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Article

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Activity of Antioxidant Enzymes in *Euphorbia heterophylla* Biotypes and their Relation to Cross Resistance to ALS and Protox Inhibitors

*Atividade de Enzimas Antioxidantes em Biótipos de **Euphorbia heterophylla** e sua Relação com a Resistência Cruzada aos Inibidores da ALS e da Protox*

ABSTRACT - The characteristics of multiple resistance in *Euphorbia heterophylla* biotypes to herbicides that are inhibitors of ALS (Acetolactate synthase) and PPO (Protoporphyrinogen oxidase) and their responsible mechanisms are still not completely elucidated. The objectives of this study were to identify cross-resistance to herbicides from different chemical groups of ALS inhibitors (imidazolinones, sulfonylureas, pyrimidyl benzoates and sulfonanilides) and also PPO inhibitors (diphenylethers, phthalamides, oxadiazoles, triazolinones and pyrimidinediones) in *E. heterophylla* biotypes with multiple resistance to these herbicides; to analyze whether the antioxidant enzymes superoxide dismutase (SOD) and peroxidase (POD) constitute mechanisms that are responsible for the resistance to PPO inhibitors. Initially, the response to doses of herbicides from these different chemical groups was determined, using doses below and above the one recommended for the species. The control of *E. heterophylla* was determined, estimating the required doses for a 50 and 80% control reduction and calculating the resistance factors. The constitutive and induced activities of the SOD and POD enzymes were also determined. The results confirmed cross-resistance for all chemical groups of ALS and PPO inhibitors in the Bom Sucesso do Sul and Vitorino biotypes. The constitutive and induced activities of the SOD and POD enzymes were superior in plants from the *E. heterophylla* biotypes Vitorino and Bom Sucesso do Sul, contributing to their resistance to PPO inhibiting herbicides.

Keywords: dose-response curves, superoxide dismutase, peroxidase, acetolactate synthase, protoporphyrinogen oxidase.

*RESUMO - As características da resistência múltipla em biótipos de **Euphorbia heterophylla** a herbicidas inibidores da ALS (acetolactato sintase) e da PPO (Protoporfirinogênio oxidase) e os mecanismos responsáveis ainda não estão completamente elucidados. Os objetivos deste estudo foram identificar a existência de resistência cruzada a herbicidas de diferentes grupos químicos de inibidores da ALS (imidazolinonas, sulfonilureias, pirimidil-benzoatos e sulfonanilidas) e também de inibidores da PPO (difeniléteres, ftalamidas, oxadiazoles, triazolinonas e pirimidinedionas) em biótipos de **E. heterophylla** com resistência múltipla a esses herbicidas; analisar se as enzimas antioxidantes superóxido dismutase (SOD) e peroxidase (POD) constituem mecanismos responsáveis pela resistência a inibidores da PPO. Inicialmente, foi determinada a resposta a doses de herbicidas desses diferentes grupos químicos, utilizando doses abaixo e acima da recomendada para a espécie. O controle de **E. heterophylla** foi determinado, estimando-se as doses necessárias para redução de 50 e 80% de controle, e calcularam-se os*

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fatores de resistência. Foram determinadas também as atividades constitutivas e induzidas das enzimas SOD e POD. Os resultados confirmaram a resistência cruzada a todos os grupos químicos de inibidores da ALS e da PPO nos biótipos Bom Sucesso do Sul e Vitorino. As atividades constitutivas e induzidas das enzimas SOD e POD foram superiores nas plantas dos biótipos de ***E. heterophylla*** *Vitorino e Bom Sucesso do Sul, contribuindo para sua resistência aos herbicidas inibidores da PPO.*

Palavras-chave: curvas de dose-reposta, superóxido dismutase, peroxidase, Acetolactato sintase, *protoporfirinogênio oxidase.*

INTRODUCTION

The use of herbicides represented a major advance for weed management in agriculture (Vidal et al., 2006), creating the basis for their control (Norsworthy et al., 2012). However, after consecutive years using of these products, many cases of resistance have emerged, currently reaching 255 resistant (R) weeds in the world (Heap, 2018).

The largest record number of single-resistance and multiple resistance weed biotypes in the world belongs to acetolactate synthase (ALS) inhibitors (Heap, 2018). The ALS enzyme, also known as acetoxyacetic acid synthase (AHAS), catalyzes the condensation of two pyruvates to form acetolactate, or one pyruvate with 2-ketobutyrate to produce acetohydroxybutyrate along the route of synthesis of the valine, leucine and isoleucine amino acids (Hess 1994). This mechanism has the highest number of herbicides registered in the market, which have gained popularity due to their broad spectrum of action, low recommended doses, low toxicity and selectivity to various crops (Monqueiro et al., 2000).

For a proper control of R weed populations, it is necessary to use herbicides with different mechanisms of action mechanisms. The herbicides inhibiting the protoporphyrinogen oxidase enzyme (PPO) are used as an alternative action mechanism to control resistant plants, mainly ALS inhibitors (Monqueiro and Christoffoleti, 2001). However, the inappropriate use of PPO inhibiting herbicides is leading to the emergence of resistant species. The PPO enzyme is responsible for catalyzing the oxidation phase of protoporphyrinogen-IX to protoporphyrin-IX in the route of chlorophyll and cytochrome synthesis (Merotto Jr and Vidal, 2001).

Euphorbia heterophylla, popularly known as wild pointsettia, is a widely disseminated weed in Brazil and has a high competitive capacity with commercial crops (Kissmann and Groth, 1992). Since the 1990s, the main selective herbicides used to control soybean in Brazil and the world have been ALS and PPO inhibitors (Merotto Jr and Vidal, 2001; Salas et al., 2016). *E. heterophylla* resistance to ALS inhibitors was detected in Brazil and Paraguay (Heap, 2018). In the State of Paraná and western Santa Catarina, wild pointsettia populations that are resistant to ALS inhibiting herbicides are widely distributed (Winkler and Vidal, 2004). In southwestern Paraná, Trezzi et al. (2005) identified two biotypes of E. *heterophylla* with multiple resistance to imazethapyr (ALS inhibitor) and fomesafen (PPO inhibitor).

In recent years, with the detection of new *E. heterophylla* biotypes suspected of multiple resistance to ALS and PPO inhibitors (Trezzi et al., 2011; Xavier et al., 2013), there is a need to prove this resistance and to investigate its responsible mechanism, in order better manage these biotypes.

