the gas exchange of the plants, but the damage in photosynthesis was little. But it is uncertain to say that gas exchange, although important in the process, can limit photosynthesis when in contact with other factors (Machado et al., 2010).

Herbicides of the class of sulfonylureas increased the levels of MDA indicating possible generation of oxidative stress. The enzyme activity changed after exposure to the herbicides, ALA-D was inhibited in almost all treatments, which could be associated with catalase inhibition and APX had its activity increased. But despite this, the herbicides addressed in this study did not negatively interfere in grain yield, which increased in the treatment with herbicides of the imidazolinone class. **Material and methods**

Plant materials

The experiment was carried out in an experimental field with Dystrophic Red Latosol at the Federal University of Fronteira Sul - UFFS, Erechim, RS, Brazil, 2018/2019 growing season. The Hyola 571 CL® canola with tolerance to imidazolinones was used.

Table 1. Treatments and doses of herbicides applied to the Clearfield® canola (Hyola 571). UFFS, Erechim, RS, 2018.

Treatment	Herbicide*	Dose (g ha^{-1}) of (i.a ou e.a)*	Commercial Name	Application Time
A	Weeded witness	----	----	----
B	Diclosulam	29.40	Spider®	Pre-emergence
C	Imazaquim	150	Nortox®	Pre-emergence
D	Sulfentrazone	525	Boral®	Pre-emergence
E	Sulfentrazone +Diuron	525+735	Boral®	Pre-emergence
F	Pendimenthalin	1500	Herbadox 400 EC®	Pre-emergence
G	Flumioxazin	25.00	Flumyzin 500®	Pre-emergence
H	Imazethapyr+imazapic	31.25+9.75	Only®	Post-emergence
I	Imazapic+imazapyr	24.5+73.5	Kifix®	Post-emergence
J	Imazethapyr	100	Imazethapyr Plus Nortox®	Post-emergence
K	Imazamox	42.00	Raptor®	Post-emergence
L	Nicosulfuron	45.00	Accent®	Post-emergence
M	Chlorimuron	15.00	Classic®	Post-emergence
N	Metsulfuron-methyl	3.96	Ally®	Post-emergence
O	Iodosulfuron	3.5	Hussar®	Post-emergence
P	Pyroxsulam	18.00	Tricea®	Post-emergence

* Doses recommended by the manufacturer for weed control.

