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Biochemical and Physiological Changes in Rice Plants Due to the Application of Herbicides[1]

Alterações Bioquímicas e Fisiológicas em Plantas de Arroz Devido à Aplicação de Herbicidas

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ABSTRACT - The application of herbicides, even if selective, can cause biochemical and physiological changes, resulting in oxidative stress. This stress comes from the accumulation of reactive oxygen species produced due to exposure to the herbicide. However, plants have developed defense strategies, which can be enzymatic or non-enzymatic. The objective of this study was to evaluate the morphological and metabolic changes such as photosynthetic parameters, oxidative damage and antioxidant enzyme activity of rice plants after applying herbicides. For this, a study was conducted in a greenhouse and laboratory and the treatments consisted of application of imazapic + imazapyr, quinclorac, bentazon, cyhalofop-butyl, penoxsulan, bispyribac-sodium and carfentrazone-ethyl, in addition to control without herbicide. The phytotoxicity in plants was strong and there was a reduction in photosynthesis, stomatal conductance and efficiency of water use in plants treated with carfentrazone-ethyl. Furthermore, the application of carfentrazone-ethyl resulted in lower chlorophylls and carotenoids and increased lipid peroxidation and proline accumulation. Changes in the activity of enzymes belonging to the antioxidant system were inspected by applying herbicides. The application of herbicide alters the physiology of rice plants, triggering responses to oxidative stress, which are more pronounced when used carfentrazone-ethyl.

Keywords: *Oryza sativa*, oxidative stress, lipid peroxidation, selectivity.

RESUMO - A aplicação de herbicidas, mesmo seletivos às culturas, podem causar alterações bioquímicas e fisiológicas, acarretando estresse oxidativo. Esse estresse é proveniente do acúmulo de espécies reativas de oxigênio produzidas em função da exposição ao herbicida. No entanto, as plantas evoluíram com estratégias de defesa, sendo estas enzimáticas ou não enzimáticas. O objetivo deste estudo foi avaliar as alterações morfológicas e metabólicas, como os parâmetros fotossintéticos, danos oxidativos e atividade das enzimas antioxidantes, de plantas de arroz após a aplicação de herbicidas. Para isso, foi realizado um estudo em casa de vegetação e laboratório, e os tratamentos foram compostos pela aplicação de imazapic + imazapyr, quinclorac, bentazon, cyhalofop-butyl, penoxsulan, bispyribac-sodium e carfentrazone-ethyl, além da testemunha sem herbicida. A fitotoxicidade nas plantas foi acentuada, bem como ocorreu redução na fotossíntese, condutância estomática e eficiência do uso da água nas plantas tratadas com carfentrazone-ethyl. Além disso, a aplicação deste herbicida resultou em menor teor de clorofilas e carotenoides, maior peroxidação lipídica e acúmulo de prolina. Alterações na atividade das enzimas pertencentes ao sistema antioxidante também foram verificadas em função da aplicação dos herbicidas. A aplicação de herbicidas altera a fisiologia das plantas de arroz, desencadeando respostas como estresse oxidativo, sendo estas mais acentuadas quando utilizado carfentrazone-ethyl.

Palavras-chave: *Oryza sativa*, estresse oxidativo, danos celulares, seletividade.

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INTRODUCTION

Rice (*Oryza sativa*) is the third most produced cereal in the world, and it is considered staple food for more than half of the world's population, being an energy and protein source in human diet. Brazil is one of the main producers, with around 13 million tons. Rio Grande do Sul alone is responsible for more than 70% of the production (Conab, 2015).

Even though Brazil is one of the main world producers of rice, the optimal productivity baseline for cultivation has not been reached yet. Problems in crop handling such as infestation by weeds that compete directly for the environment's resources such as light, nutrients and CO_2, lead to the reduction of grain productivity (Andres & Machado, 2004). In order to reduce these losses, farmers have adopted several handling methods, mainly the use of herbicides to control weeds.

The application of herbicides can cause phytotoxicity to the crops, especially if they are not used according to the recommended dosages. Even if a certain active ingredient is selective to the crop and does not cause many injuries to the plants, biochemical and physiological alterations may occur (Song et al., 2007). Some products are known to reduce productivity of crops without causing visually detectable effects, while others cause marked injuries but enable full recovery of the crop (Ferreira et al., 2005).

The formation of reactive oxygen species (ROS) has been described as a consequence of several abiotic stresses, among them the application of herbicides (Song et al., 2007). Although ROS are inevitable products of plant metabolism, in normal conditions, its production and removal is balanced (Mittler, 2002). However, in stress conditions, the production of ROS can overcome removal mechanisms, resulting in oxidative stress (Esfandiari et al., 2010). The excessive production of ROS in the plants cells is harmful to nucleic acids, proteins and lipids. In addition, changing the redox state may cause damage to the photosynthetic apparatus through photo inhibition, resulting in cell injury and chlorosis, which can be different among genotypes (Gill & Tuteja, 2010).