In several species, both tolerance and resistance to PPO inhibiting herbicides may occur through different mechanisms, such as those linked to the action site, the increase of the PPO enzyme concentration in the mitochondria (Warabi et al., 2001), the overexpression of genes that encode the PPO enzyme (Jung and Kuk, 2007) and the mutation in the gene encoding the action site (Dayan and Duke, 1997). Among the mechanisms that are not related to the herbicide action site, there is the high translocation associated with rapid metabolization (Eastin, 1971), the reduced absorption (Silva et al., 2007), the high concentration of the glutathione S-transferase enzyme (Frear and Swanson, 1973), the rapid metabolization (Thomas et al., 2005) and the high levels of antioxidants and enzymes that eliminate toxic oxygen species (Duke et al., 1997).

Some action mechanisms, such as PPO inhibiting herbicides, are capable of generating oxidative stress in plants, thus damaging cells through the production of free radicals and/or

reactive oxygen species (ROS) (Mori and Schroeder, 2004).). Plants have defense systems against stresses that involve the formation of ROS, such as membrane-associated liposoluble antioxidants, like α-tocopherol and β-carotene, water-soluble reducers like tripeptide glutathione (GSH) and ascorbate, and antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidases (POD, EC 1.11.1.7), among others (Elstner, 1982). These defense mechanisms allow reducing the damages created by the action of ROS, which could be one of the mechanisms responsible for the resistance to PPO inhibitors. The objectives of this work were: (a) to determine the cross-resistance to herbicides from the chemical groups of imidazolinones, sulfonylureas, pyrimidyl benzoates and sulfonanilides (ALS inhibitors) and the chemical groups of diphenylethers, phthalamides, oxadiazoles, triazolinones and pyrimidinediones (PPO inhibitors) in *E. heterophylla* biotypes suspected of multiple resistance to ALS and PPO inhibitors, in the state of Paraná; and (b) to determine whether the activity of the superoxide dismutase and peroxidase enzymes constitutes a mechanism that is responsible for the multiple resistance to ALS and PPO inhibitors.

MATERIAL AND METHODS

Experiment 1- Response to doses of ALS and PPO enzyme inhibitors

The experiments were conducted in a greenhouse, in a completely randomized design with four replications. Susceptible (S) and resistant (R) *E. heterophylla* biotypes and biotypes with suspected resistance to ALS and PPO inhibiting herbicides were used. The used biotypes were called Bom Sucesso do Sul (biotype with suspected resistance), Vitorino (R) and Susceptible (S), coming from Bom Sucesso do Sul (Paraná), Vitorino (Paraná) and São Paulo (São Paulo), respectively. The choice of the S biotype from São Paulo aimed at genotypically and phenotypically ensuring the expression of the susceptibility characteristic in the population.

Seeds from the biotypes were pre-germinated in gerbox boxes, containing germitest paper, in a BOD-type growth chamber; when seedlings reached 3 to 5 cm, they were transplanted to pots containing soil, with a capacity of 5 dm^3. The herbicides were applied to plants with two true leaves, using a sprayer with constant CO_2 pressure, with 110.02 fan-type nozzles and spraying volume of 200 L ha^{-1}. The environmental conditions at the beginning and at the end of the applications were the following: air temperature = 18.6 to 23.5 °C, and relative air humidity = 73.5% to 61%.

Two tests were the dose-response curves of ALS inhibitors. In the first test, the S biotype of *E. heterophylla* was used. Treatments were arranged in a 4 x 7 factorial arrangement; the first factor was constituted by ALS inhibiting herbicides [imazethapyr (imidazolinone), nicosulfuron (sulfonylurea), pyrithiobac (pyrimidyl benzoate) and diclosulan (sulfonanilide)] and the second one by concentrations of herbicides. The seven ratios of the commercial dose were: 0; 0.1; 0.16; 0.26; 0.41; 0.66; and once the commercial dose. In the second test, the *E. heterophylla* biotypes Vitorino and Bom Sucesso do Sul were used, which are resistant and suspected to be resistant to ALS/PPO inhibitors. Treatments were arranged in a 2 x 4 x 7 factorial arrangement (biotypes x herbicides x dose ratios). The same herbicides from the trial with the S biotype were used, and the commercial dose ratios of the four herbicides were 0; 1; 1.8; 3.1; 5.4; 9.4; and 16.4 times the commercial dose; each dose of the respective herbicide was applied alone. The commercial doses used in this work for imazethapyr, nicosulfuron, diclosulan and pyrithiobac were 100, 60, 35 and 42 g a.i. ha^{-1}, respectively. Only pyrithiobac had an addition of 0.5% v/v mineral oil, as recommended by the manufacturer.

Two other tests consisted of dose-response curves for PPO inhibitors. In the first test, the S biotype was used. Treatments were arranged in a 5 x 7 factorial arrangement; the factors were PPO inhibitors [fomesafen (diphenyl ether), flumiclorac (phthalamide), carfentrazone (triazolinone), oxadiazon (oxadiazol) and saflufenacil (pyrimidinedione)] and the commercial dose ratios of the herbicides (0, 0.1, 0.16, 0.26, 0.41, 0.66, and 1 times the commercial dose). The second test consisted of a 2 x 5 x 7 factorial arrangement, in which the first factor was constituted by the Vitorino and Bom Sucesso do Sul biotypes, the second one, by the same herbicides used in the test with the S biotype, and the third one, by the commercial dose ratios of the herbicides (0; 1; 1.8; 3.1; 5.4; 9.4; and 16.4 times the commercial dose). The commercial doses of fomesafen,

flumiclorac, carfentrazone, oxadiazon and saflufenacil used in this study were 250, 60, 30, 1,000 and 35 g i.a. ha^{-1}, respectively. For fomesafen, a non-ionic/anionic adhesive spreader was added at the concentration of 0.2% v/v; for carfentrazone, 0.5% v/v of mineral oil was added; for flumiclorac, 0.2% v/v of mineral oil was added; and for saflufenacil, 0.5% v/v of non-ionic adjuvant was added, as recommended by the manufacturers.

Twenty-one days after application (DAA), visual evaluations were performed, taking into account the injury, based on chlorosis, withering and necrosis in plants, through a scale where 0% represents the absence of control and 100% represents plant death (Frans et al., 1986).

