

DOSE-RESPONSE CURVES OF *Lolium multiflorum* BIOTYPES RESISTANT AND SUSCEPTIBLE TO CLETHODIM¹

Curvas de Dose-Resposta de Biótipos de Lolium multiflorum Resistente e Suscetível ao Herbicida Clethodim

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ABSTRACT - Chemical control with herbicides, especially glyphosate, is the main method used to control ryegrass. However, the repeated use of glyphosate has selected resistant ryegrass biotypes. Thus, the ACCase inhibitor herbicides have become the main alternative to control glyphosate-resistant biotypes, being widely used by farmers in Rio Grande do Sul. Repeated use of ACCase inhibitors, in turn, have selected ryegrass biotypes resistant to this herbicide mechanism. Thus, the objective of this study was to evaluate the response of ryegrass biotypes to different clethodim rates by dose-response curves. Increasing doses (0, 12, 24, 48, 72, 96, 144 and 192 g a.i. ha⁻¹) of the herbicide clethodim were applied at the 3-4 ryegrass leaf stage. The variables control at 14 and 28 days after treatment (DAT) and shoot dry weight were evaluated. The data were fitted by nonlinear regression log-logistic and C₅₀ and GR₅₀ were calculated based on the equation. The resistance factor was obtained by the ratio of C₅₀ or GR₅₀ of the resistant biotype by matching the susceptible biotype. Based on the equation parameters, the doses of GR₅₀ 64.7 and 234.5 g a.i. ha⁻¹ clethodim and C₅₀ 11.2 and 172.1 g a.i. ha⁻¹ clethodim were obtained, at 28 DAT for the susceptible and resistant biotypes, respectively. The ryegrass biotype denominated Cotril is resistant to clethodim, being controlled with a dose 15.3 times greater than that of the susceptible biotype, and a 50% reduction of this biotype occurs with a dose 3.62 times higher than that of the susceptible one.

Keywords: ryegrass, cyclohexanodiones, herbicide resistance.

RESUMO - O controle químico com herbicidas, principalmente com uso de glyphosate, é o principal método de controle do azevém. Entretanto, o uso repetido de glyphosate selecionou biótipos de azevém resistentes. Os herbicidas inibidores da enzima ACCase passaram então a ser a principal alternativa de controle de biótipos resistentes ao glyphosate, sendo usados amplamente pelos produtores no Rio Grande do Sul. Contudo, o uso repetido dos inibidores da ACCase, por sua vez, selecionou biótipos de azevém resistentes a esse mecanismo herbicida. Diante disso, o objetivo deste trabalho foi avaliar a resposta de biótipos de azevém a diferentes doses do herbicida clethodim por meio de curvas de dose-resposta. Para isso, foram utilizadas doses crescentes do herbicida clethodim (0, 12, 24, 48, 72, 96, 144 e 192 g i.a. ha⁻¹) aplicadas no estágio de 3 a 4 folhas do azevém. As variáveis avaliadas foram controle aos 14 e 28 dias após aplicação dos tratamentos (DAT) e matéria seca da parte aérea. Os dados foram ajustados por regressão não linear log-logística e, a partir da equação, calcularam-se C₅₀ e GR₅₀. O fator de resistência foi obtido pela relação do C₅₀ ou GR₅₀ do biótipo resistente pelos correspondentes ao do biótipo suscetível. A partir dos parâmetros da equação, foram obtidas as doses do GR₅₀ de 64,7 e 234,5 g i.a. ha⁻¹ de clethodim e do C₅₀ de 11,2 e 172,1 g i.a. ha⁻¹ de clethodim, aos 28 DAT, para os biótipos suscetível e resistente, respectivamente. O biótipo de azevém denominado Cotril é resistente ao clethodim, sendo controlado com uma dose 15,3 vezes maior que o respectivo suscetível, e a redução de 50% da matéria seca desse biótipo ocorre com uma dose 3,62 vezes maior, comparado ao suscetível.

Palavras-chave: azevém, ciclohexanodionas, resistência a herbicidas.

¹ Recebido para publicação em 27.8.2012 e aprovado em 26.10.2013.

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INTRODUCTION

The use of herbicides is the main tool used to control crop-infesting weeds. As a result, resistant weed populations have developed in response to situations of intense herbicide use (Powles & Yu, 2010).