Throughout time, plants have developed several protection strategies to minimize herbicides toxicity. One of these protection mechanisms is the enzymatic antioxidant system, which operates with sequential and simultaneous actions of several enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). SOD happens in several cell compartments and catalyzes dismutation of the superoxide anion into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Gill & Tuteja, 2010). H_2O_2, in turn, is removed by several other antioxidant enzymes, such as CAT and APX (Foyer & Noctor, 2000). The elimination of oxygen reactive species is necessary for the cells to survive in an environment under stress conditions (Foyer & Noctor, 2000).

In the non-enzymatic antioxidant system one can find the phenolic compounds, ascorbic acid, glutathione (GSH), chlorophylls, carotenoids, proteins and amino acids. Carotenoids are present in the non-enzymatic antioxidant compounds, and they are pigments responsible for the photo protection of the photosynthetic membranes, acting as auxiliary pigments. They also act in the dissipation of the excited state of chlorophyll and neutralization of ROS, once they are antioxidants with low molecular weight (Kreslavski et al., 2013). Among the amino acids, proline has a fundamental role in the response of plants to oxidative stress, which has been shown in experiments in which exogenous proline was applied (Ozden et al., 2009) or in which the synthesis or degradation of proline was genetically manipulated (Molinari et al., 2007).

The partition between these two systems under stress conditions can be regulated by the concentration of oxygen in the system (Blokhina et al., 2003). The cellular antioxidant system works as an ROS accumulation sensor (Srivalli et al., 2003), and any disturbance in the balance between the formation and elimination of ROS affects the cell's homeostasis. The activity of the antioxidant enzymes is frequently used as a biomarker for several abiotic stresses (Song et al., 2007). Considering that, the objective of this study was to evaluate the morphologic alterations, the photosynthetic parameters, the oxidative

damage, the activity of the antioxidant enzymes and the alterations in the metabolism of rice plants after applying the herbicides.

MATERIAL AND METHODS

The experiment was conducted in a greenhouse belonging to the Herbology Center (CEHERB) of the Federal University of Pelotas, in a completely randomized experimental design with four replicates. The treatments included the application of post-emergent herbicides: imazapic + imazapyr (24.5 + 73.5 g i.a. ha^{-1}), quinclorac (375 g i.a. ha^{-1}), bentazon (960 g i.a. ha^{-1}), cyhalofop-butyl (315 g i.a. ha^{-1}), penoxsulan (60 g i.a. ha^{-1}), bispyribac-sodium (50 g i.a. ha^{-1}) and carfentrazone-ethyl (200 g i.a. ha^{-1}), besides control without application.

The used cultivar was Puitá INTA-CL, in population of six plants per experimental design, that being constituted by a vase with volumetric capacity of three liters, filled with soil coming from a paddy crop, classified as Solodic eutrophic Albaqualf. The application of herbicides was done when the plants were at a V_4stage, using a backpack sprayer pressurized with CO_2 and bar with four Teejet 110.015 nozzles, fan-like, spaced in 0.5 m, with spray volume of 120 L ha^{-1}.

The variables phytotoxicity, height, liquid photosynthesis, transpiration rate, stomatal conductance and CO_2 substomatal concentration were evaluated 120 hours after spraying the treatments (HAP). The phytotoxicity evaluation was done through visual notes, following a scale of zero (0) to 100, in which zero meant the absence of symptoms and 100 the death of plants. The height was measured with the help of a graduated ruler, measuring all the plants of the experimental unit, measuring the length from the soil level until the apex, with distended leaf limb. The physiologic variables were measured using infrared gases analyzer (IRGA), from brand LI-COR, model LI 6400. For that, the average third of the last completely expanded lead was used, and the readings were done in the morning, between 7 and 9 o'clock. Carboxylation efficiency was calculated by the liquid photosynthesis/substomatal CO_2 concentration ratio, and the water use efficiency was calculated by the liquid photosynthesis/transpiration rate ratio.

Leaf samples were collected 24 and 120 hours after spray and stored at a temperature of -80 °C until the moment of the enzymatic activity analysis of the oxidative damage and the secondary metabolites. The variables analyzed were: content of chlorophyll and carotenoid, content of hydrogen peroxide, lipid peroxidation, content of total proteins, catalase enzyme activity, ascorbate peroxidase and superoxide dismutase and content of proline.

The total contents of chlorophyll and carotenoid were determined according to the methodology described by Arnon (1949), with modifications. 0.1 g samples were macerated in a mortar in the presence of 5 mL of acetone at 80% (*v/v*). The material was centrifuged at 12,000 rpm for 10 minutes, completing the volume to 20 mL with acetone at 80% (*v/v*). The theories of chlorophyll *a*, *b*, totals and total carotenoids were calculated by the use of the Lichtenthaler formulas (1987), from the absorbance of the solution obtained by spectrophotometry at 647, 663 and 470 nm, and the results were expressed in mg g^{-1} of fresh mass (FM).

