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Full Length Research Paper

Oxidative stress in *Emilia coccinea* (Asteraceae) caused by a mixture of Clomazone + Ametryn

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Abstract

The objected was to assess the relationship of physiological parameters to the susceptibility of *Emilia coccinea* to a mixture of ametryn and clomazone applied post-emergence. The herbicide mixture was applied in four doses 30 days after sowing (DAS). The physiological parameters studied were photosynthetic pigments, chlorophyll fluorescence, soluble proteins, superoxide dismutase (SOD) activity, ascorbate peroxidase (APX) activity, electrolyte leakage and lipid peroxidation (MDA) 72 hours after application (HAA). Twenty-five days after application, the frequency of dead plants and dry matter accumulation per plant were recorded. The data were analyzed by analysis of variance and linear or nonlinear regression analysis. The total chlorophyll content (CHL), carotenoids (CAR) and maximum quantum efficiency of PSII (Fv / Fm) decreased exponentially with increasing doses of the herbicide mixture. The SOD activity decreased linearly. There was no significant linear increase in APX activity. There were linear increases in the electrolyte leakage and lipid peroxidation with increased doses of the mixture. In relation to plant mortality, a dose of 10 L ha^{-1} eliminated 100% of the *E. coccinea* population. Given these observations, we conclude that the membrane damage appeared 72 HAA of a mixture of clomazone + ametryn was closely related to mortality at 25 DAS.

Keywords: Chlorophyll fluorescence, photosynthetic pigments, antioxidant enzymes, herbicide susceptibility.

INTRODUCTION

Worldwide, the use of herbicide mixtures has increased every year, mainly as a tool to prevent the emergence of resistant biotypes and to assist in controlling species considered difficult to control (Beckie and Reboud, 2010). Among these mixtures, we highlight the combination of molecules that interfere with the electron flow between photosystems with molecules that inhibit the synthesis of photosynthetic pigments.

Ametryn (2-(ethylamino)-4-isopropylamino-6-methyl-thio-s-triazine) inhibits the electron flow in photosystem II (PSII) by blocking the transfer of electrons from the electron donor, QA, to the mobile electron carrier, QB (Holt et al., 1993). Clomazone (2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone) is a pigment inhibitor that blocks the formation of chloroplastic isoprenoids, including photosynthetic pigments, carotenoids, tocopherol and electron carriers, in higher plants (Norman et al., 1990). Carotenoids play a role in protecting plants against the harmful effects of excessive radiation (Havaux and Niyog, 1999), and a decrease in their biosynthesis could cause damage to plants. Although the behavior of both herbicide molecules is well described, the literature regarding the physiological effects of the mixture of these two herbicides on weeds is

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Table 1. Chemical analysis of the substrates used in the experiment in which *E. coccinea* was exposed to different doses of a mixture of clomazone + ametryn

pH (water)	5.6
Na (mg dm^{-3})	21
P (mg dm^{-3})	2
K (mg dm^{-3})	84
Ca+Mg ($cmol_c$ dm^{-3})	2.9
Ca ($cmol_c$ dm^{-3})	1.6
Mg ($cmol_c$ dm^{-3})	1.3
Al ($cmol_c$ dm^{-3})	0.02
H^+ ($cmol_c$ dm^{-3})	5.5
Na (%)	1.1
Organic Matter	2.09
Fe (mg dm^{-3})	210.5
Cu (mg dm^{-3})	1.72
Zn (mg dm^{-3})	1.56
Mn (mg dm^{-3})	2.33

scarce.

Herbicides can also lead the plants to increase production of reactive oxygen species (ROS). These radicals can react with various macromolecules in biological membranes, such as lipids, proteins and DNA, causing serious damage that may lead to tissue death. Monitoring the activity of enzymes that catalyze the removal of these compounds, such as superoxide dismutase (SOD) (EC 1.15.1.1) and ascorbate peroxidase (APX) (EC 1.11.1.11) can reveal the stress level in plants. The levels of these enzymes can also be related to tolerance and / or resistance to herbicide molecules in plants (Mittler, 2002; Pyon et al., 2004; Hassan and Alla 2005).

Thus, the objective of this work was to relate the oxidative stress caused by the mixture of clomazone and ametryn with the susceptibility of *Emilia coccinea* (Sims) G. Don, a species that occurs quite often in the sugarcane plantations of Brazil.