In general, it can be said that the selection of herbicide-resistant weed populations is the result of misusing herbicides. Evidence suggests that herbicide resistance appears in a plant population because of the selection of preexisting resistant biotypes which find favorable conditions to multiply as a result of the selection pressure exerted by repeated applications of the same active ingredient (Silva & Silva, 2007).

Among the species that have resistance issues due to the mechanism of change in the site of action is ryegrass (*Lolium multiflorum*), an outcrossing annual weed of the family Poaceae. It is adapted to low temperatures, and grows only in winter and spring (Kissmann, 2007). The species is well adapted to low and average fertility soils, and it has good response to fertilization and easy dispersion; therefore, it is considered as a weed, and it is present in almost all winter crops, orchards and vineyards in Southern Brazil (Vargas et al., 2007).

Ryegrass is usually controlled by the application of glyphosate. The repeated use of this herbicide caused the emergence of resistant biotypes in the state of Rio Grande do Sul (Roman et al., 2004). Thus, herbicides that inhibit enzyme *Acetyl-coenzyme A carboxylase* (ACCCase) are used as an alternative control. A major ACCCase-inhibiting herbicide is clethodim, a cyclohexanedione herbicide used for weed control in annual and perennial liliopsida at post-emergence which causes limited damage to non-target species (Cox & Allen, 2008).

In most cases, weed resistance to herbicides causes changes to resistant biotypes compared to susceptible ones. One way to measure the level of resistance is using dose-response curves, which allow the identification of the dose of the herbicide that provides control of 50% of the population (C_{50}) or 50% reduction in the production of shoot

dry weight (GR_{50}) (Christoffoleti, 2002). Knowledge of these variables yields the resistance factor (RF), which refers to how many times the dose required for controlling the resistant population is greater than the dose that causes the same effect on susceptible populations (Hall et al. 1998).

In recent years, there has been reduced efficiency control of ryegrass with the use of herbicides inhibiting the enzyme ACCCase. This leads to believe that, in addition to resistance to inhibitors of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), this species is also resistant to ACCCase inhibitors.

Thus, this study aims to evaluate the response of ryegrass biotypes to different doses of the herbicide clethodim by dose-response curves.

MATERIAL AND METHODS

In order to verify the resistance of ryegrass to the herbicide clethodim, an experiment was conducted in a greenhouse, between February and April 2010, in a completely randomized design with four replications. The ryegrass biotype identified as Cotril, from the city of Carazinho, Rio Grande do Sul, was used. It initially survived field application with a dose of 400 mL ha⁻¹ of herbicide Select (96 g a.i. of clethodim) and was selected in a mass selection in the greenhouse, when a dose of 500 mL ha⁻¹ of herbicide Select was used (120 g a.i. of clethodim). The seeds came from plants of the biotype that survived the herbicide clethodim (Select at the dose of 500 mL ha⁻¹) in an assay at the greenhouse. They were later isolated in mini-greenhouses to avoid cross-pollination, and cultured until harvest. The control was comprised of seeds from plants from an area where clethodim had never been used; thus, they were considered to be susceptible.

The experimental units consisted of plastic pots with volumetric capacity of 500 mL, which contained commercial substrate. Six seeds were sown per pot. Thinning was done later, when the plants had two leaves, which resulted in one plant per pot.

To determine the values of C_{50} or GR_{50} , compared to those of untreated plants, increasing doses of the herbicide clethodim were applied (0, 12, 24, 48, 72, 96, 144 and 192 g a.i. ha⁻¹), and 96 g ha⁻¹ was the recommended dose for the control of ryegrass (Brasil, 2010). The treatments were applied at post-emergence, when the plants were at the three/four-leaf stage. A CO₂-pressurized backpack sprayer outfitted with flat fan spray nozzles (110.015), and a spray volume equivalent to 165 L ha⁻¹. Adjuvant Lanza at a dose of 0.5 v/v. was also used.

The variables assessed were control and shoot dry weight. The control was visually assessed by two raters, at 14 and 28 days after treatment (DAT), using a percentage scale, where 0 represented no symptoms and 100, plant death (Frans et al., 1986).