The cellular damage in the tissues were determined in terms of hydrogen peroxide content (H_2O_2), according to what was described by Sergier et al. (1997), and the thiobarbituric acid reactive species (TBARS), via accumulation of the malonic aldehyde (MDA), as described by Heath & Packer (1968). For both analyses, 0.2 g of leaves were macerated with liquid nitrogen, homogenized in 2 mL of trichloroacetic acid (TCA) 0.1% (*m/v*) and centrifuged at 14,000 rpm for 20 minutes. To quantify H_2O_2, aliquots of 0.2 mL of the supernatant were added in 0.8 mL of phosphate buffer 10 mM (pH 7.0) and 1 mL of Potassium iodide 1M, followed by agitation in vortex. The solution was kept at rest for 10 minutes at room temperature and, after that, the absorbance was read at 390 nm. The H_2O_2 concentration was determined through a standard curve with known concentrations of H_2O_2 and expressed in mM g^{-1} of MF. To determine TBARS, aliquots of 0.5 mL od the

supernatant previously described were added to 1.5 mL of thiobarbituric acid (TBA) 0.5% (*m/v*) and trichloroacetic acid 10% (*m/v*) and incubated at 90 °C for 20 minutes. The reaction was paralyzed in ice bath for 10 minutes. Afterwards, the absorbance was determined at 532 nm, discounting the unspecific absorbance at 600 nm. The MDA concentration was calculated by using the absorptivity coefficient of 155 mM cm^{-1} and the results expressed in nM MDA g^{-1} of MF.

To determine the activity of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidases (APX), first there was an extraction in which 0.2 g of the sample was macerated in a porcelain mortar, in the presence of liquid nitrogen. Then, 900 µL of phosphate buffer 200 mM (pH 7.8), 18 µL of EDTA 10 mM, 180 µL of ascorbic acid 200 mM and 702 µL of ultrapure water and centrifuged at 14,000 rpm at 4 °C for 20 minutes was added. From that extract, the protein of the samples was quantified by the Bradford method (1976), in which 60 µL of the extract were added at 2 mL of Bradford solution, and there was an absorbance reading in the wavelength of 595 nm. The results were calculated in function of the standard curve of casein and expressed in milligrams of protein per mL (casein mL^{-1}).

The SOD activity was determined according to the methodology adapted from Peixoto (1999), from Del Longo (1993) and Giannopolitis & Ries (1977). By this method, there was certain inhibition in the reduction of NBT (ñ-nitro blue tetrazolium) by the enzymatic extract, avoiding the formation of chromophore. In this essay, a SOD enzymatic activity unit was considered a necessary enzyme to obtain 50% of the inhibition to reduce NBT by SOD contained in the enzyme extract. For the reaction 20 µL of the enzyme extract was added in a test tube containing 1 mL of potassium phosphate buffer 100 mM (pH 7.8), 400 µL of methionine 70 mM, 20 µL of EDTA 10 µM, 390 µL of ultrapure water, 150 µL of NBT 1 mM and 20 µL of riboflavin 0.2 mM. After that, the tubes were incubated in a 15 Watts fluorescent lamp for 10 minutes, then reading the absorbance at 560 nm. For calculation, the white of the reaction was considered as the tubes that did not contain extract, exposed and not exposed to the light. The activity was determined by the calculation of the extract that inhibits 50% of the reaction of NBT and expressed in UA mg^{-1} protein $minute^{-1}$.

The CAT activity was determined according to the methodology described by Azevedo et al. (1998), by the consumption of H_2O_2 (extinction coefficient of 39.4 mM cm^{-1}). A 20 µL aliquot of the extract was added to a 1 mL potassium phosphate buffer 200 mM (pH 7.0), 880 µL of ultrapure water and 100 µL of hydrogen peroxide 250 mM. The absorbance reading in spectrophotometer (Ultrospec 6300 Pro UV/Visible – Amersham Bioscience) in the wavelength of 240 nm was done for 90 seconds, with readings in the interval of 7 seconds.

The APX activity was determined according to the methodology described by Azevedo et al. (1998), by the consumption of H_2O_2 (extinction coefficient of 2.9 mM cm^{-1}). A 20 µL aliquot of the extract was added to a 1 mL potassium phosphate buffer 200 mM (pH 7.0), 780 µL of ultrapure water and 100 µL of ascorbic acid 10 mM and 100 µL of hydrogen peroxide. The absorbance reading in the wavelength of 290 nm was done for 90 seconds, with readings in the interval of 7 seconds. Both for the CAT and the APX activity, for calculation, it was considered that the decrease of an absorbance unit is equal to an active unit (AU). The activities of total extract were determined by the calculation of the amount of extract that reduced the reading of absorbance in one AU and expressed in UA mg^{-1} protein $minute^{-1}$.

The proline content was determined according to the methodology described by Bates et al. (1973), with modifications. For that, 0.2 g of plant tissue was macerated in liquid nitrogen and 2 mL of sulfosalicylic acid 3% (m/v) was added. It was centrifuged at 10.000 rpm for 10 min at room temperature. 1 mL of the supernatant was collected, and then 1 mL of acid ninhydrin (1.25 g of ninhydrin; 30 mL of glacial acetic acid; 20 mL of phosphoric acid 6M) and 1 mL of glacial acetic acid was added. It was incubated at 95 degrees for one hour, and then it was cooled in ice bath for 10 minutes. 3 ML of toluene was

added, shaken in a vortex, and only the upper phase of the sample was collected for absorbance reading at 520 nm. The results were expressed in μmol of proline g^{-1} MF, through the elaboration of a standard curve of proline with known concentrations.

The data obtained was analyzed as to its normality and homoscedasticity and later submitted to the variance analysis ($p \leq 0.05$). Having proven statistical significance, the effects of the herbicides were evaluated by the Duncan test ($p \leq 0,05$).

