

Full Length Research Paper

## Resistance characterization of Italian ryegrass (*Lolium multiflorum*) biotypes to clethodim herbicide

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The objective of this study is to determine the dose required to control 50% population ( $C_{50}$ ) and to reduce 50% dry matter production ( $GR_{50}$ ) of resistant biotypes of ryegrass in comparison to that of a susceptible biotype as well as to evaluate the mechanism of resistance by cyt-P450 inhibitor application. The study was conducted in a greenhouse in Rio Grande do Sul, Brazil on plants that survived clethodim herbicide application, which were suspected of possessing resistance. For plants surviving field application, the biotypes were 50% controlled with herbicide dose of 28.4- and 29.5-times greater compared to that of susceptible biotypes; 50% of dry matter reduction occurred with doses of 540- and 574-times greater than the susceptibility dose of a biotype, since the dose required to reduce 50% of susceptible biotype was 0.2 g a.i. ha<sup>-1</sup>. The biotypes showed metabolism of clethodim herbicide as regards the inhibition by piperonyl butoxide, indicating that metabolism is the probable cause of control failures in the field.

**Key words:** *Lolium multiflorum*, acetyl coenzyme A carboxylase, weed, mechanism of resistance; metabolization.

### INTRODUCTION

Resistance is the inherent capacity of some biotypes to survive and reproduce after exposition to herbicide dose, which is otherwise lethal to the susceptible population of the same species (Christoffoleti and López-Ovejero, 2008). The onset of resistance to a particular herbicide in

a plant population occurs as a result of selection of pre-existing resistant biotypes under selection pressure exerted by repeated applications of the same active ingredient (Roman, 2007).

In the last growing season (2012) in Brazil, a reduction

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of efficiency of ryegrass control with ACCase-inhibiting herbicides was reported. Failure was observed in areas treated with herbicides aryloxyphenoxypropionates and cyclohexanediones on isolated plants (Fraga et al., 2013). Case analysis did not reveal any plausible explanation for the failure, since the application factors such as the herbicide dose, vegetative stage, environmental conditions, and equipment aids used were suitable. Furthermore, faults occurred in only one species present in the area, *Lolium multiflorum*, and applications with accurate spraying in greenhouse showed similar results.

The quantitative characterization of the resistance level can be performed by dose–response curves, which allow identification of the herbicide dose that provides 50% of population control ( $C_{50}$ ) or 50% reduction in dry matter (DM) production ( $GR_{50}$ ) (Burgos et al., 2013). With the knowledge about these variables, the resistance factor (RF) can be calculated, which refers to the number of times of dose application required for controlling the resistant population was greater than the dose that induced the same effect on a susceptible population (Hall et al., 1998).

Herbicide resistance can be endowed by target-site and/or non-target site-resistance. The target-site resistance can occur by gene point mutations resulting in amino acid changes in a target enzyme, which prevents or reduces herbicide binding (Powles and Yu, 2010). The non-target-site resistance can be conferred by enhanced activities of metabolic enzymes, reducing the amount of herbicide reaching the target-site below the lethal level and/or protecting plants from herbicide damage (Yu and Powles, 2014). Cytochrome P450 monooxygenase, glycosyl transferase (GT), glutathione S-transferase (GST) are the major super-families involved in herbicide metabolism (Yuan et al., 2007).

The cytochrome P450 inhibitors as piperonyl butoxide (PBO) can inhibit *in vivo* the metabolism of some herbicides, thus reversing resistance (Preston et al., 1996; Wang et al., 2013). We aimed to determine the parameters of  $C_{50}$  and  $GR_{50}$  of ryegrass clethodim resistant and susceptible biotypes and to investigate the response of these biotypes to clethodim application after treatment with the metabolic inhibitor of cyt-P450 monooxygenase.