MATERIALS AND METHODS

The trial was conducted in greenhouse located at the Agrarian Sciences Center of the Federal University of Alagoas (09 º 28'02"S, 35 º 44'43"W, 127 m). The seeds used were obtained from sugarcane plantations and were initially sown in 7 L pots, with a surface area of 615.75 cm^2, containing sifted soil as a substrate. The chemical characteristics of the soil used in the experiment are presented in Table 1. Fifteen days after seedling emergence, the plants were thinned and stabilized at a density of 200 plants m^{-2}.

Thirty days after sowing, when the plants had 4-6 leaves, the commercial product Sinerge CE® was applied (FMC Agricultural Products, made by mixing clomazone + ametryn, whose registered dose is 5 L ha^{-1}). The treatments consisted of herbicide doses defined in relation to the registered dose: T1- 0 L ha^{-1} (control), T2- 2.5 L ha^{-1} (0.5 kg ha^{-1} of clomazone + 0.75 kg ha^{-1} of ametryn), T3- 5 L ha^{-1} (1 kg ha^{-1} of clomazone + 1.5 kg ha^{-1} of ametryn), T4- 10 L ha^{-1} (2 kg ha^{-1} of clomazone + 3 kg ha^{-1} of ametryn). The product was applied by a sprayer pressurized with CO_2, equipped with medium / high flow nozzles (Teejet XR 110.04 VS) and calibrated to deliver 350 L ha^{-1}. The trial was performed in six replicates.

Seventy-two hours after application (HAA) of the herbicide the following variables were analyzed: photosynthetic pigment content, chlorophyll fluorescence, electrolyte leakage, soluble proteins, superoxide dismutase activity, ascorbate peroxidase activity and malonaldehyde content (MDA) in the leaf tissue.

The chlorophyll (CHL) and carotenoid (CAR) contents were determined by extraction with acetone (Hendry and Grime, 1993). Samples of 20 mg of fresh leaf (the uppermost leaf of the plant) were placed into screw-cap test tubes containing 5 mL of 80% acetone (v / v), protected from light with aluminum foil and left in the refrigerator for 72 hours. Each repetition was extracted in triplicate.

The fluorescence measurements were performed with a modulated fluorometer portable halogen lamp (35W) (Opti-Sciences, model OS1-FL, Hudson, USA) with a

saturating light pulses 0.5 s in duration. Each replicate was the average of two readings taken in the same leaf (the third leaf from the top) in the early morning (06:00).

For soluble proteins, 20 mg of plant tissue (from the second leaf from the top) was homogenized in 2 mL of 80 mM Tris-HCl (pH 7.4) (Alla et al., 2008) and the supernatant was collected as the soluble protein fraction with Coomassie blue reagent (Bradford, 1976).

The SOD activity was determined by adding 40 µL of the crude enzyme extract to a solution containing 13 mM L-methionine, 0.075 mM nitro blue tetrazolium chloride (NBT), 0.1 mM EDTA and 0.002 mM riboflavin in 52.5 mM potassium phosphate buffer (pH 7.8). The reaction took place in assay tubes after illumination with a 30 W fluorescent lamp at 25 ºC for 15 min (Giannopolitis and Ries, 1977). The activity was expressed as units per mg soluble protein per min (UA mg^{-1} protein min^{-1}).

The ascorbate peroxidase activity was determined by adding 40 µL of the crude enzyme extract to 1 mL of a solution containing 0.5 mM sodium ascorbate diluted in 50 mM potassium phosphate buffer (pH 6.0) and 0.1 mM H_2O_2. The decrease in absorbance at 290 nm was monitored for 1 min, a modification of the protocol described by Nakano and Asada (1981). The enzyme activity was expressed as UA mg^{-1} protein min^{-1}. All determinations were assayed in triplicate.

The electrolyte leakage was determined according to the methodology proposed by Flint et al. (1966) with modifications. Ten discs of 6 mm in diameter were collected from leaf tissue (the second leaf from the top of the plant) and immediately washed in deionized water. After that, the leaf discs were placed with the adaxial surface upwards in 50 mL of deionized water and covered. After 6 hours, we took the first reading with a conductivity meter (CD-850, Instrutherm, São Paulo, Brazil). The covers were then replaced by a layer of aluminum foil and sealed with Parafilm®, and the containers were left in a drying oven at 90 ºC for 2 hours. The last reading was done after cooling the solutions to room temperature. The percentages of electrolyte leakage were calculated from the equation: (electrical conductivity after 6 h / electrical conductivity after heating to 90 ºC) * 100