Data were submitted to analysis of variance by F test ($p<0.05$). The relations between dependent variables and herbicide concentrations were adjusted through the non-linear regression model, using the three-parameter logistic model (Equation 1), with the help of the SigmaPlot 10.0 software:

$$Y = \frac{a}{1 + \left(\frac{x}{x_0}\right)^b} \quad \text{(eq. 1)}$$

where: y = dependent variable; a = maximum asymptote; x = herbicide concentration; x_0 = concentration that provides 50% control; and b = slope of the curve.

The standard error of the mean was calculated by the quotient between standard deviation and square root of the sample size. The doses required to provide 50 and 80% control (C_{50} and C_{80}) were also calculated through the Microsoft Excel software. The resistance factors (RF) were calculated by the quotient between C_{50} of the R and S biotypes.

Experiment 2- Determination of the activity of the superoxide dismutase and peroxidase enzymes

The activities of the superoxide dismutase (SOD) and peroxidase (POD) enzymes were determined in plants treated with PPO inhibitors. In the pots, extra plants were transplanted to harvest samples of *E. heterophylla* seedlings grown in greenhouse, which were carried out 0, 24, 48 and 72 hours after application (HAA) of the herbicides, by cutting plants close to the soil, with immediate identification, wrapping in aluminum foil and freezing in liquid nitrogen (-196 °C). Afterwards, they were taken to the laboratory, where they were stored for two days in a freezer at -40 °C for further determination of the activities of SOD and POD enzymes. SOD activity was determined by the inhibition of the photoreduction of nitrotetrazolium blue chloride (NBT). Fifty-µL aliquots of the enzyme extract were transferred to test tubes containing 3 mL of 0.05 M potassium phosphate buffer, pH 7.8, with 100 nM EDTA, 13 mM methionine, 75 µM NBT and 2 µM riboflavin; this solution was prepared under conditions of minimum illumination. The reaction was conducted at 25 °C in a reaction chamber, under the illumination of a 15 W fluorescent lamp. After five minutes, the reaction was stopped by turning off the lights and readings were performed with a spectrophotometer (Shimadzu 1800 UV) at 560 nm. Controls (100% photoreduction) received all reagents, except the sample (enzyme extract), and were submitted to the light, in order to determine the total NBT photoreduction. One activity unit (AU) was considered as the amount of enzyme required to inhibit 50% of the NBT photoreduction, compared to the reaction medium without the enzyme extract. The specific activity of SOD was expressed as U μg^{-1} of protein. This methodology was proposed by Giannopolitis and Ries (1977), Beauchamp and Fridovich (1971) and Del Longo et al. (1993).

The activity of the POD enzyme was analyzed by the addition, in a cuvette, of 900 µL of 0.1 M potassium phosphate buffer pH 6.0, containing, for each 100 mL of buffer, 250 µL of 0.2 M guaiacol and 306 µL of hydrogen peroxide, and 100 µL of the enzyme extract. The activity was determined by increasing the absorbance to 470 nm, at 30 °C for 3 minutes, at 60-second intervals. The POD activity was expressed as a variation of absorbance units (UA min^{-1} μg^{-1} of proteins), according to the method described by Lusso and Pascholati (1999). The activities of SOD and POD were expressed in the constitutive form, which is the activity of the enzyme without herbicide

application, and the induced form, which is the activity of the enzymes after the application of the herbicides. Activity in the induced form was determined only when the herbicides were applied, not considering the constitutive activity in this expression, and it was relativized in relation to the dose 1x, with the S biotype. Data were submitted to analysis of variance by F test ($p<0.05$). The standard error of the mean was calculated by the quotient between standard deviation and square root of the sample size.