## MATERIALS AND METHODS

To achieve the first objective of this work, was conducted a trial of dose–response curve from August to November 2013 with ryegrass (*Lolium multiflorum*) plants from the commercial fields of the municipality of Coqueiros do Sul, Rio Grande do Sul (S 28°11'13"W 52°44'34"). Eight biotypes that survived 96 g a.i. of clethodim (Select 240 EC<sup>®</sup>) were collected and the trials were conducted in greenhouse at Federal University of Pelotas – Capão do Leão, Rio

Grande do Sul. The collected plants had between 6 and 8 tillers, were separated after collection and originated plants (clones) were evaluated in subsequent experiments. The ryegrass plants that extracted with tillers were isolated for further study. The tillers were transplanted to pot soil and submitted to mass selection in a greenhouse with a dose of 108 g a.i. of clethodim. In this experiment, biotypes showing less phytotoxic effect of the herbicide were nominated as COQ 6 and COQ 7. In a second separation of tillers from the mother-plants, clones were obtained for the formation of experimental units for conducting the dose–response trial. Plants from the same collection sites of resistant biotypes were evaluated, but those that did not survive the herbicide application in the first selection were used to provide the susceptible plants.

The experimental design was a randomized block with four replications, and the experimental units were composed of 550-mL-pots, with one plant per pot. The treatments were arranged in a factorial design, where the factor A tested ryegrass biotypes susceptible (SUS) and resistant (COQ 6 and COQ 7), while the factor B was compared with the effect of increasing doses of the clethodim herbicide (Select 240EC<sup>®</sup>).

To determine the  $C_{50}$  and  $GR_{50}$  values, increasing doses of clethodim herbicide were applied (0, 13.5, 27, 54, 108, 216, 432, and 864 g a.i. ha<sup>-1</sup>), considering 108 g a.i. ha<sup>-1</sup> as the dose to control ryegrass. Treatments were applied post-emergence, when the clone plants were in their four-leaf stage. For this purpose, we used backpack precision sprayer pressurized with CO<sub>2</sub>, equipped with nozzle-type fan 110.015 that distributed spray volume of 120 L ha<sup>-1</sup>. The adjuvant Lanza<sup>®</sup> was added to the water at a dosage of 0.5% (v/v).

The evaluated variables included control and DM of shoots of all biotypes. The control was visually assessed by two raters at 20 and 32 days after herbicide application (DAHA) on the percentage scale, where zero (0) represents no symptoms and hundred (100) represents plant death (Frans et al., 1986). At 32 DAHA, the plants were collected to determine the DM production. For this purpose, the dry plant material was subjected to stove treatment with forced air circulation in an oven at 60°C until constant weight is achieved. The dry weight was converted into percentage form and the materials obtained after treatments with herbicide were compared with the control.

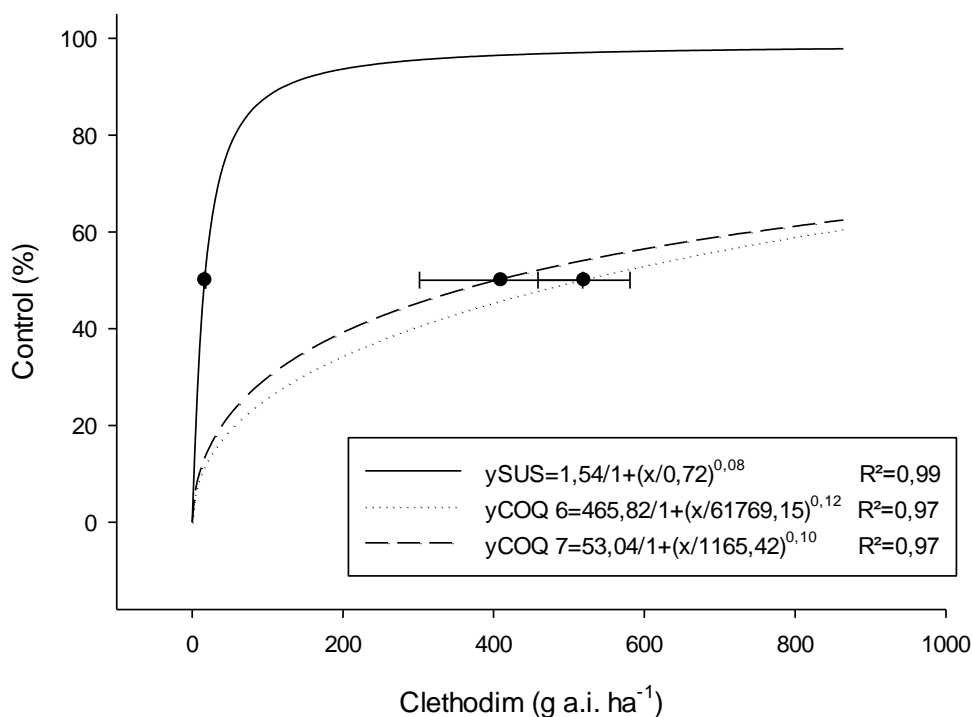
The data were analyzed for normality (Shapiro–Wilk test) and then subjected to the analysis of variance ( $p \leq 0.05$ ). For data found to be statistically significant, regression analysis was performed for the dose factor, and the biotype factor was preceded by comparing  $C_{50}$  or  $GR_{50}$  of resistant and susceptible biotypes.