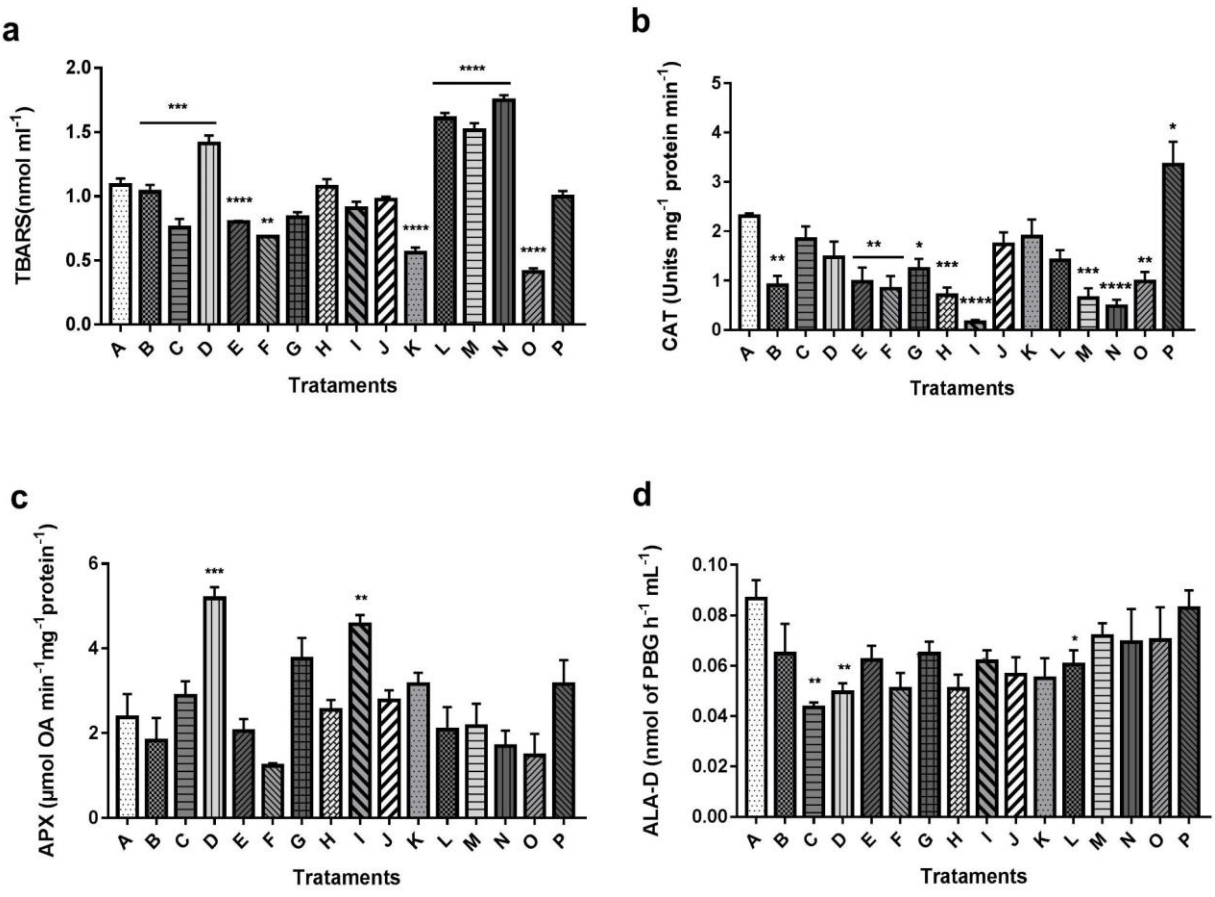

Fig 1. Effect of herbicide application on lipid peroxidation (a), catalase (b), ascorbate peroxidase (c) and on δ ácido aminolevulinato desidratase (d) of canola Hyola 571CL. A: Weeded witness, B: Diclosulam, C: Imazaquim, D: Sulfentrazone, E: Sulfentrazone+Diuron, F: Pendimenthalin, G: Flumioxazin, H: Imazethapyr+imazapic, I: Imazapic+imazapyr, J: Imazethapyr, K: Imazamox, L: Nicosulfuron, M: Chlorimuron, N: Metsulfuron-methyl, O: Iodosulfuron, P: Pyroxsulam. 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.

Table 2. Mechanisms of action and chemical groups of herbicides used in the *Clearfield®* canola (Hyola 571).

Mechanisms of action	Chemical group	Herbicide	Treatment
Inhibitors of acetolactate synthase (ALS)	Triazolopyrimidine	Diclosulam	B
	Imidazolinone	Imazaquim	C
Inhibitors of protoporphyrinogen oxidase (PROTOX)	Triazinone	Sulfentrazone	D
Inhibitors of PROTOX + Inhibitors of photosynthesis at photosystem II	Triazinone + Urea	Sulfentrazone +Diuron	E
Inhibitors of microtubule assembly	Dinitroaniline	Pendimenthalin	F
Inhibitors of PROTOX	N-phenylphthalimide	Flumioxazin	G
Inhibitors of ALS	Imidazolinone	Imazethapyr+imazapic	H
	Imidazolinone	Imazapic+imazapyr	I
	Imidazolinone	Imazhetapyr	J
	Imidazolinone	Imazamox	K
	Sulfonylurea	Nicosulfuron	L
	Sulfonylurea	Chlorimuron	M
	Sulfonylurea	Metsulfuron-methyl	N
	Sulfonylurea	Iodosulfuron	O
	Triazolopyrimidine	Pyroxulam	P