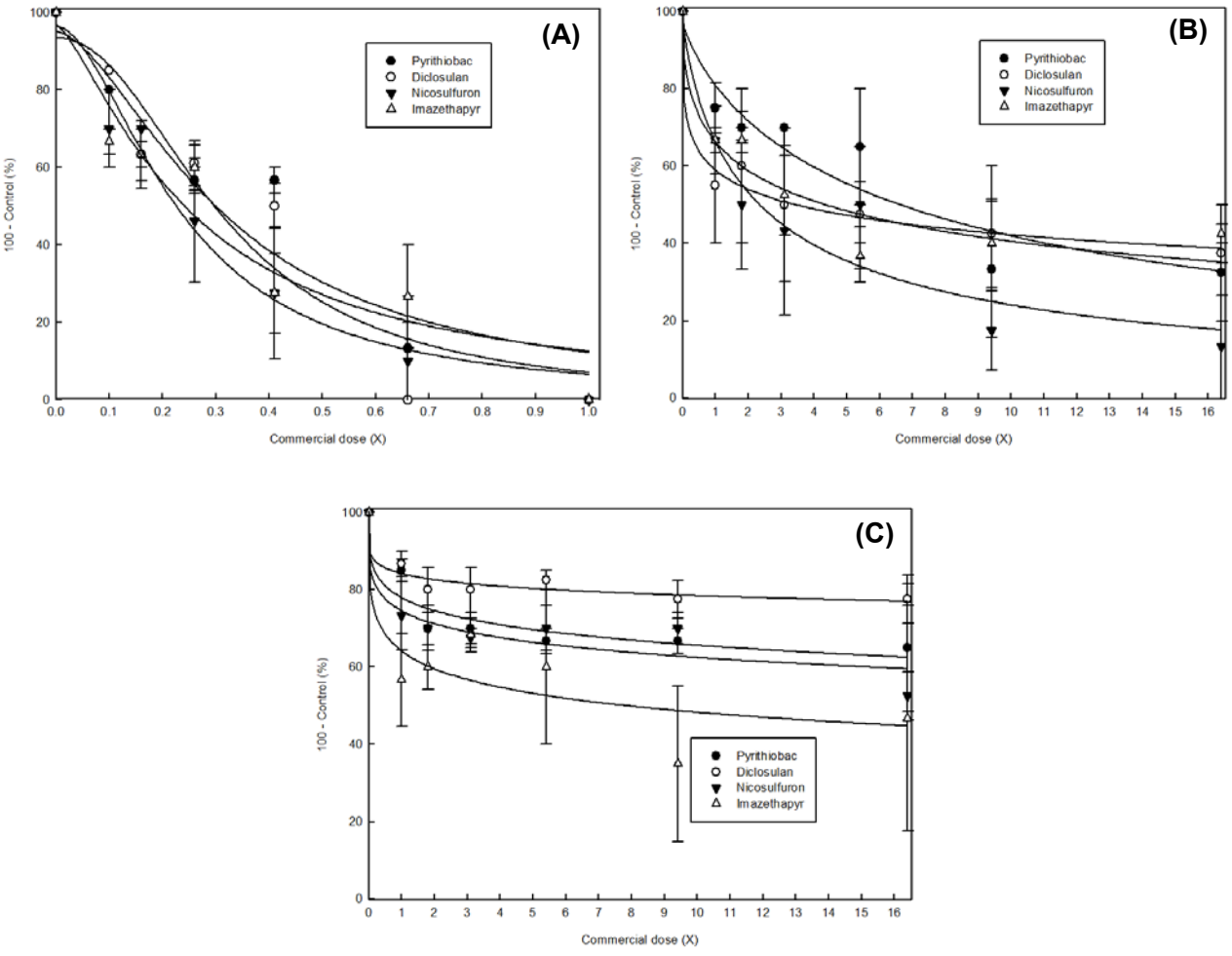
RESULTS AND DISCUSSION

Experiment 1 - Response to doses of ALS and PPO enzyme inhibitors

In both experiments, there was an increase in the control levels with higher doses of ALS and PPO inhibitors, with significantly higher values for the S biotype in relation to the R ones (Figures 1 and 2).

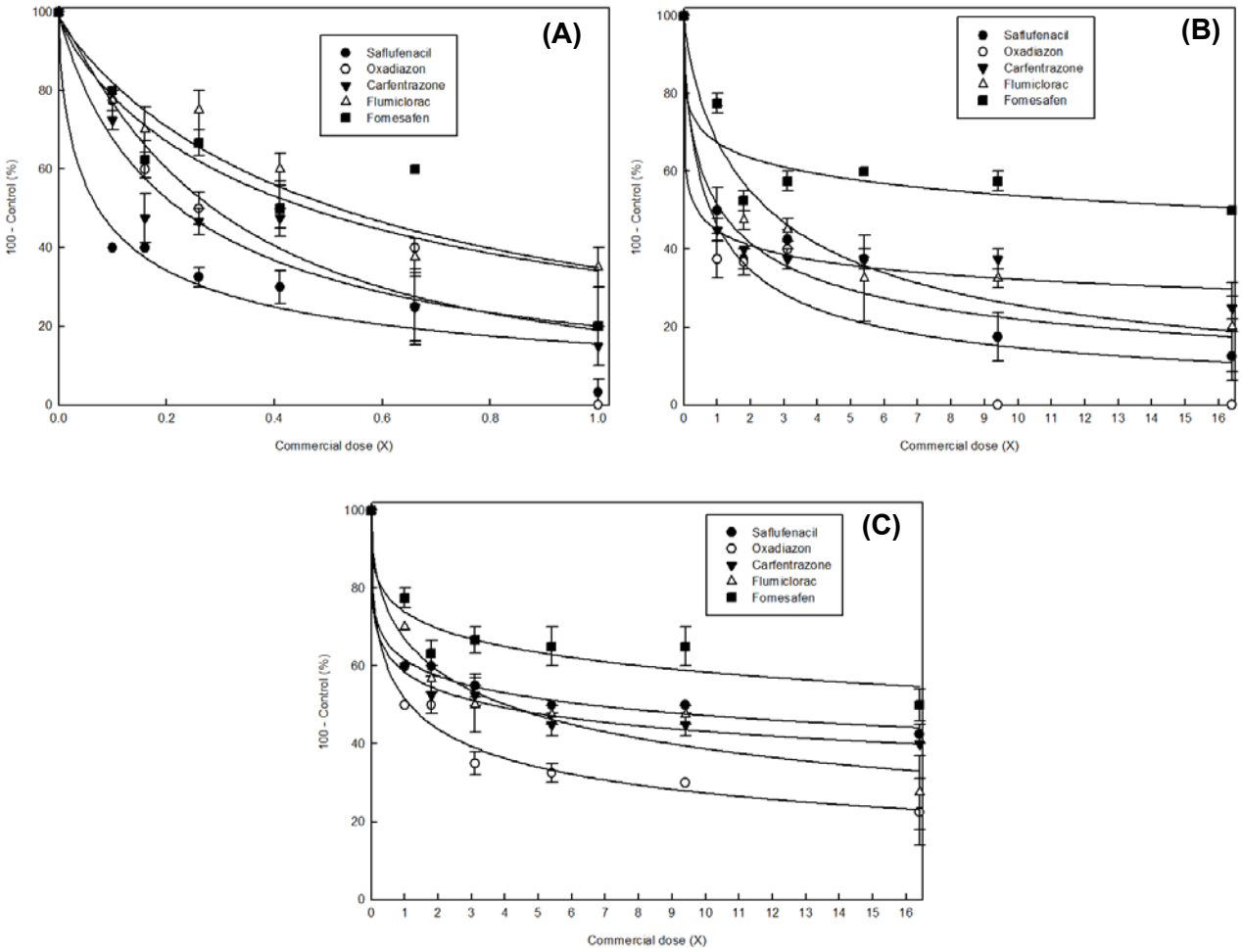
The 1x dose of all tested ALS inhibitors, common to all biotypes, resulted in 100% control of plants from the S biotype (Figure 1A). For this dose, R biotypes had a maximum control level of 45%, which occurred with the application of diclosulan on the Vitorino biotype (Figure 1B). With the higher dose used on R biotypes, the maximum control level was 85% for the Vitorino biotype with the use of nicosulfuron (Figure 1B).

The C_{50} and C_{80} parameters of the Vitorino and Bom Sucesso do Sul biotypes were higher than those obtained by the S biotype. The RF's of the Vitorino biotype were higher than 10 and



Vertical bars represent the standard error of the mean of each treatment.

Figure 1 - Control [100- Control(%)] of susceptible (A), Vitorino (B) and (C) Bom Sucesso do Sul *E. heterophylla* biotypes, 21 days after the application of different ALS inhibiting herbicides.



Vertical bars represent the standard error of the mean of each treatment.

Figure 2 - Control [100 - Control (%)] of susceptible (A), Vitorino (B) and Bom Sucesso do Sul (C) biotypes, 21 days after the application of different PROTOX inhibiting herbicides.

those of the Bom Sucesso do Sul biotype were higher than 33 (Table 1). These RF's, together with the control data, confirm the resistance of these biotypes to the four chemical groups of the tested ALS inhibitors.