RESULTS AND DISCUSSION

A statistical significance was seen in the treatments for the variables phytotoxicity, height, liquid photosynthesis, substomatal CO_2 concentration, stomatal conductance and efficiency of the use of water and carboxylation. When the plants were evaluated 24 hours after spraying (HAS), there was statistical significance for all the variables evaluated. In turn, 120 after pulverization, there was not statistical significance for the variable ratio chlorophyll A/B.

The greater phytotoxicity, as well as the reduction in height, was observed when the carfentrazone-ethyl herbicide was applied (Table 1). The other herbicides presented low phytotoxicity to the plants, showing to be more selective. However, even with low selectivity, quinclorac and bispyribac-sodium caused reduction in the height of rice plants.

Currently, the most used herbicides in the irrigated rice crops are those whose action mechanisms involve the inhibition of ALS and ACCase enzymes. It was observed that the herbicides belonging to these action mechanisms, in general, caused little injury regarding the morphology and symptomatology of rice crops, showing its high selectivity to the crop.

Regarding the physiologic variables, there was less liquid photosynthesis and higher substomatal CO_2 concentration in the plants treated with carfentrazone-ethyl (Table 2). Higher concentrations of CO_2 in the substomatal cavity suggest that the plants are not being able to assimilate the available CO_2 and convert into more energetic products. The higher concentration of CO_2 results in a blockage of the electrons transport chain and, consequently, in the interruption of ATP and NADPH, which are used as a source of energy for fixation of CO_2 (Weller, 2003). In this case, it is believed that the substomatal CO_2 concentration happened due to the stomatal closing in response to the application of the herbicide.

The stomatal closure is directly related to the carbon fixation and, as a consequence, to the production of the plant's biomass (Gonçalves et al., 2013). In addition, the results obtained may be connected to the action mechanism of the carfentrazone-ethyl herbicide, which acts by inhibiting the PROTOX enzyme and consequently the chlorophyll synthesis, and it can compromise the photosynthesis, resulting in the reduction of the plant's capacity of enjoying the environment resources.

There was reduction in the stomatal conductance due to the application of carfentrazone-ethyl (Table 2). The stomatal control is an important property through which the plants limit the loss of water, affecting gas changes. This characteristic can be influenced by several factors, including the stress (Paiva et al., 2005), and it can be an indication of smaller photosynthetic efficiency.

The efficiency of the use of water and carboxylation was also reduced in the plants

Table 1 - Phytotoxicity (%) and height (cm) of rice plants subjected to the application of post-emergent herbicide 120 hours after spraying (HAS). FAEM/UFPel, Capão do Leão/RS, 2013

Treatment	Phytotoxicity (%)	Height (cm)
Control	0 c	24.9 ab
Imazapyr + Imazapic	3.0 b	21.8 bc
Quinclorac	2.5 bc	18.9 cde
Bentazon	2.2 bc	22.8 ab
Cyhalofop-butyl	2.7 bc	21.4 bcd
Penoxsulan	2.5 bc	26.2 a
Bispyribac-sodium	2.7 bc	18.2 of
Carfentrazone-ethyl	78.0 a	17.8 e
CV (%)	14.8	10.27

Means followed by different letters in the column differ by the Duncan test ($p \leq 0.05$).

Table 2 - Liquid photosynthesis (A) (ì mol CO_2 m^{-2} s^{-1}), stomatal conductance (Gs) (mol H_2O m^{-2} s^{-1}), substomatal CO_2 concentration (Ci) (ì mol CO_2 mol^{-1}), efficiency of water use (EWU) (mol CO_2 mol H_2O^{-1}) and carboxylation efficiency (CE) (ì mol m^{-2} s^{-1}) of rice plants subjected to the application of post-emergent herbicides, evaluated 120 hours after spraying (HAS). FAEM/UFPel, Capão do Leão/RS, 2013

Treatment	A	Gs	Ci	EWU	CE
Control	9.9 a	0.42 a	331 b	1.35 a	0.029 a
Imazapyr + Imazapic	12.4 a	0.44 a	332 b	1.32 a	0.037 a
Quinclorac	12.3 a	0.44 a	334 b	1.28 a	0.036 a
Bentazon	11.1 a	0.41 ab	341 b	1.08 a	0.032 a
Cyhalofop-butyl	11.1 a	0.40 ab	342 b	1.43 a	0.033 a
Penoxsulan	10.1 a	0.38 ab	336 b	1.20 a	0.030 a
Bispyribac-sodium	9.6 a	0.36 ab	335 b	1.16 a	0.028 a
Carfentrazone-ethyl	1.7 b	0.27 b	378 a	0.13 b	0.002 b
CV (%)	3.5	19.0	1.8	34.2	25.7

Means followed by different letters in the column differ by the Duncan test ($p \leq 0.05$).

subjected to the application of carfentrazone-ethyl, in a similar way to the one seen in the other variables (Table 2). These results come from alterations in the liquid photosynthesis and in the substomatal CO_2 concentration. This indicates that the crop, when subjected to stress by herbicide, presented smaller efficiency in the absorption and water flow by the transpiration current, besides greater transpiration during the stomatal opening periods, resulting in the reduction of the variables values.