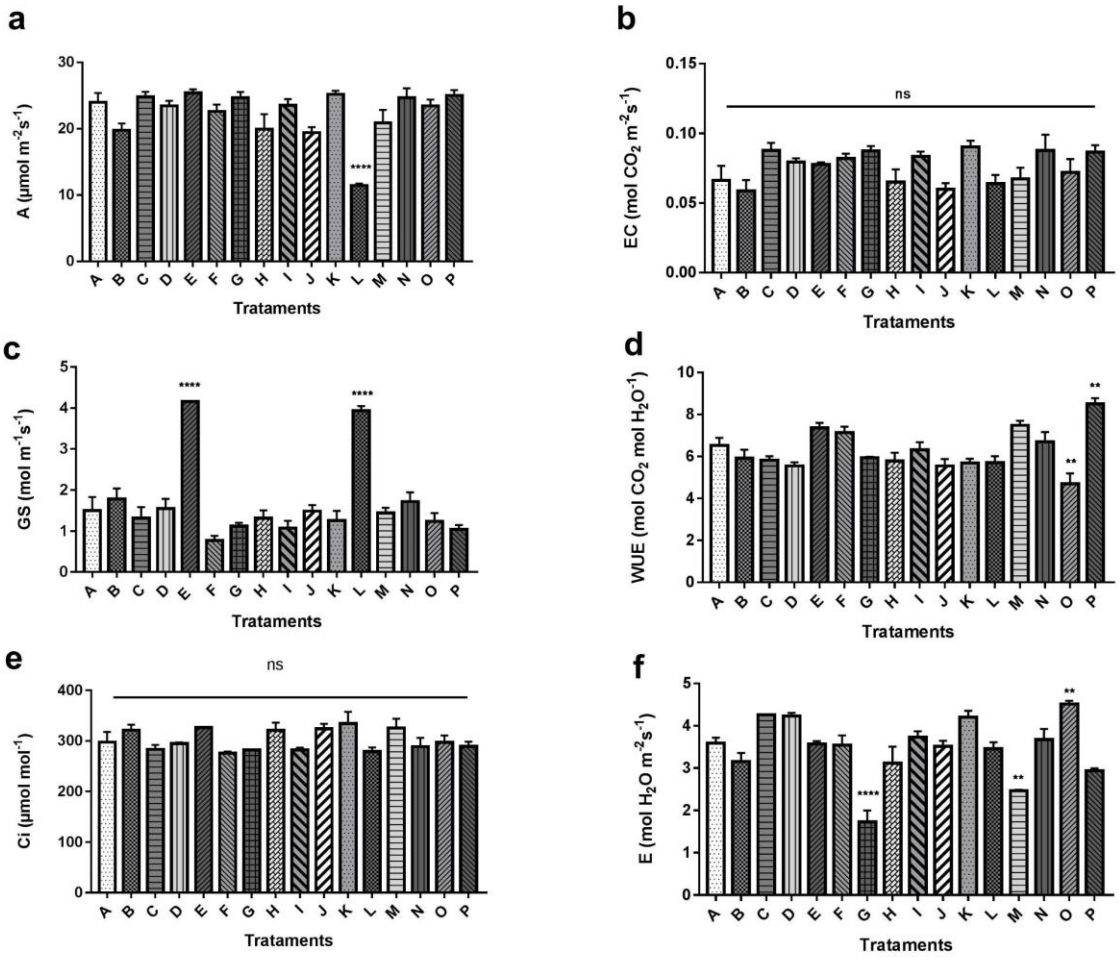

Fig 2. Effect of herbicide application on photosynthetic rate (A) (a), carboxylation efficiency (CE) (b), efficiency of stomatal conductance of gases (Gs) (c), water use efficiency (WUE) (d), internal CO_2 concentration (Ci)(e) and transpiration rate (E) (f) of canola Hyola 571CL. A: Weeded witness, B: Diclosulam, C: Imazaquim, D: Sulfentrazone, E: Sulfentrazone+Diuron, F: Pendimenthalin, G: Flumioxazin, H: Imazethapyr+imazapic, I: Imazapic+imazapyr, J: Imazethapyr, K: Imazamox, L: Nicosulfuron, M: Chlorimuron, N: Metsulfuron-methyl, O: Iodosulfuron, P: Pyroxsulam. 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.

Table 3. Chemical and physical soil characteristics properties of soil.