Lipid peroxidation was evaluated in terms of the concentration of thiobarbituric acid-reactive substances (TBARS) (Cakmak and Horst, 1991). Fresh leaves were homogenized in 1 mL of 0.1% (w/v) trichloroacetic acid (TCA) at 4 ºC. The homogenate was centrifuged at 20,000 × *g* for 15 min, and 0.5 mL of the supernatant was added to 3 mL of 0.5% (v/v) TBA in 20% TCA. The mixture was incubated at 95 ºC for 50 min. The samples were then centrifuged at 9000 × *g* for 10 min, and the absorbance of the supernatant was read at 532 nm.

Twenty-five days after the herbicide application the percentage of mortality was recorded, and these data were subjected to the chi-square test (χ^2). The CHL, CAROT, Fv / Fm, soluble proteins, SOD, APX, electrolyte leakage and MDA data were subjected to analysis of variance (ANOVA). If a significant difference was obtained, the data were then submitted to linear regression analysis ($y = a + bx$) or non-linear logistic type analysis ($y = a / (1 + (x / b) c)$), adapted from Streibig (1988).

RESULTS

The doses of the herbicide mixture (clomazone + ametryn) had highly significant effects on the physiological parameters evaluated 72 hours after application (Table 2). The chlorophyll and carotenoid contents decreased exponentially as the applied doses increased (Figures 1a and 1b).

The maximum quantum efficiency of PSII (Fv / Fm) also decreased exponentially as a function of doses of the herbicide mixture (Figure 2a). The soluble protein content increased linearly as a function of doses of the applied product (Figure2b).

The SOD values decreased exponentially with increasing doses of the herbicide mixture (Figure 3a). Because the SOD activity was repressed within 72 hours of application, the APX activity showed no significant linear increase (Figure 3b).

The electrolyte leakage and MDA content also showed linear increases (Figure 4a and 4b). Regarding the percentage of dead and live plants, the doses differed by a χ^2 test (Table 3). The population of the species studied was 100% eliminated by application of the herbicide mixture at twice the recommended rate (10 L ha^{-1}).

DISCUSSION

The decrease in pigment after application of the clomazone + ametryn herbicide mixture was due in part to the inhibition or reduction of the accumulation of pigments in the plastids of susceptible plants caused by the clomazone molecule (Ferhatoglu and Barett, 2006). This inhibition results in symptoms known as bleaching (Norman et al., 1990). The toxicity and bleaching observed in plants susceptible to clomazone are caused by metabolic activation of the clomazone to 5-OH clomazone and subsequently to 5-keto clomazone, which is actively toxic (Ferhatoglu and Barett, 2006). The 5-keto clomazone decrease the pigment content by inhibiting the synthesis of chloroplastic isoprenoids. This molecule

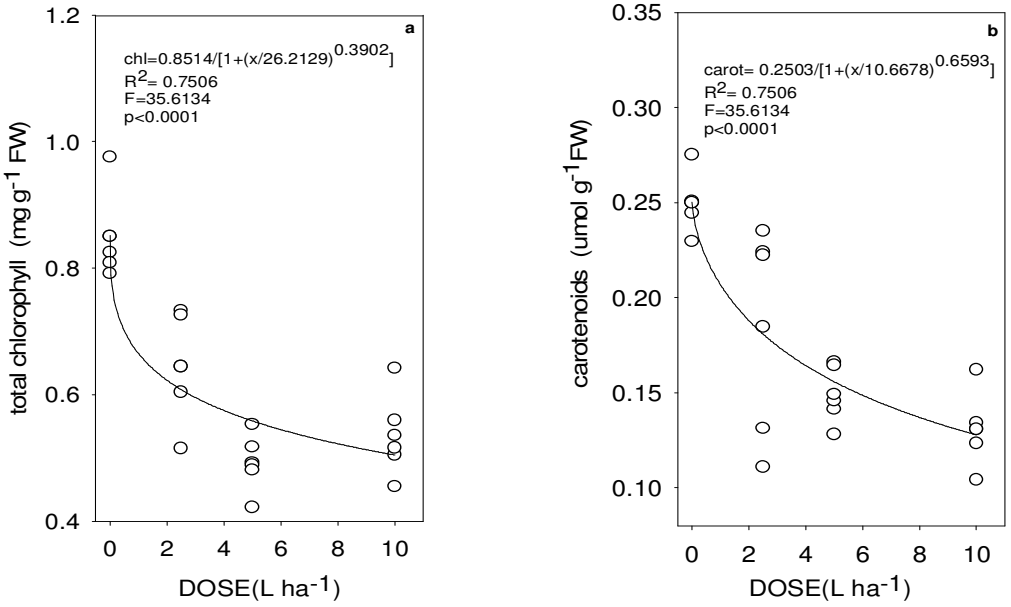

Figure 1. Chlorophyll (a) and carotenoids (b) content of *E. coccinea* seventy-two hours after post-emergence application of a mixture of clomazone + ametryn.