The water efficiency is directly related to the stomatal opening time because when the plant absorbs CO_2 required for photosynthesis it loses water due to transpiration with variable intensity, according to the current of water potentials (Concenço et al., 2007). Moreover, the smaller efficiency values of carboxylation obtained for carfentrazone-ethyl suggest that the available CO_2 is not being efficiently converted into sugars. The results obtained for the variables related to the photosynthesis corroborate the ones obtained for phytotoxicity, where it was seen that, as phytotoxicity caused by herbicides increased, there was reduction in the liquid photosynthesis, stomatal conductance and efficiency of the water use and carboxylation.

Generally, 24 hours after spraying, there was reduction in the contents of chlorophyll *a* when applying herbicide carfentrazone-ethyl, but there was no statistical difference regarding the control and the herbicides penoxsulan and bispyribac-sodium (Table 3). The other herbicides did not reduce the contents of the photosynthetic pigments, corroborating previous studies where the effect of bentazone, cyhalofop-butyl and penoxsulam was observed in rice plants (Nohatto, 2014). Chlorophyll *b*, total chlorophyll and carotenoids showed reduction when compared to the control in plants treated with carfentrazone-ethyl. These results lead to a greater A/B ratio observed in the plants subjected to the application of carfentrazone-ethyl.

Photosynthetic pigments, such as chlorophyll *a*, are used by the plants to capture the lighting energy, producing reducing power for the fixation and assimilation process of CO_2 in the Calvin cycle. The porphyrins synthesis is crucial for the production of chlorophylls in plants and heme in plants and animals. The main differences refer to the pathway feed, done from glutamate in plants (Nelson & Cox, 2000). Several agents (biotic and abiotic) reduce concentration of chlorophyll *a* in photosynthetic tissues, both for the increase in degradation and the inhibition of biosynthesis (Gan, 2007).

Among the abiotic factors, important synthetic compounds used as herbicides act in the inhibition of the synthesis of chlorophyll in the porphyrinic portion, main site of action of PROTOX inhibitors (Wakabayashi & Boger, 1999). These compounds act through

Table 3 - Chlorophyll content *a* (Cha) (mg g^{-1}), chlorophyll *b* (Chb) (mg g^{-1}), total chlorophyll (Chtot) (mg g^{-1}), carotenoids (CR) (mg g^{-1}) and A/B ration in rice plants subjected to the application of post-emergent herbicides, evaluated 24 hours after spraying (HAS). FAEM/UFPel, Capão do Leão/RS, 2013

Treatment	Cha	Chb	Chtot	CR	A/B
Control	1.77 ab	0.46 ab	2.24 ab	0.52 a	3.90 b
Imazapyr + Imazapic	1.91 a	0.62 a	2.53 ab	0.58 a	3.08 b
Quinclorac	1.88 a	0.57 ab	2.46 ab	0.55 a	3.30 b
Bentazon	2.03 a	0.63 a	2.66 a	0.53 a	3.35 b
Cyhalofop-butyl	1.88 a	0.57 ab	2.46 ab	0.59 a	3.33 b
Penoxsulan	1.64 ab	0.44 abc	2.08 abc	0.50 a	4.02 b
Bispyribac-sodium	1.55 ab	0.40 bc	1.95 bc	0.53 a	4.13 b
Carfentrazone-ethyl	1.37 b	0.25 c	1.63 c	0.27 b	6.56 a
CV (%)	16.4	24.7	16.8	17.3	32.1

Means followed by different letters in the column differ by the Duncan test ($p \leq 0.05$).

inhibition of common enzyme between the pathways of the chlorophyll and cytochromes synthesis, which results in the accumulation of intermediate tetrapyrrolic, paralyzing the formation of this pigment (Matringe et al., 1989).

The chlorophyll molecules are the main pigments responsible for the capture of light for photochemical reactions, present in the photosystems reaction centers (Taiz & Zeiger, 2009) and, consequently, the decline of these compounds to compromise the photosynthetic activity, harming the development of plants. The photosynthesis rate usually decreases during exposure to several stress sources in superior plants (Chaves et al., 2009). The main reason is the stomatal closure, which leads to the decrease in the internal concentration of CO_2 and can lead to the transfer of electrons to O_2, one of the main causes of production of ROS (Rodziewicz et al., 2014).

This variable can be influenced by the application of herbicides and environmental factors such as water availability, light and energy (Ometto et al., 2003). Similar results were observed by Corniani et al. (2006), who observed that the sunflower plants subjected to water stress had a reduction in the photosynthetic rate and increase in the substomatal CO_2 concentration. It is suggested that in stress situations caused by herbicides the available CO_2 is not converted into photosynthetic products, increasing its concentration in the substomatal cavity.

When the content of chlorophylls and carotenoids in 120 HAS, it was seen reduction caused by herbicide carfentrazone-ethyl for the variable chlorophyll *a* and total chlorophyll and carotenoids, regarding the control, while the contents of chlorophyll *b* did not differ from the control (Table 4).