Regression analysis was performed by using the SigmaPlot 10.0 program and adjusting the data to the sigmoidal regression equation of the logistic type, as follows:

$$y = a / [1 + (x / x_0)^b]$$

Where,  $y$  = percentage control;  $x$  = herbicide dose;  $e$   $a$ ,  $x_0$   $e$   $b$  = equation parameters,  $a$  is the difference between the maximum and minimum points in the curve,  $x_0$  is the dose that promotes 50% of response of variable, and  $b$  is curve declivity.

The values of  $C_{50}$  and  $GR_{50}$  were obtained by calculation of necessary value to promote 50% of control or dry matter reduction, according to parameters generated in the curves equations. From the values of  $C_{50}$  and  $GR_{50}$ , the RF for each biotype with suspected resistance was obtained by comparison with the results of the susceptible plants. For this was necessary to check the interval of confidence ( $p \geq 0.95$ ) of susceptible biotype in relationship to resistant biotypes calculated by standards errors and standard



**Figure 1.** Control (%) of *Lolium multiflorum* biotypes resistant and susceptible, by applying different clethodim herbicide dose, evaluated at 20 days after herbicide application (DAHA). Capão do Leão/RS, 2013. Error bars correspond to interval of confidence in 95% of probability of error of the dose that causes 50% of plant control.

deviation from the curves equations. The overlap of interval of confidence of susceptible biotype in relation to resistant biotypes evaluated indicates that there was no significance difference between  $C_{50}$  and  $GR_{50}$  of biotypes (Avila et al., 2005).

For the realization of the trial of clethodim metabolism conducted from August to October 2014, plants that survived (COQ 8) clethodim application in the fields of Coqueiros do Sul city (same location as in the first experiment) were collected. Therefore, 120 g a.i.  $ha^{-1}$  of clethodim in was applied a 6-ha area by collecting the surviving plants at 30 DAHA. For result confirmation, repeated application was performed at the greenhouse, according to the methodology used in the first experiment.

The treatments were arranged in a factorial design with the factor A composed of ryegrass biotypes (COQ 8 and SUS) and factor B composed by application of cytochrome P450 monooxygenase inhibitor Piperonyl Butoxide (PBO) alone and by preceding 30 min before the clethodim application, clethodim alone, and a no-treated control.