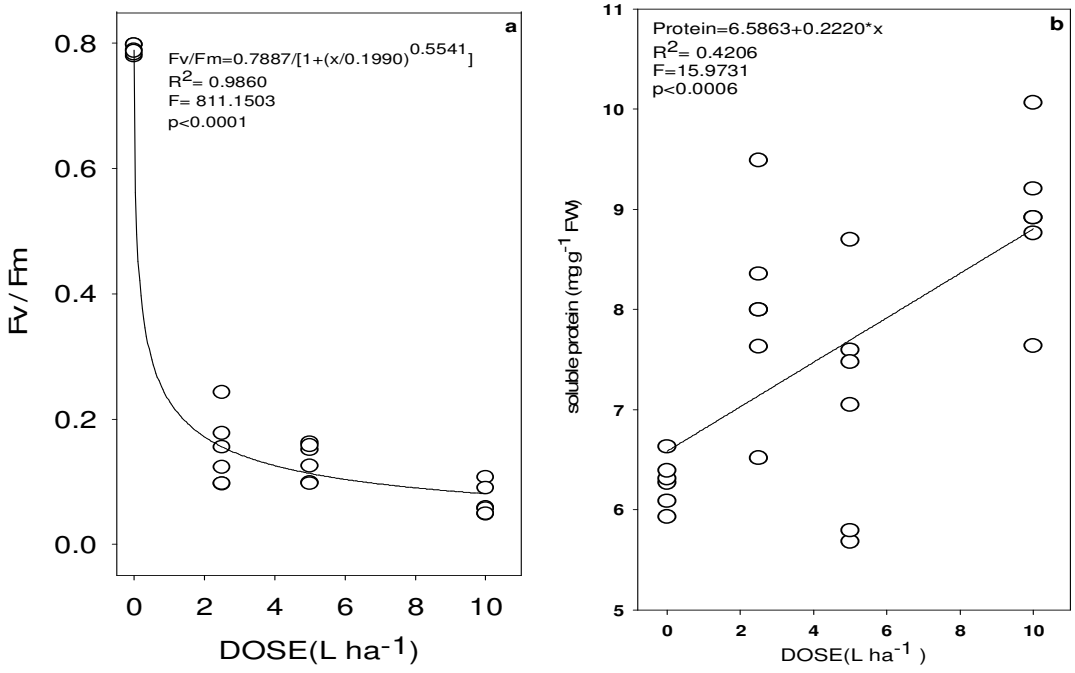

Figure 2. Maximum quantum efficiency of PSII (a) and soluble proteins (b) of *E. coccinea* seventy-two hours after post-emergence application of mixture clomazone + ametryn