In literature, different values of RF are found in tests with *E. heterophylla* R biotypes and different ALS inhibiting herbicides, as observed in this study (Table 1). Testing the effect of chlorimuron-ethyl and imazethapyr herbicides on *E. heterophylla*, Gelmini et al. (2001) found RF's of 19 and 26 for the control variable, and of 22 and 23 for the phytomass, respectively. Trezzi et al. (2005) calculated the RF's for ALS and PPO inhibitors in biotypes collected in the municipalities of Vitorino and Pato Branco. The application of imazethapyr resulted in an RF above 24 for the Vitorino biotype, while for the Pato Branco biotype, it was 15. In other words, previously conducted studies had already confirmed the resistance to ALS and PPO inhibitors in the Vitorino biotype, but the cross-resistance to different chemical groups has not been determined yet.

Reports from owners of areas with resistance problems indicate that control difficulties with ALS inhibitor started in 2002 and 2003, respectively, for the Vitorino and Bom Sucesso do Sul biotypes, both with a history of intensive use of ALS inhibitors. Most cases of the studied resistance to ALS inhibitors show cross-resistance to the selecting herbicide and also to several chemical groups of herbicides with the same action mechanism (Rizzardi et al., 2002; Tranel et al., 2016). This is explained by the fact that most cases of ALS-resistant biotypes present as a resistance mechanism the alteration in the active site of the ALS enzyme, which makes it insensitive to

Table 1 - Parameters of the logistic equation[(1)] used to determine the dose of ALS inhibiting herbicides (g a.i. ha^{-1}) required to obtain 50% and 80% control, (C_{50}) and (C_{80}), for susceptible and resistant biotypes (Vitorino and Bom Sucesso do Sul), 21 days after the application (DAA) of treatments, and resistance factors (RF)

Biotype	Herbicide	Parameters[(1)]				C_{50}**	C_{80}**	FR
		A	B	C_{50}*	R^2			
Susceptible	Pyrithiobac	95	1.68	13.4	0.95	12.6	30	-
	Diclosulan	93	2.18	11.1	0.95	10.5	20	-
	Nicosulfuron	96	1.81	14.1	0.99	13.6	29	-
	Imazethapyr	97	1.38	25.3	0.96	24	67	-
Vitorino	Pyrithiobac	98	0.80	294	0.96	278	>689	22
	Diclosulan	100	0.30	121	0.99	121	>574	12
	Nicosulfuron	100	0.80	143	0.97	141	810	10
	Imazethapyr	100	0.46	428	0.96	435	>1640	18
Bom Sucesso do Sul	Pyrithiobac	100	0.27	>689	0.95	>689	>689	>54
	Diclosulan	100	0.17	>574	0.97	>574	>574	>54
	Nicosulfuron	100	0.25	>984	0.95	>984	>984	>72
	Imazethapyr	100	0.28	796	0.91	780	>1640	33

[(1)] Logistic equation of 3 parameters $f= a/(1+(x/x_0)^b)$; A = Maximum asymptote, B = Curve slope. C_{50} = Dose (g a.i.ha^{-1}) giving 50% control. C_{80} = Dose (g a.i.ha^{-1}) required to generate 80% control. *Values adjusted through the SigmaPlot software. ** Values estimated through the Microsoft Excel software.

herbicides from different chemical groups (Christoffoleti, 2001; Xavier et al. 2013). However, there are cases of cross-resistance to ALS inhibitors in which the resistance mechanism is not related to the action site, such as absorption difficulty (Plaza et al., 2006), reduced translocation (Riar et al., 2013) and high metabolization of the herbicide (Park et al., 2004); therefore, other mechanisms should be investigated.

Changes in the action site usually result from mutations in the genes encoding the enzyme, which alters its conformation and activity (Devine and Shukla, 2000), resulting in a reduction in its affinity for herbicides (Tranel and Wright, 2002). Several cases of ALS mutations that cause weed resistance to ALS inhibitors are described in literature. Mutations are found at the following positions: alanine 122 (Ala122), alanine 205 (Ala205), proline 197 (Pro197), serine 653 (Ser653), tryptophan 574 (Trp574), arginine 377 (Arg 377), aspartate 376 (Asp 376) and glycine 654 (Gly 654) (Tranel et al., 2016).

In a study conducted with the same R biotypes used in this study, Xavier et al. (2013) detected a reduced effect of the herbicides imazapyr, imazethapyr and nicosulfuron on the ALS activity of the Vitorino and Bom Sucesso do Sul biotypes and, in opposition, a high effect on the activity of the susceptible biotype, indicating insensitivity of the ALS enzyme in resistant biotypes. However, the mutation responsible for the resistance mechanism in these biotypes has not been determined so far. Nonetheless, because of the cross-resistance to all tested chemical groups, it can be inferred that the possible responsible mutations may be in tryptophan at position 574, in alanine at position 205, in aspartate at position 376, and in serine at position 653 (Tranel et al., 2016).

The 1x dose of the PPO inhibitors, which is common to all biotypes, resulted in control levels of the S biotype above 65% (Figure 2A). For this dose, R biotypes had a maximum control level of 62%, obtained with the application of oxadiazon on the Vitorino biotype (Figure 2B). For the 16.4x dose, the control levels of R biotypes were at least 50% with the application of fomesafen on Bom Sucesso do Sul and 100% at most, that is, plant death, with the application of oxadiazon on Vitorino (Figure 2B, C).

For the control variable, the RF's of the Vitorino and Bom Sucesso do Sul biotypes reached a range of minimum values between 1.9 and 3.9 and maximum values between 38.7 and 88.7, respectively (Table 2). These RF's confirm the resistance of these biotypes to the five tested chemical groups of PPO inhibitors. In all the evaluated biotypes, the RF's for saflufenacil and fomesafen were higher than those of the other herbicides, standing out because of very high

Table 2 - Parameters of the logistic equation[(1)] used to determine the dose of PROTOX inhibiting herbicides (g a.i. ha^{-1}) required to obtain 50% and 80% control, (C_{50}) and (C_{80}), for susceptible and resistant biotypes (Vitorino and Bom Sucesso do Sul), 21 days after the application (DAA) of treatments, and resistance factors (RF)