The plants were harvested at 28 DAT to determine the production of shoot dry weight. For this purpose, the plant material was dried in a forced air oven at 60 °C until constant weight. Dry weight was fitted to percentage values by comparing the material obtained in the treatments with the herbicide to the weight obtained in the control, considered as 100%.

Data were analyzed for normality (Shapiro-Wilk test) and then subjected to analysis of variance ($p \leq 0.05$). If statistical significance was found, regression analysis was performed for the dose factor and, for the biotype factor, a comparison was made between C_{50} or GR_{50} values of the biotypes under study.

Regression analysis was performed by fitting the data to the equation for sigmoidal logistic regression, as follows:

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

where: y = the percentage of control; x = dose of the herbicide; a , x_0 and b = parameters of the equation, where a is the difference between the maximum and minimum points of the curve; x_0 , the dose which provides 50% of the response of the variable; and b , the slope of the curve.

The values of C_{50} and GR_{50} yielded the RF for the susceptible and resistant biotypes of the species. To use the RF, the confidence interval (≥ 0.95) had to be checked in the

susceptible biotype, compared to the resistant one. The overlap of the confidence interval of the susceptible biotype compared to the resistant one indicates no significant difference between the C_{50} and GR_{50} values of the biotypes (Avila et al., 2005).

RESULTS AND DISCUSSION

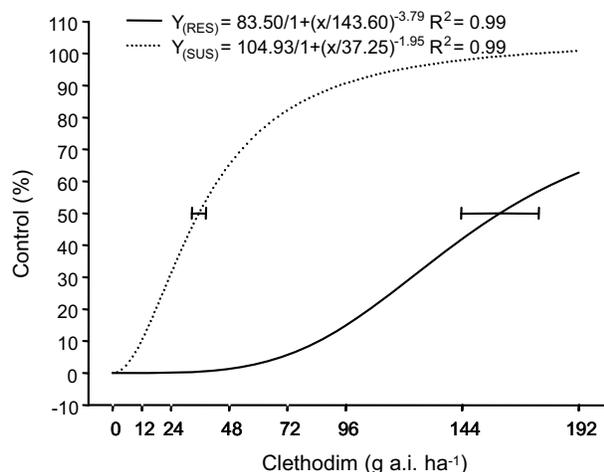
There was interaction between biotype and dose for all variables. The Shapiro-Wilk test showed that the transformation of data was not required.

Ryegrass control by the herbicide clethodim was fitted to the equation for sigmoidal logistic regression at the two assessment periods; the values of the coefficient of determination (R^2) ranged from 0.97 to 0.99, on average, for the susceptible and resistant biotypes, respectively, indicating satisfactory adjustment of the data to the model (Figures 1 and 2). From the equations, the values of C_{50} for the resistant and susceptible biotypes of ryegrass could be calculated.

The analysis of the charts of the control of the biotypes resistant and susceptible to the herbicide clethodim showed the behavior of sigmoidal curves in both assessment periods (Figures 1 and 2). At 14 DAT, it was observed that for a control of 50% of the susceptible biotype, a dose of 35.5 g a.i. ha⁻¹ of clethodim was needed, while the resistant biotype required a dose of 159.6 g a.i. ha⁻¹ (Figure 1, Table 1).

The analysis of the chart of the control at 28 DAT showed that the dose of 11.2 g a.i. ha⁻¹ of clethodim was enough to control 50% of the susceptible ryegrass biotype. The resistant biotype required a dose of 172.1 g a.i. ha⁻¹ for the same control group (Figure 2 and Table 1). It was also observed that the dose of 72 g a.i. ha⁻¹, corresponding to 75% of the registered dose of the product, was enough to control over 95% of the susceptible biotype. This biotype is 100% controlled by the registered dose of the product (96 g a.i. ha⁻¹) while the dose of 192 g a.i. ha⁻¹, which corresponds to 2x the registered dose, was the highest dose tested in this experiment, and it obtained control below 70% of the resistant biotype (Figure 2).