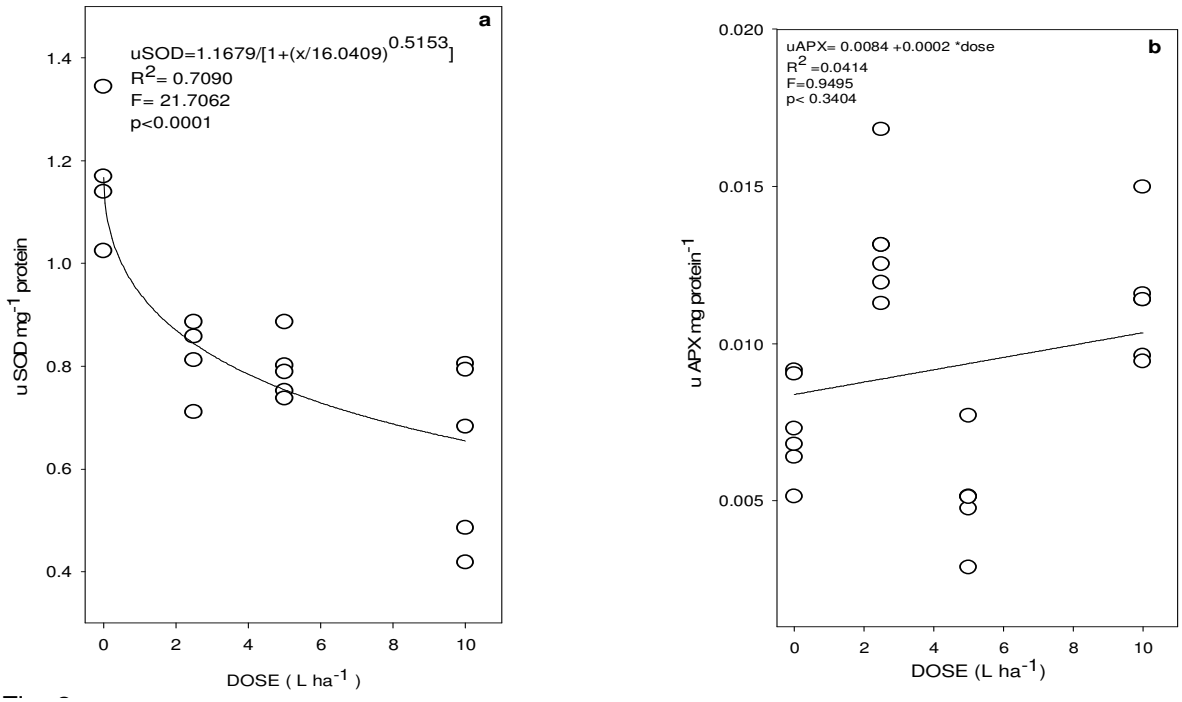

Figure 3. Superoxide dismutase (a) and ascorbate peroxidase (b) activity of *E. coccinea* seventy-two hours after post-emergence application of a mixture of clomazone + ametryn.

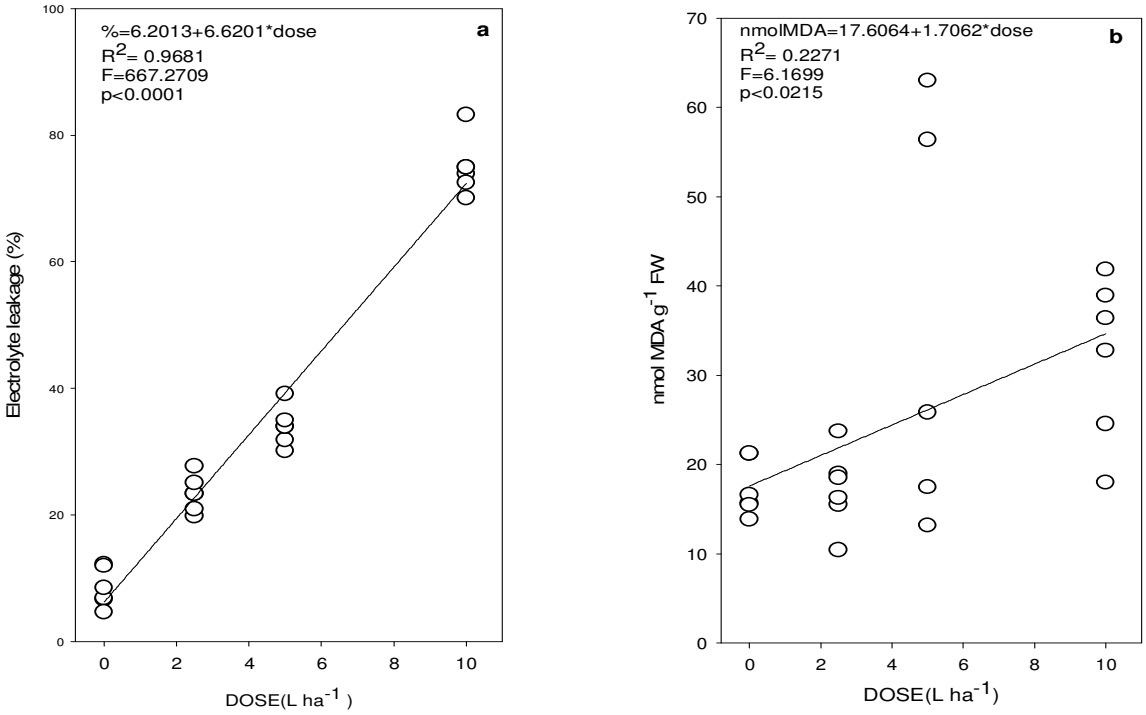

Figure 4. Electrolyte leakage (a) and Malonyldialdehyde (MDA) content (b) of *E. coccinea* seventy-two hours after post-emergence application of a mixture of clomazone + ametryn.