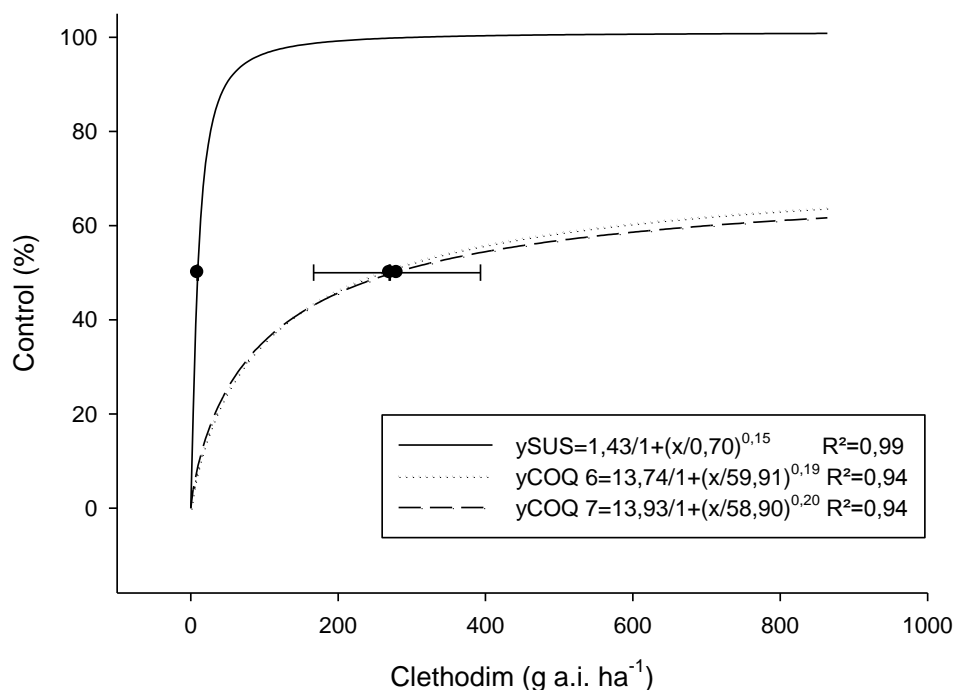
The plants were subjected to herbicide treatment when at the four-leaf stage with a tiller. At 30 min before application of clethodim, the PBO metabolism inhibitor was applied at a dose of 2100 g a.i.  $ha^{-1}$  (Preston et al., 1996) under the same mode as that in the dose-response trial.

The variables analyzed included control at 14 and 28 DAHA and DM of shoots at 28 DAHA, as for the first experiment. The data were analyzed for normality by the Shapiro-Wilk test, followed by analysis of variance ( $p \leq 0.05$ ). For statistically significant results, the Tukey test ( $p \leq 0.05$ ) was performed for biotypes factors and inhibitors.

## RESULTS AND DISCUSSION

For the dose-response curve trial, an interaction was considered between the biotope factors and dose for all variables. While assessing the control at 20 DAHA, resistant biotypes were less affected at the same dose as for the susceptible plants (Figure 1). It was observed that, for 50% control of SUS, 17 g a.i.  $ha^{-1}$  clethodim was needed, and 520 and 410 g a.i.  $ha^{-1}$ , respectively, were needed for the resistant biotypes COQ 6 and COQ 7. The recommended dose of clethodim (108 g a.i.  $ha^{-1}$ ) resulted in the control of 88% of the SUS and 32 and 35% for resistant biotypes COQ 6 and COQ 7, respectively.

Evaluation at 32 DAHA for the SUS revealed 50% control at a dose of 9.5 g a.i.  $ha^{-1}$  of clethodim. As for the resistant biotypes COQ 6 and COQ 7, the dose required was 270 and 280 g a.i.  $ha^{-1}$ , respectively, to achieve 50% control (Figure 2). The dose of 54 g a.i.  $ha^{-1}$  corresponding to half dose of clethodim to control ryegrass was sufficient to achieve >95% control in the susceptible biotype. This biotype was controlled at the maximum (100%) with of 108 g a.i.  $ha^{-1}$ . The resistant biotypes COQ 6 and COQ 7 were controlled by 68 and 66%, respectively, at the dose of 864 g a.i.  $ha^{-1}$ , 8 times



**Figure 2.** Control (%) of *Lolium multiflorum* biotypes resistant and susceptible, by applying different clethodim herbicide dose, evaluated at 32 days after herbicide application (DAHA). Capão do Leão/RS, 2013. Error bars correspond to interval of confidence in 95% of probability of error of the dose that causes 50% of plant control.

the registered and highest dose evaluated in this study.

The genetic variability of weed population is affected by several evolutionary factors such as the production system, interactions between culture and weed, gene flow, dispersal, and natural selection (Huangfu et al., 2009). Thus, the differences between the evaluated ryegrass biotypes may arise from intrinsic genetic characteristics of the biotypes that influence the response to herbicides. The genetic constitution can determine susceptibility or resistance to herbicides (Hartwig et al., 2008). However, considering that both the susceptible biotype as resistant biotypes share the same origin, it is possible that the differences observed between the evaluated biotypes occur due to cultural practices, especially by the herbicide action.