Biotype	Herbicide	Parameters[(1)]				C_{50}**	C_{80}**	FR
		A	B	C_{50}*	R^2			
Susceptible	Saflufenacil	100	0.65	2.6	0.98	2.6	21.7	-
	Oxadiazon	99	1.17	293.9	0.94	293.9	950	-
	Carfentrazone	100	0.93	6.7	0.97	6.7	30	-
	Flumiclorac	98	0.96	32.3	0.97	31.3	>60	-
	Fomesafen	99	0.86	118.6	0.91	116.3	>250	-
Vitorino	Saflufenacil	100	0.58	39.3	0.98	38.9	430.5	15.1
	Oxadiazon	100	0.70	824	0.94	810	5900	2.8
	Carfentrazone	100	0.23	12.5	0.99	12.5	>492	1.9
	Flumiclorac	100	0.78	150.3	0.98	154.2	900	4.9
	Fomesafen	100	0.25	>4103	0.93	>4103	>4103	38.7
Bom Sucesso do Sul	Saflufenacil	100	0.25	228.8	0.99	228.8	>574	88.7
	Oxadiazon	100	0.45	1160	0.99	1160	>16413	3.9
	Carfentrazone	100	0.27	107.8	0.99	107.8	>492	16
	Flumiclorac	100	0.51	242.3	0.98	242.3	>985	7.8
	Fomesafen	100	0.31	>4103	0.96	>4103	>4103	65.6

[(1)] Logistic equation of 3 parameters $f = a/(1+(x/x_0)^b)$; A = Maximum asymptote, B = Curve slope. C_{50} = Dose (g a.i. ha^{-1}) giving 50% control. C_{80} = Dose (g a.i. ha^{-1}) required to generate 80% control. * Values adjusted through the SigmaPlot software. ** Values estimated through the Microsoft Excel software.

values. The resistance to saflufenacil is emphasized, as this herbicide was recently registered in Brazil and was not used in the sampled properties.

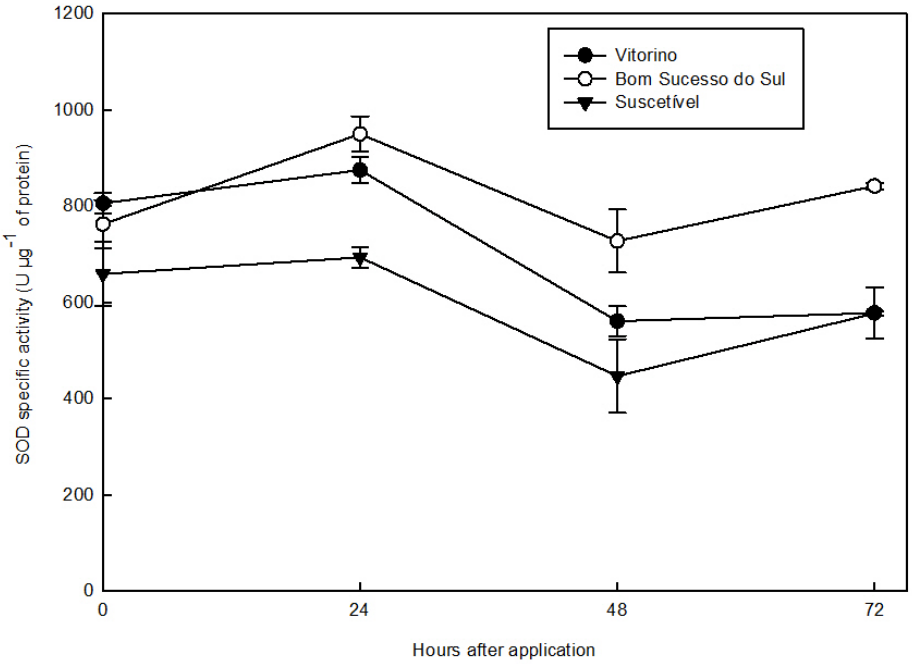
RF values for fomesafen applications on the Vitorino and Bom Sucesso do Sul biotypes were 38.7 and 65.6, and the RF for Vitorino is close to 39, as previously reported by Trezzi et al. (2005) for this same biotype. Therefore, the resistance of the Bom Sucesso do Sul biotype to PPO inhibitors is confirmed, as well as the cross-resistance of this biotype to different chemical groups.

Cross resistance to herbicides in weeds is a frequent behavior, resulting from the combination or isolated effect of two factors, such as the existing mutation that encodes the enzyme gene and the mechanism responsible for resistance in plants (Rizzardi et al., 2002). According to the type of change in the enzyme caused by the substitution of amino acids, changes in the structure and function of the enzyme may occur, allowing herbicides that inhibit the same mechanism of action to bind to the same site or at sites close to the active site of the enzyme (Landstein et al., 1993). This contributes to cross-resistance cases of herbicides from the same chemical group and chemical groups that are distinct but with the same mechanism of action.

Experiment 2- Determination of the activity of the superoxide dismutase and peroxidase enzymes

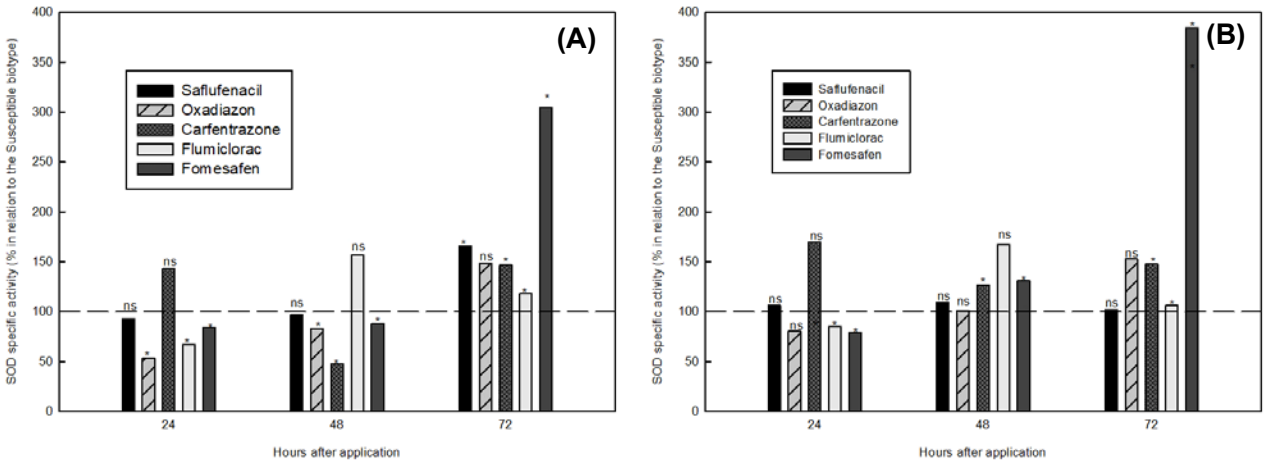
The antioxidative action of the superoxide dismutase (SOD) and peroxidase (POD) enzymes was primarily evaluated by its constitutive activity, that is, it was determined without herbicide presence, and, later, by the activity measured after the application of PPO inhibitors. R biotypes Vitorino and Bom Sucesso do Sul presented constitutive activities of the SOD enzyme above those of the S biotype (Figure 3). Therefore, the higher activity of the antioxidant enzyme may be responsible, at least in part, for the lower action of PPO inhibitors on R biotypes.