Table 2. ANOVA parameters of the experiment with *E. coccinea* submitted to doses of ametryn + clomazone in post-emergence

DOSE (L ha^{-1})	CHL	CAR	Fv/Fm	PROT	SOD	APX	LEAK.	MDA
CV (%)	10.25	16.63	11.99	11.34	18.73	19.16	9.74	47.18
F	36.592	18.751	589.723	10.793	16.551	25.857	415.95	3.735
p<	0.0001	0.0001	0.0001	0.0002	0.0001	0.0001	0.0001	0.0289

CHL = Chlorophyll content (mg g^{-1} FW); CAR= carotenoids (µmol g^{-1} FW); Fv/Fm= Maximum quantum efficiency of PSII; prot= Soluble proteins (mg g^{-1} FW); SOD= Superoxide dismutase activity (uSOD mg $^{-1}$ protein); APX= ascorbate peroxidase activity (uAPX mg $^{-1}$ protein); LEAK= Electrolyte leakage (% of total leaked); MDA= Malonyldialdehyde (nmol MDA g^{-1} FW)

Table 3. Average percentage of live and dead plants from *E. coccinea* 25 days after application of a mixture of clomazone + ametryn, with the result of the χ^2 test.

DOSE(L ha^{-1})	LIVE (%)	DEAD (%)
2.5	36.25	63.75
5.0	16.25	83.75
10.0	0.00	100.00
Total(%)	17.57	82.43
χ^2	36.20	
p<	0.0001	

blocks the activity of 1-deoxy-D-Xylulose-5-P (DOXP) synthase in the 2-C-Methyl-D-erythritol-4-P (MEP) pathway (Mueller et al., 2000). However, this fact has been reported too, by the effect of several molecules in different species, including *Hordeum vulgare* (Kana et al., 2004), *M. urundeuva* (Duarte et al., 2006) and *Ananas comosus* (Catunda et al., 2005).

The observed decrease in photosynthetic pigments was related to ametryn, the second active ingredient of the herbicide mixture used in this study. Ametryn blocks the electron transport between Q_A and Q_B, leading to the formation of free radicals (O_2^- and Chl^3) by unused energy from the photosynthetic process. These free radicals react with important molecules such as chlorophyll in the chloroplast and unsaturated lipids, leading to a decline in the content of photosynthetic pigments (Devine and Shukla, 2000).

Ametryn is likely the cause of the decline in Fv / Fm. Ametryn is a triazine; molecules belonging to this group inhibit the passage of electrons in photosystem II (PSII), possibly by linking quinone B and thereby preventing the oxidation of quinone A (Holt, 1993). Matouškova et al. (1999) argue that even more importantly than blocking the flow of electrons, the herbicides that act on PSII inhibit the rapid turnover of the D1 protein. Damage to this protein cannot be repaired; the protein must be replaced by a complete re-synthesis of peptides followed by their re-assembly in PSII, which is inhibited by ametryn molecule. Several authors have reported a decrease in quantum efficiency linked to environmental stresses (Baker, 2008), but clomazone alone caused small changes in the quantum efficiency of primary leaves of barley subjected to doses of 0.25 and 0.5 mM (0.0599 g L^{-1} and 0.1198 g L^{-1}), showing that the direct damage to PSII is caused by the ametryn contained in the herbicide mixture (Kana et al., 2004).

Increases in the protein contents of plants can be related to various stresses. Abiotic stresses, such as drought, high or low temperatures and nutrient deficiency, can lead to the accumulation of low-molecular-weight proteins (100 kDa) that assist in maintaining the survival of species under adverse conditions. These proteins play roles in the synthesis of other proteins as well as the removal of non-functional and degraded proteins (Waters et al., 1996). Zhu et al. (2009) studied gene expression in soybean in relation to the application of PSII-inhibiting herbicides (atrazine and bentazon) to investigate the resistance of this species to bentazon. The authors noted that the differentially expressed genes included those involved in xenobiotic detoxification; antioxidants, such as cytochrome P450s, glutathione S-transferase, SOD and peroxidases; as well as genes related to cell recovery,

such as ribosomal components.