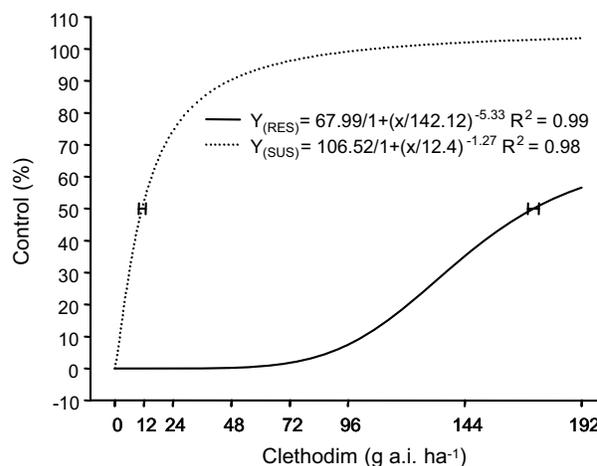
Error bars correspond to the confidence interval at 95% probability of error in the dose causing 50% toxicity in the plant.

Figure 1 - Control (%) of resistant and susceptible *Lolium multiflorum* biotypes, due to the application of different doses of the herbicide clethodim, assessed at 14 days after treatment (DAT).

Based on the lack of overlap of the confidence interval (CI) of the susceptible biotype and the CI of the resistant biotype (Figures 1 and 2), it was possible to establish the FR for both assessment periods. In the last assessment period at 28 DAT, the FR value was 15.3 (Table 1), indicating that the dose of the herbicide clethodim required to promote 50% control of the resistant biotype is 15.3 times higher than the dose needed to produce the same effect on the susceptible biotype. Thus, according to the result obtained, it can be considered that the ryegrass biotype Cotril is resistant to the herbicide clethodim.

ACCase inhibiting herbicides have been an alternative for controlling ryegrass biotypes resistant to glyphosate, but their repeated use has generated problems of resistance. In studies that evaluated the resistance of ryegrass to this mechanism of action, resistance to the herbicide clethodim was observed when it was applied at the dose of 96 g a.i. ha⁻¹ (Rauch et al., 2010).

As for shoot dry weight, there was a decrease in sigmoidal curves as there was an increase in the dose of the herbicide clethodim for both biotypes, and such increase was more pronounced in the susceptible biotype. The variable was fitted to the equation for



Error bars correspond to the confidence interval at 95% probability of error of the dose causing 50% toxicity in the plant.

Figure 2 - Control (%) of resistant and susceptible *Lolium multiflorum* biotypes, due to the application of different doses of the herbicide clethodim, assessed at 28 days after treatment (DAT).

sigmoidal logistic regression and the values of the coefficient of determination (R^2) ranged from 0.96 to 0.98 for the susceptible and resistant biotypes, respectively, demonstrating satisfactory adjustment of the data to the model (Figure 3).

The dose that caused 50% reduction in shoot dry weight (GR_{50}) of the susceptible biotype was 64.7 g a.i. ha⁻¹ of clethodim,

Table 1 - Values of C_{50} with confidence intervals (CI) and resistance factor of resistant and susceptible *Lolium multiflorum* biotypes, in response to the application of different clethodim doses (0, 12, 24, 48, 72, 96, 144 and 192 g a.i. ha⁻¹), evaluated at 14 and 28 days after treatment (DAT)

Biotype	C_{50}^1	Resistance factor ²	
	(g a.i. ha ⁻¹)	95% CI	
14 DAT			
Resistant	159.6	143.6 – 175.6	4.50
Susceptible	35.5	32.6 – 38.4	
28 DAT			
Resistant	172.1	169.8 – 174.5	15.3
Susceptible	11.2	9.8 – 12.7	

¹ C_{50} = dose required to achieve 50% control. ² Factor of resistance to the herbicide clethodim of *Lolium multiflorum* biotypes, obtained by dividing the C_{50} value of the resistant biotype by that of the susceptible biotype to clethodim.

and that of the resistant biotype was 234.5 g a.i. ha⁻¹. Considering the value of the RF (3.62), evidence was confirmed that the ryegrass biotype Cotril is resistant to the herbicide clethodim (Table 2). For *Digitaria ciliaris* biotypes resistant to ACCase inhibitors, FR values of 16.15 and 10.56 were observed for the herbicides sethoxydim and tepraloxidim, respectively (López-Ovejero et al. 2005). In the case of ryegrass resistant to clethodim, plants were harvested for assessment at 28 DAT. If the period had been longer, the RF value would have been higher, as the resistant biotype would have continued to develop normally.