Reductions in the content of pigments resulting from the action of PROTOX inhibitors were observed by several authors (Triphathy et al., 2007) as a consequence of the oxidative stress, leading to the reduction of photosynthesis and indicating that the content of chlorophyll may be a biomarker for the plants growth. This consequence may be attributed to the fact that damages in the photosynthetic system reflect on the reduction of the levels of chlorophyll and carotenoids (Santos et al., 2011). As the oxidative stress increases in function of time of exposure to the light, the thylakoids are damaged and lose their capacity to do photosynthesis due to damage in the photosynthetic apparatus (Tripathy et al., 2007), which can explain the smaller liquid photosynthesis observed in plants treated with carfentrazone-ethyl.

Â-carotene and zeaxanthin as well as tocopherols have an important photo protector role, whether for dissipation of the excess of energy as heat or by ROS cleaning (Gill & Tujeta, 2010; Kreslavski et al., 2013). Carotenoids such as Neoxanthin and lutein were associated to the process of ROS removal

Table 4 - Chlorophyll content *a* (Cha) (mg g^{-1}), chlorophyll *b* (Chb) (mg g^{-1}), total chlorophyll (Chtot) (mg g^{-1}) and carotenoids (CR) (mg g^{-1}) in rice plants subjected to the application of post-emergent herbicides, evaluated 120 hours after spraying the treatments (HAS). FAEM/UFPel, Capão do Leão/RS, 2013

Treatment	Cha	Chb	Chtot	CR
Control	1.42 ab	0.46 ab	1.88 a	0.48 b
Imazapyr + Imazapic	1.69 ab	0.39 ab	2.09 a	0.57 ab
Quinclorac	1.83 a	0.52 a	2.35 a	0.64 a
Bentazon	1.77 ab	0.55 a	2.32 a	0.57 ab
Cyhalofop-butyl	1.36 b	0.44 ab	1.80 a	0.49 b
Penoxsulan	1.76 ab	0.51 ab	2.27 a	0.63 a
Bispyribac-sodium	1.46 ab	0.46 ab	1.91 a	0.50 b
Carfentrazone-ethyl	0.71 c	0.21 b	0.92 b	0.28 c
CV (%)	18.7	40.8	21.8	15.1

Means followed by different letters in the column differ by the Duncan test ($p \leq 0.05$).

(Bonnecarrère et al., 2011). The reduction in the carotenoids contents due to the application of herbicides brings marking consequences to plants, such as smaller growth and development and higher phytotoxicity, as observed in this study. This happens because carotenoids are important pigments for the absorption of light during photosynthesis of the plants and, therefore, their development and growth.

Reduction was seen in the contents of H_2O_2 cm^{-1} at 24 HAS when applying herbicides quinclorac, bentazon and carfentrazone-ethyl (Table 5). It is not possible to affirm what is the reason for the reductions, seeing that there were no differences in the SOD activity regarding the control. The results could be attributed to H_2O_2 conversion into another free radical, such as hydroxyl ($^{*}OH$). This radical has great oxidative potential and attacks quickly and with not discrimination of macromolecules, leading to serious cellular damage and causing lipid peroxidation, protein denaturation and DNA mutation, which can lead to irreparable metabolic dysfunctions and even cellular death (Scandalios et al., 200).

Similar to the other variables, it was observed in the 24 HAS evaluation that there was an increase in lipid peroxidation in plants treated with carfentrazone-ethyl (Table 5). As mentioned previously, the PROTOX inhibition may lead to the formation of ROS, causing membrane lipid peroxidation and subsequent oxidative stress.

There was elevation in the contents of proline when applying bispyribac-sodium and carfentrazone-ethyl, in comparison to the control with no application (Table 5). Proline has a protecting function against stress in plants due to its capacity of eliminating free radicals and establishing subcellular structures (Verbruggen & Hermans, 2008). The accumulation of this amino acid is mainly reported in salt stress situations (Ashfaque et al., 2014), but it can perform an important role against xenobiotics as well (Song et al., 2007). In addition, the proline accumulated under adverse growth conditions act as a sign

Table 5 - Content of hydrogen peroxide (H_2O_2) (mM g^{-1}), lipid peroxidation in terms of thiobarbituric acid reactive species (TBARS) (nM MDA g^{-1} of MF) and content of proline (PROL) (mg proline g^{-1} MF) in rice plants after application of post-emergent herbicides, evaluated 24 hours after application (HAS). FAEM/UFPel, Capão do Leão/RS, 2013

Treatment	H_2O_2	TBARS	PROL
Control	11.29 a	14.49 b	0.135 c
Imazapyr + Imazapic	8.97 abc	11.80 b	0.143 c
Quinclorac	8.68 bc	9.73 b	0.170 bc
Bentazon	7.37 cd	9.49 b	0.166 bc
Cyhalofop-butyl	9.00 abc	9.58 b	0.147 c
Penoxsulan	10.03 ab	10.75 b	0.166 bc
Bispyribac-sodium	9.07 abc	10.47 b	0.199 b
Carfentrazone-ethyl	6.25 d	34.31 a	0.292 a
CV (%)	16.0	32.9	11.7

Means followed by different letters in the column differ by the Duncan test ($p \leq 0.05$).

of memory for the next generation (Zhang et al., 2013).