Properties	Value
pH	4.8
SOM (g dm^{-3})	35
P (mg dm^{-3})	4.0
K (mg dm^{-3})	117.0
Al^{3+} ($cmol_c$ dm^{-3})	0.6
Ca^{2+} ($cmol_c$ dm^{-3})	4.7
Mg^{2+} ($cmol_c$ dm^{-3})	1.8
Effective cation exchange capacity ($CEC_{(t)}$) ($cmol_c$ dm^{-3})	7.4
Total cation exchange capacity ($CEC_{(T)}$) ($cmol_c$ dm^{-3})	16.5
H+Al ($cmol_c$ dm^{-3})	9.7
Sum of bases (SB) ($cmol_c$ dm^{-3})	6.8
Base saturation (V) (%)	41
Clay (g dm^{-3})	600

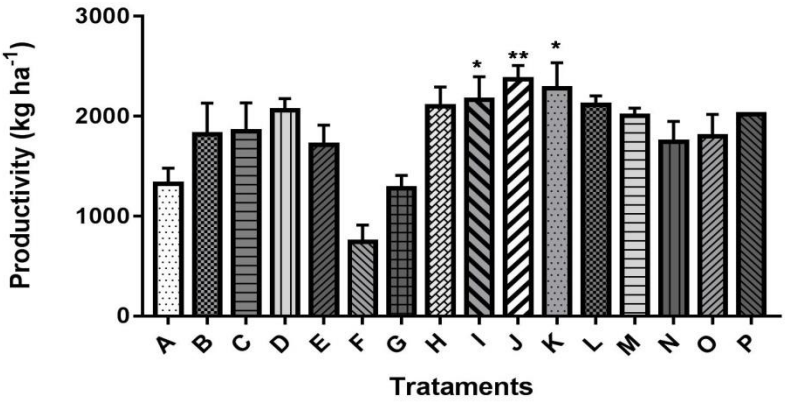

Fig 3. Effect of herbicide application on productivity of grains canola Hyola 571 CL. A: Weeded witness, B: Diclosulam, C: Imazaquim, D: Sulfentrazone, E: Sulfentrazone+Diuron, F: Pendimenthalin, G: Flumioxazin, H: Imazethapyr+imazapic, I: Imazapic+imazapyr, J: Imazethapyr, K: Imazamox, L: Nicosulfuron, M: Chlorimuron, N: Metsulfuron-methyl, O: Iodosulfuron, P: Pyroxsulam. Asterisks indicate statistical significance when treatments differ by Dunnet test compared to the control without herbicide: p <0.05 (*); p <0.005 (**). The bars represent the standard error of the means of four replicates.

Table 4. Toxicity (%) on the canola hybrid Hyola 571 CL as a function of herbicide application. UFFS/Erechim/RS, 2019.

Treatments	Phytotoxicity to canola (%)			
	7 DAT[1]	14 DAT	21 DAT	28 DAT
Weeded witness	0.00 e[2]	0.00 d	0.00 b	0.00 c
Diclosulam	8.00 d	8.00 d	8.00 b	14.33 c
Imazaquin	5.67 d	1.67 d	0.00 b	0.00 c
Sulfentrazone	5.67 d	6.00 d	5.00 b	1.33 c
Sulfentrazone+diuron	19.00 b	36.67 b	14.00 b	5.67 c
Pendimenthalin	65.00 a	55.00 a	55.00 a	60.00 a
Flumioxazin	68.33 a	41.67 b	63.33 a	65.00 a
Imazethapyr+imazapic	1.67 e	3.00 d	0.00 b	2.67 c
Imazapic+imazapyr	0.00 e	5.33 d	4.33 b	4.33 c
Imazethapyr	4.33 d	6.67 d	1.67 b	1.00 c
Imazamox	0.00 e	2.00 d	0.00 b	0.00 c
Nicosulfuron	8.00 d	5.33 d	8.00 b	3.67 c
Chlorimuron-ethyl	14.00 c	33.33 b	55.00 a	56.67 a
Metsulfuron-methyl	18.67 b	33.33 b	56.67 a	41.67 b
Iodosulfuron	11.00 c	20.00 c	45.00 a	30.00 b
Pyroxsulam	5.00 d	8.00 d	11.33 b	11.33 c
Overall average	14.65	16.63	20.46	19.65
CV (%)	25.43	28.41	32.92	40.78

[1] DAT: days after treatment application. [2] Means followed by the same letters in columns, do not differ according to Scott-Knott's at 5% probability.