The RF at 20 DAHA was 30.5 and 24.11 for the resistant biotypes COQ 6 and COQ 7, respectively (Table 1). At 32 DAHA, last time of evaluation, the value of the RF for the biotypes COQ 6 and COQ 7 was 28.4 and 29.5, respectively (Tab. 1). These high RF values can be attributed to the times of plant collection to determine the shoot DM, since, at 32 DAHA, the resistant biotypes showed strong growth, while the SUS was controlled at 20 DAT.

The evaluation of the DM of shoot after 32 DAHA

confirmed the control results observed, owing to the decrease in DM with increasing dose of clethodim herbicide for the resistant and susceptible ryegrass biotypes, which were more pronounced for the SUS (Figure 3).

The dose required to reduce 50% of DM ( $GR_{50}$ ) was 0.2 g a.i. ha<sup>-1</sup> of clethodim for the susceptible biotype and 108 and 115 g a.i. ha<sup>-1</sup> of clethodim, respectively, for the resistant biotypes COQ 6 and COQ 7. Considering the values of RF 540 and 575 for biotypes COQ 6 and COQ 7, respectively, confirms that the assessed biotypes are resistant to the herbicide clethodim (Table 2). For *Lolium multiflorum*-resistant biotype to ACCase inhibitor herbicides showed a value of RF 400 for the herbicide diclofop and 165 for the herbicide clodinafop (Kuk et al., 2008).

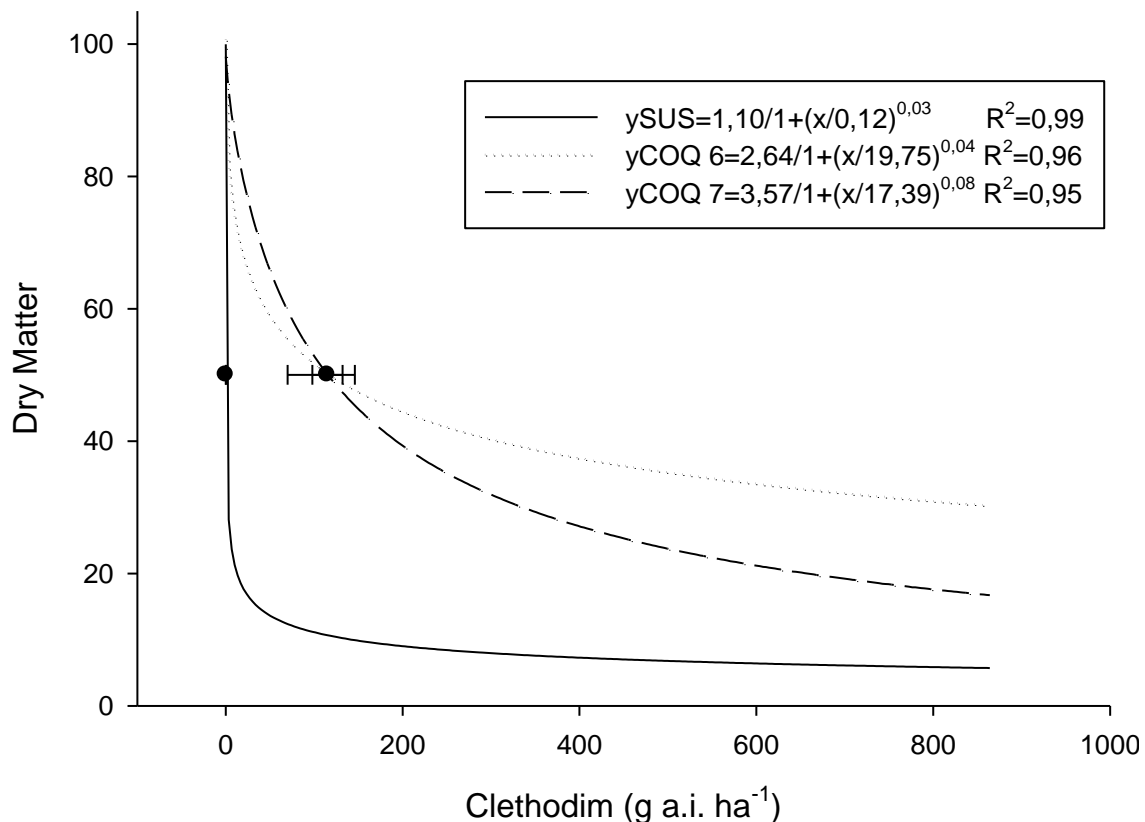
The isolated application of clethodim herbicide at 14 DAHA provided control of approximately 52% for the SUS, but lower values for the resistant biotype COQ 8 (Table 3). However, at 28 DAHA, the SUS biotype control evolved to approximately 71%, while the resistant biotype was still lesser at 39%. The application of the cyt-P450 inhibitor PBO alone did not show any difference among the evaluated biotypes.