The differences observed between the C₅₀ values of the biotypes may arise from genetic

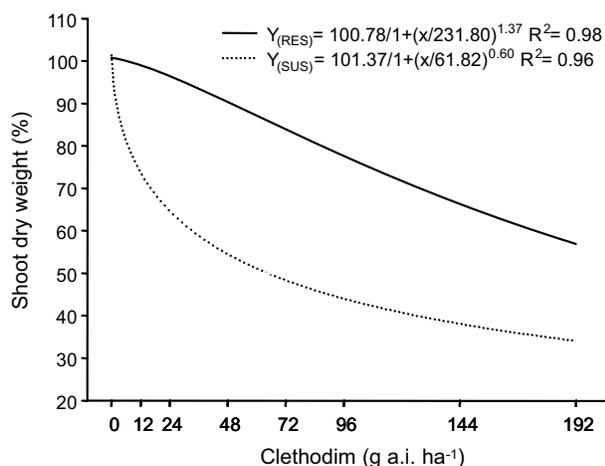


Figure 3 - Shoot dry weight (%) of resistant and susceptible *Lolium multiflorum* biotypes, due to the application of different doses of the herbicide clethodim, assessed at 28 days after treatment.

Table 2 - GR50 values with confidence intervals (CI) and resistance factor of resistant and susceptible *Lolium multiflorum* biotypes, in response to the application of different clethodim doses (0, 12, 24, 48, 72, 96, 144 and 192 g a.i. ha⁻¹), evaluated at 28 days after treatment (DAT)

Biotype	C ₅₀ ^{1/}	Resistance factor ^{2/}	
	(g a.i. ha ⁻¹)	95% CI	
Resistant	234.5	219.4 – 249.6	3.62
Susceptible	64.7	54.1 – 75.3	

^{1/} G₅₀ = dose required to achieve 50% reduction of dry weight. ^{2/} Factor of resistance to the herbicide clethodim of *Lolium multiflorum* biotypes, obtained by dividing the G₅₀ value of the resistant biotype by that of the susceptible biotype to clethodim.



traits between ryegrass biotypes, possibly influencing their responses to the herbicide clethodim. The genetic makeup of a species can determine varying degrees of resistance or susceptibility to herbicides (Hartwing et al., 2008). However, because there is no previous information about the management and genetic characterization of the species, it cannot be said that the differences in responses between the ryegrass herbicides investigated in this study are caused by these factors.

Genetic diversity is especially common among annual weeds and favors the selection of herbicide resistance. This explains, at least in part, why most biotypes worldwide which are resistant to herbicides are from annual species. Large weed infestation favors increased risk of selection for resistance (Winkler et al. 2002).

In most weeds showing resistance to inhibitors of the enzyme ACCase, this is attributed to alterations in the site of action of the enzyme (Volenberg & Stoltenberg, 2002). However, there are reports correlating resistance to ACCase inhibitors with metabolic processes (Bravin et al. 2001). When the mechanism of resistance is due to a change in the site of action, mutations can be grouped as follows: high resistance to sethoxydim and low to other herbicides (e.g., biotypes of *Setaria* spp. and *Avena fatua*); high resistance to fluazifop-p-butyl and low resistance to other herbicides (e.g. *Lolium rigidum*, *Eleusine indica* and *Alopecurus myosuroides*); relatively high resistance to aryloxyphenoxypropionate (APP) and very low or no resistance to cyclohexanodione (CHD) herbicides (e.g. *Lolium rigidum*, *Avena fatua* and *Lolium multiflorum*); one or more mutations give intermediate levels of resistance to both herbicide groups (e.g. *Avena fatua*) and high resistance to both herbicide groups (e.g. *Avena fatua*) (Bourgeois et al., 1997, Devine, 1997).

More studies are needed to clarify the mechanisms involved in differential response of ryegrass to ACCase inhibiting herbicides and also the possible effects of these mechanisms on the activity of other herbicides. This situation indicates that additional field experiments are needed to

verify if environmental conditions were considered adequately. This information is essential so that management alternatives can be identified and recommended to farmers. With the information currently available, it can be stated that the ryegrass biotype known as Cotril is resistant to the herbicide clethodim, and it is controlled with a dose 15.3 times higher than its susceptible counterpart, and 50% reduction in dry weight of this biotype occurs with a 3.62-fold higher dose, compared to the susceptible one.

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