The relative activity of the SOD enzyme with the presence of herbicides presented a variable behavior, according to the herbicide and the tested biotype (Figure 4). In the determinations carried out at 24 and 48 HAA, the SOD activity of the Vitorino R biotype with the presence of herbicides was significantly lower than that of the S biotype for oxadiazon, flumiclorac and fomesafen. However, this effect did not occur for carfentrazone (24 HAA) and flumiclorac (48 HAA). In the evaluation carried out at 72 HAA, the SOD activity of the Vitorino biotype exceeded that of the S biotype for all herbicides, except oxadiazon (Figure 4). In the Bom Sucesso do Sul biotype, SOD activity at 24 HAA was significantly lower than that of the S biotype for flumiclorac and



Vertical bars represent the standard error of the mean of each activity.

Figure 3 - Constitutive activity of the SOD enzyme (U µg^{-1} of protein) of susceptible and resistant *E. heterophylla* biotypes (Vitorino and Bom Sucesso do Sul) after periods of 0, 24, 48 and 72 hours of incubation.



* and ^{ns}: significant and not significant by t test ($p<0.05$).

Figure 4 - Activity of the superoxide dismutase (SOD) enzyme (% in relation to the susceptible biotype) of the resistant *E. heterophylla* biotypes (A) Vitorino and (B) Bom Sucesso do Sul, 24, 48 and 72 hours after incubation, with the label dose of PROTOX inhibiting herbicides.

fomesafen, but not for saflufenacil and carfentrazone. However, the SOD activity at 48 HAA and 72 HAA in this biotype was higher than that of the S biotype for carfentrazone and fomesafen (Figure 4).

It is important to highlight that all the R or suspect biotypes tested in this work come from crop areas with an intensive use of PPO inhibitors and, therefore, were selected by the application of herbicides with this action mechanism. In all biotypes, fomesafen became the main herbicide used on the properties, after finding the effectiveness loss of ALS inhibitors, which characterizes a cascade system (Trezzi et al., 2005).

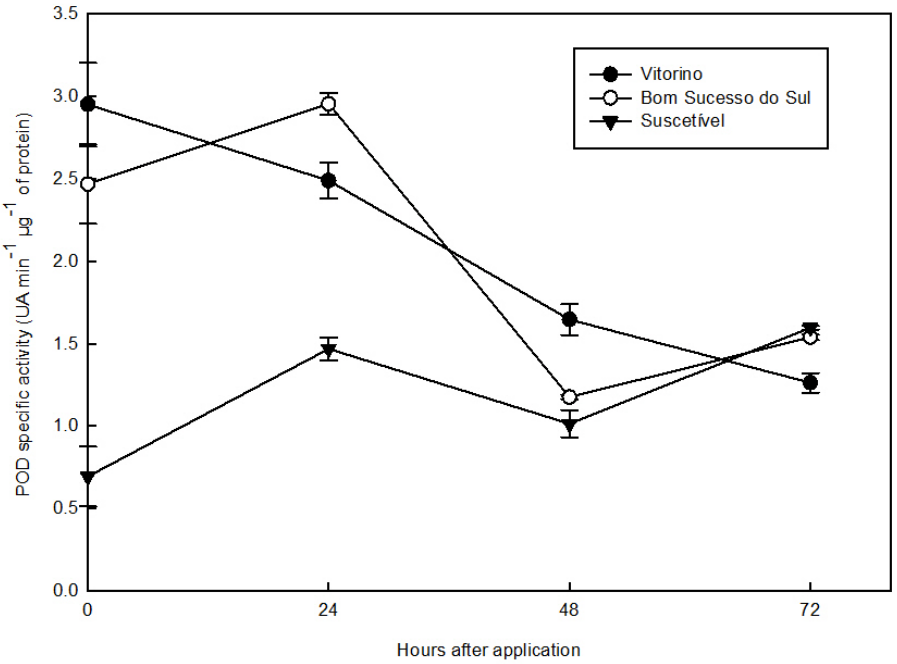
Considering all herbicides and incubation periods, the highest SOD activity in the Vitorino and Bom Sucesso do Sul biotypes occurred with fomesafen at 72 HAA, surpassing the S biotype, respectively, by 200 and 280% (Figure 4A, B). Fomesafen was the least effective herbicide in the control of R biotypes and the one with the highest RF values, together with saflufenacil, indicating a close relation between SOD activity and resistance to PPO inhibitors. The lower control efficiency of the R biotypes and the lower response rate of antioxidant enzymes to fomesafen are due to the fact that this herbicide was the resistance selector in both R populations, used for many years as an alternative herbicide in the control of ALS resistant populations.

Within a cell, SOD is considered the first defense line against reactive oxygen species (ROS) (Alscher et al., 2002), particularly the superoxide radical (O_2^{-}*), transforming it into hydrogen peroxide (H_2O_2) (Asada, 1999). Studies demonstrate that SOD acts against the oxidative stress induced by oxyfluorfen, which is also a PPO inhibitor, on soybean plants (Cataneo et al., 2005).

Some studies have shown a correlation between plant resistance to the oxidative stress generated by herbicides and SOD activity (Kraus and Fletcher, 1994; Casano et al., 1999). Thus, the rapid and superior action of SOD allows PPO inhibitors to reduce their effect on plants. In the R biotypes investigated in this study, SOD presented a higher constitutive activity and, with the presence of herbicides, its induced activity increased in order to fight the free radicals produced by these herbicides. Thus, there are indications that this enzyme constitutes, at least partially, the mechanism of resistance to PPO inhibitors in these biotypes.