The association of clethodim and PBO resulted in

**Table 1.** Values of  $C_{50}$  with confidence intervals (CI) and resistance factor (RF) of *Lolium multiflorum* biotypes resistant and susceptible, by applying different clethodim herbicide dose (0, 13,5, 27, 54, 108, 216, 432 e 864g a.i. ha<sup>-1</sup>) evaluated at 20 and 32 days after herbicide application (DAHA). Capão do Leão/RS, 2013.

Biotype	$C_{50}^1$	CI	RF <sup>2</sup>
	g a.i. ha <sup>-1</sup>	95%	
20 DAHA			
COQ 6	520	208.4 – 330.6	30.5
COQ 7	410	170.9 – 387.1	24.11
SUS	17	16.3 – 17.7	-
32 DAHA			
COQ 6	270	211.3 – 328.7	28.4
COQ 7	280	166.8 – 393.1	29.5
SUS	9.5	8.8 – 10.2	-

<sup>1</sup> $C_{50}$  = dose that provide 50% of population control; <sup>2</sup>Resistance factor of *Lolium multiflorum* resistant biotypes, obtained by division of  $C_{50}$  of resistant biotype in relation to susceptible biotype to clethodim.



**Figure 3.** Dry matter production (%) of *Lolium multiflorum* biotypes resistant and susceptible, by applying different clethodim herbicide dose, evaluated at 32 days after herbicide applications (DAHA). Capão do Leão/RS, 2013. Error bars correspond to interval of confidence in 95% of probability of error of the dose that causes 50% of dry matter reduction.

**Table 2.** Values of clethodim concentration that reduced above-ground dry matter by 50% (GR<sub>50</sub>) with confidence intervals (CI) and resistance factor (RF) of *Lolium multiflorum* biotypes resistant and susceptible, by applying different clethodim herbicide dose (0, 13.5, 27, 54, 108, 216, 432 e 864 g a.i. ha<sup>-1</sup>), evaluated at 32 days after herbicide application (DAHA). Capão do Leão/RS, 2013.

Biotype	GR <sub>50</sub> <sup>1</sup>	CI	RF <sup>2</sup>
	g i.a. ha <sup>-1</sup>	95%	
COQ 6	108	70.1 – 145.9	540
COQ 7	115	97.9 – 132.1	575
SUS	0.2	0.1 – 0.3	-

<sup>1</sup>GR<sub>50</sub> = dose that provide 50% of dry matter production; <sup>2</sup>Resistance factor of *Lolium multiflorum* resistant biotypes, obtained by division of C<sub>50</sub> of resistant biotype in relation to susceptible biotype to clethodim.

**Table 3.** Control (%) at 14 and 28 days after herbicide application (DAHA) and dry matter production (g) of *Lolium multiflorum* biotypes resistant (COQ 8) and susceptible (SUS), submitted to clethodim application, alone or preceding in thirty minutes of the application of cyt-P450 monooxygenase inhibitor (PBO) and control without application. Capão do Leão/RS, 2014.

Treatments	Biotypes					
	SUS			COQ 8		
<b>Control (%) at 14 DAHA<sup>1</sup></b>						
Control	A <sup>2</sup>	0.0	b <sup>2</sup>	A	0.0	c
PBO	A	1.2	b	A	2.5	c
Clethodim	A	52.5	a	A	33.7	b
PBO + Clethodim	A	48.7	a	B	45.2	a
C.V (%)			18.64			
<b>Control (%) at 28 DAHA</b>						
Control	A	0.0	b	A	0.0	c
PBO	A	1.0	b	A	0.0	c
Clethodim	A	71.5	a	B	39.5	b
PBO + Clethodim	A	75.0	a	A	77.0	a
C.V (%)			9.44			
<b>Dry matter production (g plant<sup>-1</sup>)</b>						
Control	A	3.705	a	A	3.785	a
PBO	A	3.332	a	A	3.215	a
Clethodim	B	1.452	b	A	2.985	a
PBO + Clethodim	A	0.925	b	A	1.307	b
C.V (%)			22.39			