In 120 HAS there was an increase of the H_2O_2 content in plants treated with bispyribac-sodium in relation to the control, but not differing from penoxsulam (Table 6). On the other hand, the smaller values were observed in plants subjected to the application of carfentrazone-ethyl. Due to the data obtained in the other analyses that indicate the stress caused by herbicides, the hypothesis is that the smaller content of H_2O_2 in plants treated with carfentrazone-ethyl is not related to the smaller stress, but to the possible formation of free radicals, more harmful to the cell than H_2O_2.

The over production of superoxide anion (O_2^{-*}) and H_2O_2 is usually considered an answer to the stress inducers (Song et al., 2007). The increase in the levels of O_2^{-*} due to environmental stress lead to the formation of other oxygen reactive species, such as $^{*}OH$ (Halliwell, 2006). Once accumulated in the cells, H_2O_2 activate the calcium passage channels in the vacuole membrane, increasing its concentration in cytosol (Kohler & Blatt, 2002), which leads to the depolarization of the guard cells, potassium efflux, turgor loss and, as a consequence, it can cause the closing of stomata.

Table 6 - Content of hydrogen peroxide (H_2O_2) (mM g^{-1} MF), lipid peroxidation in terms of thiobarbituric acid reactive species (TBARS) (nM MDA g^{-1} of MF) and content of proline (PROL) (mg proline g^{-1} MF) in rice plants after application of post-emergent herbicides, evaluated 120 hours after spraying of the treatments (HAS). FAEM/UFPel, Capão do Leão/RS, 2013

Treatment	H_2O_2	TBARS	PROL
Control	10.33 bc	9.66 c	0.099 c
Imazapyr + Imazapic	8.98 c	4.86 d	0.105 bc
Quinclorac	9.21 c	12.86 c	0.117 bc
Bentazon	10.10 bc	10.58 c	0.140 b
Cyhalofop-butyl	9.04 c	14.46 c	0.125 bc
Penoxsulan	11.77 ab	9.98 c	0.109 bc
Bispyribac-sodium	12.59 a	19.56 b	0.105 bc
Carfentrazone-ethyl	2.82 d	75.99 a	0.364 a
CV (%)	14.5	13.0	16.2

Means followed by different letters in the column differ by the Duncan test ($p \leq 0.05$).

H_2O_2 is beginning to be accepted as a secondary messenger for signals generated by the ROS due to its considerably long half-life and high permeability through the membranes (Quan et al., 2008). However, the biological effects of H_2O_2 have shown to be dependent not only on the concentration, but also on the production site, on the development stage of the plant and on the previous exposure of the plant to other types of stress (Petrov & van Breusegem, 2012). Therefore, more studies must be done in order to ratify the results observed in this paper and establish the H_2O_2 effect when applying herbicides.

When lipid peroxidation is evaluated at 120 HAS, it was observed the accumulation of malonic aldehyde (MDA) in plants treated with bispyribac-sodium and carfentrazone-ethyl, while the other treatments presented similar or inferior value to the control (Table 6). The smaller accumulation of MDA was observed in the plants subjected to the application of imazapyr + imazapic, showing high tolerance of cultivar Puitá INTA CL to the group of imidazolinones, an expected result since this cultivar is capable of metabolizing the herbicide.

The greatest content of proline in 120 HAS was observed in the treatment with carfentrazone-ethyl, being superior to the treatment with bentazon, and they are superior to the control (Table 6). The hypothesis is that, besides being less selective, these herbicides are the product of the action of contact and they have a faster effect on the plants. Moreover, bentazon (FS II) and carfentrazone-ethyl (PROTOX) have as a consequence of the action mechanism the production of ROS and, therefore, induce the increase of proline as a way to defend the plants.

When evaluating the contents of proteins in the plants, both at 24 and at 120 HAS, it was seen greater reduction in the plants treated with herbicide carfentrazone-ethyl (Tables 7 and 8). This reduction can be attributed both to the smaller synthesis and the hydrolysis and formation of the proline amino acid.

Regarding SOD activity, there was no increase regarding the control, suggesting that the herbicides did not activate the defense

Table 7 - Protein content (PROT) (mg casein g^{-1} MF), activity of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (UA mg^{-1} protein $minute^{-1}$) in rice plants subjected to the application of post-emergent herbicides, evaluated 24 hours after spraying of the treatments (HAS). FAEM/UFPel, Capão do Leão/RS, 2013

Treatment	PROT	SOD	CAT	APX
Control	28.55 a	2.00 a	0.130 a	0.338 b
Imazapyr + Imazapic	26.92 a	2.19 a	0.108 bcd	0.346 b
Quinclorac	25.30 a	2.12 a	0.102 cd	0.372 b
Bentazon	25.60 a	1.41 b	0.095 d	0.568 a
Cyhalofop-butyl	28.05 a	2.20 a	0.117 abc	0.304 bc
Penoxsulan	28.35 a	1.94 b	0.125 ab	0.359 b
Bispyribac-sodium	27.35 a	2.33 a	0.112 abcd	0.341 b
Carfentrazone-ethyl	20.80 b	2.50 a	0.114 abcd	0.245 c
CV (%)	10.9	17.3	10.9	11.1

Means followed by different letters in the column differ by the Duncan test ($p \leq 0.05$).