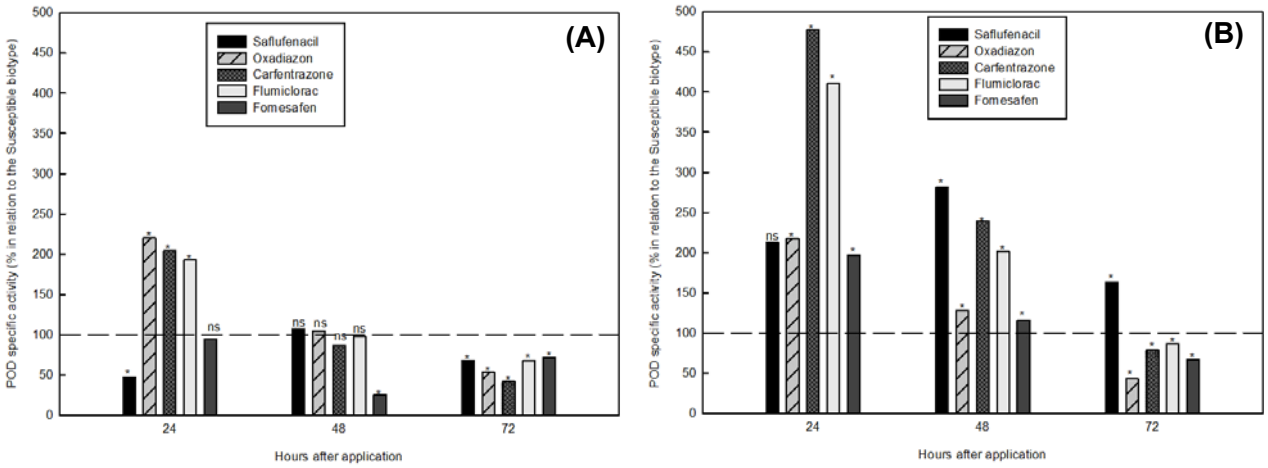
R biotypes Vitorino and Bom Sucesso do Sul showed a constitutive activity of the peroxidase enzyme (POD) higher than that of the S biotype up to 48 HAA, and did not differ from the Bom Sucesso do Sul biotype at 72 HAA (Figure 5). POD are enzymes capable of catalyzing the oxidation of H_2O_2 to form water (Groppa et al., 1999). Their activity can be used as a biochemical indicator of stress resulting from biotic and abiotic factors (Barbosa et al., 2014).

The relative activity of the POD enzyme with the presence of herbicides was also variable according to the herbicide and to the tested biotype (Figure 6). The POD activity of the Vitorino biotype was higher than that of the S biotype only in the determinations carried out 24 hours after the application (HAA) of oxadiazon, carfentrazone and flumiclorac, but for fomesafen, the activities of both biotypes were similar and lower in Vitorino for saflufenacil. Compared to 24 HAA, POD activity in the Vitorino biotype was reduced in the following periods, being equal to or lower



Vertical bars represent the standard error of the mean of each activity.

Figure 5 - Constitutive activity of the peroxidase (POD) enzyme (UA min^{-1} μg^{-1} of protein) of susceptible and resistant *E. heterophylla* biotypes (Vitorino and Bom Sucesso do Sul) after periods of 0, 24, 48 and 72 hours of incubation.



* and ^{ns}: significant and not significant by t test ($p<0.05$).

Figure 6 **-** Activity of the peroxidase (POD) enzyme (% in relation to the susceptible biotype) of the resistant *E. heterophylla* biotypes (A) Vitorino and (B) Bom Sucesso do Sul at 24, 48 and 72 hours after incubation with the 1x dose of PROTOX inhibiting herbicides.

than S, in the periods of 48 and 72 HAA, respectively (Figure 6). In the Bom Sucesso do Sul biotype, POD activity at 24 HAA and 48 HAA was higher than that of the S biotype, for all herbicides, and, in general, higher than the Vitorino biotype. The POD activity for Bom Sucesso do Sul was reduced to levels below those of the S biotype at 72 HAA, except for saflufenacil (Figure 6). In a metabolic sequence, the POD enzyme activates defense mechanisms, breaking down the H_2O_2 molecule. The ability to respond to plant stresses can vary according to the time at which the mechanisms are activated, and it is expected that in R biotypes, which use these defense mechanisms, they will have shorter response times. The rapid increase in the POD activity may indicate its capacity to avoid an initial accumulations of H2O2; however, it is not known whether other functions are activated in this process, such as lignification and suberization (Almagro et al., 2009; Singh et al., 2013).

The high activity of POD in the initial periods after the application of herbicides is due to the fact that this is often the first enzyme to have its activity altered, regardless of the used substrate or the applied stress, whether biotic or abiotic (Siegel, 1993; Lima et al., 1999). Probably, the maximum activity of POD a few hours after the application of the herbicides and its subsequent reduction occur because the enzyme cannot fight all the produced free radicals, due to the action of the herbicide. The fact that POD has a higher induced activity and that it is maintained for a longer period in the Bom Sucesso do Sul biotype compared to the Vitorino biotype indicates the existence of variability among R biotypes. With this, the higher RF values of the Bom Sucesso do Sul biotype for all PPO inhibiting herbicides in relation to Vitorino (Table 2) indicates a positive correlation between the POD enzyme activity and the resistance to PPO inhibiting herbicides.

This enzyme is used as an indicator of oxidative stress after the exposure to herbicides. Changes in the POD activity of wheat and bird's-foot trefoil were observed after the oxidative stress caused by the application of chlorimuron-ethyl and phosphinothricin, respectively (Wang and Zhou, 2006; Savic et al., 2010).

The results confirm the multiple resistance to ALS and PPO inhibitors in *E. heterophylla* biotypes coming from the state of Paraná. Bom Sucesso do Sul and Vitorino biotypes present cross-resistance to herbicides from the chemical groups of imidazolinones, sulfonylureas, pyrimidyl benzoates and sulfonanilides (ALS inhibitors) and from the chemical groups of diphenylethers, phthalamides, triazolinones, oxadiazol and pyrimidinedione (PPO inhibitors). These biotypes showed resistance to herbicides that had never been previously applied. The analysis of an enzyme alone, in general, does not allow the determination of the resistance mechanism. The constitutive and induced activities of the superoxide dismutase and peroxidase enzymes present a complementary action, contributing to the resistance of *E. heterophylla* biotypes to PPO inhibitors.

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