<sup>1</sup>Days after herbicide application. <sup>2</sup>Means followed by the same letter in the lines (uppercase) and in the columns (lowercase), does not differ significantly by Tukey's test (p≤0.05).

increased control in biotype COQ 8 at 14 and 28 DAHA. The control values were 48 and 75% for SUS biotype and 45 and 77% for COQ 8 biotypes at 14 and 28 DAHA,

respectively. Thus, the PBO increased the phytotoxicity of herbicide-resistant biotype at the same level as that for SUS, resulting in a synergistic effect. Biotypes of *Lolium*

*rigidum* and *Alopecurus myosuroides* showed better control by the herbicide chlorotoluron and fenoxaprop-ethyl, respectively, when subjected to a prior application of PBO (Burnet et al., 1993; Yu et al., 2014a).

At 14 and 28 DAHA, the application of clethodim alone and clethodim + PBO affected distinctly the COQ 8 biotype, as for these treatments, the control was close to 33 and 45% at 14 DAHA and 39% and 77% at 28 DAHA, respectively, (Table 3). In biotypes of *A. myosuroides*, the addition of PBO inhibitor reduced the resistance factor for the fenoxaprop-ethyl herbicides clodinafop and haloxyfop (Letouze and Gasquez, 2003).

In the DM variable it was observed that in both biotypes the isolated application of PBO did not reduce in comparison with the control (Table 3). In the isolated application of clethodim, the SUS reduced DM on 61% reduction in comparison with the control, while, for biotype COQ 8, the reduction was not significant compared to control treatment. However, the application of PBO+clethodim resulted in 56% reduction of DM in resistant biotype as compared to isolated application of the herbicide, which resulted in a synergistic effect by the association (Table 3). These results corroborate to the control results at 14 and 28 DAHA, when the application of clethodim, preceded by the application of the cyt P450 inhibitor PBO induced biotype control increment of resistance in COQ 8, resulting in a synergistic effect.

Analyses of DM revealed that the application of clethodim preceded by PBO application resulted in lower DM as compared to other treatments for biotype COQ 8 (Table 3). *E. phyllopogon* plants previously treated with inhibitor of cyt-P450 showed an increase in control and lower fresh weight as compared to plants treated only with penoxsulam, suggesting possible involvement of metabolism as a resistance mechanism (Yasuor et al., 2009).

Among the major enzyme systems, P450 monooxygenase (cyt P450) and GST have greater importance for metabolism of herbicides as a cause of resistance. However, the expression of these enzymes which confer resistance by metabolization can be influenced by environmental factors or by epigenetic factors, which do not result in the passage of this trait to the progeny (Milder et al., 2007; Gressel, 2009).

Biotypes of *Lolium multiflorum* evaluated in this study showed changes in the metabolism pattern, which can explain the mechanism of resistance to the herbicide clethodim observed in Brazilian south fields. The increased control and reduction of DM in the resistant biotype submitted to clethodim + PBO compared with clethodim alone, indicates that the resistance of these biotypes to ACCase inhibitor herbicide (clethodim) could be due to the increased P450 enzyme activity as verified in an early work conducted by Yu et al. (2013). However,

other studies about metabolism of clethodim in ryegrass need to be conducted to be performed aimed at obtaining more concrete confirmation of the results with the use of other inhibitors of cyt-P450, as different inhibitors act by inhibiting the P450 isoenzymes differently.

## Conclusion

The ryegrass biotypes COQ 6 and COQ 7 are resistant to the herbicide clethodim. The Resistance Factor for COQ 6 and COQ7 biotypes were 28.4 and 29.5, with 50% reduction of DM with doses 540- and 575-times greater, respectively, than that necessary for biotype susceptible. The PBO reverses the insensitivity of ryegrass biotype COQ 8 to clethodim herbicide, indicating that metabolism is the likely cause of insensitivity of this biotype to clethodim.

## Conflict of Interests

The authors have not declared any conflict of interests.

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