Table 8 - Protein content (PROT) (mg casein g^{-1} MF), activity of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (UA mg^{-1} protein $minute^{-1}$) in rice plants subjected to the application of post-emergent herbicides, evaluated 120 hours after spraying of the treatment (HAS). FAEM/UFPel, Capão do Leão/RS, 2013

Treatment	PROT	SOD	CAT	APX
Control	24.05 abc	1.81 d	0.094 b	0.521 a
Imazapyr + Imazapic	26.72 a	2.16 cd	0.114 ab	0.493 a
Quinclorac	23.35 bc	2.36 c	0.117 ab	0.434 a
Bentazon	22.69 c	2.01 cd	0.131 a	0.236 b
Cyhalofop-butyl	22.83 c	2.82 b	0.094 b	0.588 a
Penoxsulan	26.43 ab	2.16 cd	0.098 b	0.516 a
Bispyribac-sodium	24.77 abc	2.22 cd	0.109 ab	0.545 a
Carfentrazone-ethyl	11.60 d	4.55 a	0.042 c	0.456 a
CV (%)	8.2	11.3	13.2	17.6

Means followed by different letters in the column differ by the Duncan test ($p \leq 0.05$).

system or that in 24 hours it is not enough to have this response (Table 7). Similar results were observed for CAT, where there was no enzyme activation by the application of herbicides, and the values obtained were inferior to the control (Table 7). Differently from the other enzymes, there was an increase in the APX activity at 24 HAS in the plants treated with bentazon, while this activity was reduced in plants subjected to the application of carfentrazone-ethyl (Table 7). This can be a result of the smaller observed CAT activity in the plants treated with bentazone and the greater accumulation of H_2O_2 due to the application of carfentrazone-ethyl, since H_2O_2 is the substrate of the APX enzyme. While APX had greater affinity with the substrate compared to CAT, the accumulation of H_2O_2 was not enough to activate the defense system. It is worth mentioning that this smaller accumulation of H_2O_2 does not indicate less oxidative stress after all, this radical may have been converted into another radical, more harmful to the cell, as explained previously.

Contrary to the evaluation at 24 HAS, when SOD was evaluated in 120 HAS, an increase in the activity of the enzyme was observed due to the application of quinclorac, cyhalofop-butyl and carfentrazone-ethyl (Table 8). As suggested, 24 hours cannot be enough time to activate the defense system, especially when subjected to the application

of systemic products. The higher activity of SOD in plants subjected to the application of cyhalofop-butyl was not expected, because this herbicide is known to be selective to rice crops. However, this selectivity may be related precisely to the efficient antioxidant system of plants in response to this herbicide.

In a similar study, there was an increase in SOD activity of up to 243% in rice plants when atrazine was applied, compared to the control treatment (Zhang et al., 2014). Wheat plants treated with paraquat (Ekmekci & Terzioglu, 2005), of rice, with glyphosate (Ahsan et al., 2008) and fluroxipyr (Wu et al., 2010), and soybeans, with lactofen (Ferreira et al., 2010), also had an SOD increase. The elevation in SOD activity in response to the stress imposed by certain herbicides comes from the accumulation of ROS, especially under conditions that can lead to the death of a cell.

The greater CAT activity rm 120 HAS was observed in plants treated with bentazon, probably due to the fast accumulation of H_2O_2 observed in products with contact action (Table 8). Similarly, rice plants treated with atrazine also resulted in higher CAT activity (Zhang et al., 2014). However, the less accumulation of this radical in the plants treated with carfentrazone-ethyl led to less enzyme activity. On the other hand, regarding APX, generally there was no alteration in the enzyme activity due to the herbicide treatments.

Generally, the greatest alterations were caused by herbicides inhibitors of PROTOX. These herbicides may have their action reduced by the increase in the activity of some antioxidant enzymes, such as SOD, CAT and APX, which have the power to mitigate the oxidative stress (Jung et al., 2008). Rice plants treated with PROTOX inhibitors of several chemical groups (Acifluorfen, oxyfluorfen, carfentrazone-ethyl and oxadiazon) showed an increase of SOD, CAT and APX activity (60, 17 and 68%, respectively) compared to non-treated plants (Jung et al., 2008). However, the impact of the antioxidant system in the effectiveness of the herbicides inhibitors of PROTOX is still complex (Matzenbacher et al., 2014).

It is worth mentioning that, throughout time, the plants developed sophisticated strategies to support the adverse effect of herbicides and reduce their phytotoxicity through the multiple detoxification system (Kawahigashi, 2009). Several studies have shown that ROS (such as H_2O_2) can serve as indicating molecules involved in the response of plants to biotic and abiotic stresses (Mittler, 2002; Gill & Tuteja, 2010), and they can activate enzymes of the antioxidant system (Jiang & Yang, 2009).

We can conclude that the application of herbicides, even if selective to the rice crop, results in alterations of the photosynthetic parameters, oxidative damage and activation of the defense system of plants. Among the herbicide studied, the greater the phytotoxicity, the greater the damage to the photosynthetic apparatus and the greater lipid peroxidation resulted from the application of carfentrazone-ethyl; the dosage used in this study must not be recommended for post-emergence use, even with a license. The other herbicides, in general, have shown to be selective to rice crops.

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