Conduction of study and experimental design
The correction of the pH and soil fertilization were carried out according to the chemical analysis (Supplementary Table 3), following the technical recommendations for the crop (CQFS, 2016). The experimental design adopted was randomized blocks, with four repetitions.

Treatments
Pre-emergent herbicides were applied immediately after planting and post-emergent, after 28 days after emergence. Herbicide treatments and doses are presented at Supplementary Table 1.
The mechanism of action and the chemical group of all herbicides applied are shown in Supplementary Table 2. Remaining weeds into plots, after herbicides application, were mechanically removed as needed to allow assessment of herbicide phytotoxicity to canola plants, without the effect of competition with weeds. Herbicides were applied with a precision sprayer, pressurized by CO_2, equipped with four nozzles DG 110.02, under constant pressure of 2.0 $kg.cm^{-2}$ and moving speed of 3.6 $km.h^{-1}$, spreading 200 $L.ha^{-1}$ of solution.

Traits measured

Herbicide phytotoxicity
Seven, 14, 21 and 28 days after herbicide treatment (DAT), the toxicity of herbicides on canola hybrid Hyola 571 CL was assessed. We adopted the percentage scale where zero (0%) corresponded to absence of symptoms and one hundred (100%) corresponded to plant death (SBCPD, 1995).

Determination of the antioxidant system status
Sample collection
After 7 days of application of post-emergent herbicides, totaling 40 days of application of pre-emergent, five plants of each treatment were collected, in liquid nitrogen and kept in an ultra-freezer at -80ºC until biochemical analysis.

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 previously established by Havir and McHale (1987). 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). The extract for malondialdehyde (MDA) contents was obtained according to Hodges et al. (1999).
Enzymatic activity
CAT activity was determined using the method of Havir and McHale (1987) with modifications. APX and GPX activity were assessed following the method of Nakano and Asada (1981). The activity of ALA-D was determined according to Sassa (1982) and Morsch et al. (2002). MDA content was measured according to the method proposed by Hodges et al. (1999).

Determination of gas exchange
Photosynthetic rate, internal CO_2 concentration, transpiration rate, stomatal conductance, carboxylation efficiency and also the water use efficiency were measured using a infrared gas analyzer (IRGA), brand ADC, model LCA PRO according to Alves et al. (2019).

Productivity Determination
Quantification of canola grain yield was obtained through manual harvesting of plants in an area of 3 x 1.5 m, of each experimental unit, when grain humidity reached 18%. After grain weighing, humidity was determined and corrected to 10 %. Values were expressed in $kg.ha^{-1}$.

Statistical analysis
For herbicide phytotoxicity on the canola hybrid, the data set was submitted to analysis of variance by the F-test, and when significant, treatments were grouped by Scott-Knott's at $p<0.05$. For the other data sets, the data were submitted to analysis of variance (ANOVA) using GraphPad Prism 7.0 software, where Dunnet's test was applied at $p<0.05$.

Conclusion

Pendimethalin, flumioxazin, chlorimuron-ethyl, metsulfuron-methyl, and iodosulfuron caused the highest phytotoxicity to canola hybrid Hyola 571 CL. The commercial mixtures of [imazethapyr+imazapic] and [imazapic+imazapyr], as well as imazamox, were the most selective ones for canola Hyola 571 CL. The application of herbicides, even over tolerant canola, results in changes in the plant's homeostasis. Imazethapyr + imazapic, imazapic + imazapyr, chlorimuron, and metsulfuron-methyl caused greater inhibitory effects on CAT, especially the ALS inhibitors. Imazethatapyr was the most satisfactory treatment, not changing the enzymatic activity, and increased grain yields of Clearfield canola, similarly to imazamox. In contrast, sulfentrazone was the one that promoted the greatest negative interference, causing oxidative stress, followed by its mixture with diuron; in third place were nicosulfuron, chlorimuron, and metsulfuron-methyl. Nicosulfuron was the only one that affected photosynthesis. Given the results, it can be obtained that the hybrid shows sensitivity to herbicides and that the differences in gas exchange rates and other analyzes for the types of treatment do not result in productivity, which remains balanced.

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Competing interests
The authors declare that there are no conflict interests in this work.

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