On the other hand, in 10-day-old seedlings of wheat and maize, Alla et al. (2008) observed a decrease in the formation of proteins as well as a shortage in ammonia assimilation and accumulation of soluble nitrogen caused by the herbicide metribuzin, which is also an inhibitor of PSII. The effect of metribuzin on protein synthesis was responsible for a decrease in the activities of glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.7.1). Although these results contrast with those of the present study, it is noteworthy that the PSII inhibitor molecule used in the study mentioned is a triazine, a different chemical group than the herbicides used in this study. The studies also differed in terms of plant species and age.

Biotic, abiotic and xenobiotic factors can alter the activity of SOD in several species; for example, exposure of wheat seedlings to ozone caused an initial increase in SOD activity, but this increase was reversed when the time of exposure to O_3 was increased (Rao et al. 1995). On the other hand, drought caused an increase in the generation of ROS and consequently an increase in SOD activity in maize seedlings (Jiang and Zhang, 2002). SOD acts as the first line of defense against the harmful effects of ROS, in particular O_2^-, transforming it into less reactive compounds (Asada, 1999).

A decrease in SOD activity, which occurred in this study, was also observed in other studies of PSII-inhibiting herbicides (linuron, atrazine), singly or in combination with other active ingredients. Malenčić et al. (2008) studied the effects of pre-emergence application of a mixture of linuron (an inhibitor of PSII) and dimethenamid (an inhibitor of cell division) in soybean plants and some weed species. They found that SOD activity increased significantly in *Chenopodium album*, *Convolvulus arvensis* and *Ambrosia artemisiifolia*. However, enzyme activity in *Glycine max* was reduced by the herbicide mixture. The post-emergence application of atrazine (an inhibitor of PSII) to beans (*Vicia faba*) and maize (*Zea mays*) also decreased the activity of SOD in both species (Hassan and Alla, 2005).

Like SOD, the activity of APX is also affected by various environmental causes and in various species, and the behavior of this enzyme activity is usually related to the SOD (Bowler et al., 1992). In studies Alla and Hassan (2005), where SOD activity was decreased, the APX activity also decreased in 10-day-old seedlings of maize and beans, two days after the application of PSII-inhibiting herbicides. However, in studies with paraquat-resistant and susceptible biotypes of *Erigeron canadensis*, the activities of SOD and APX in the resistant biotypes showed similar behavior, with increases 12 hours after herbicide application (Pyon et al., 2004). The decreases in SOD and APX activities may have reduced the scavenging of ROS generated by the mixture of clomazone + ametryn. This accumulation of free radicals may also have reflected the reduction in pigment content.

According to Bowler et al. (1992) the activity of all isoforms of SOD together with other enzymes acting in the scavenging of ROS, such as CAT and GR, decreased when plants entered senescence. Thus, at 72 HAA, the plants already showed signs of necrosis, and the leaf tissue could have been in an advanced state of degradation. Increasing doses of the herbicide mixture led to greater induced senescence. With the decrease in SOD and APX activities, the ability to maintain acceptable levels of cellular ROS was lost, and important biomolecules for cell function, such as lipids, were degraded. Thus, further studies should be performed at shorter intervals after application of this herbicide mixture, to better clarify the roles of SOD and APX in the susceptibility of *E. coccinea*.

Biological membranes are generally regarded as selective, allowing the entry and exit of substances according to their size and nature. The loss of selectivity may be indicative of damage to the structures of the unsaturated fatty acids that make up the biological membranes of cellular organelles due to the action of ROS on these membranes. Various environmental stresses, such as cold, drought and high temperatures, can cause a loss of membrane stability (Bajji et al., 2001).

The increase in MDA content has been directly related to lipid peroxidation in various species due to environmental factors such as cold stress in rice seedlings (Kuk et al., 2003) and boron toxicity in tomato (Cervilla et al., 2007). Alla and Hassan (2005) also found an increase in the MDA content of maize and bean seedlings after exposure to PSII-inhibiting herbicides. The increased levels of MDA corroborated that electrolyte leakage had occurred. *E. coccinea* showed high electrolyte leakage and MDA content, thus emphasizing that the main deleterious effect of the herbicide mixture clomazone + ametryn is damage to the membranes.

The dose-dependent electrolyte leakage presented by *E. coccinea* was related to plant mortality at 25 days after application. Thus, this parameter can be a good indicator of the damage caused by the mixture of ametryn + clomazone.

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