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Article

Influence of Density and Development Stage of Ryegrass on Glyphosate Effectiveness

Influência da Densidade e Estádio de Desenvolvimento do Azevém na Eficácia de Glyphosate

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ABSTRACT - Glyphosate is one of the main herbicides used to control ryegrass. The objective of this research was to evaluated whether the glyphosate dose-response curve is affected by density of ryegrass plants in two phenological growth stages. To attend this objective, two experiments were carried out in greenhouse (one with *Lolium multiflorum* and another with *Lolium rigidum*) and two under field conditions. In the greenhouse experiments, the experimental design was a completely randomized design in a factorial scheme, with factor A as plant density and factor B as glyphosate doses. In the field, two experiments were carried out in a randomized block design in a split-plot scheme. Plots consisted of glyphosate doses (0, 40, 80, 120, 160, 250, and 350 g ha^{-1}), and the subplots consisted of three ryegrass densities (8 plants m^{-2}, 140 plants m^{-2} with thinning before herbicide application, and 140 plants m^{-2} with thinning after herbicide application). In one experiment, glyphosate was sprayed at 28 days after emergence (DAE), whereas in another the application was carried out at 51 DAE. High-density ryegrass plants required a higher herbicide dose to obtain the same level of control of low-density plants. In addition to the density effect, an increased glyphosate dose was necessary for the late application to obtain the same level of control when compared to the initial application. Herbicide effectiveness was affected by population density and plant development stage.

Keywords: *Lolium multiflorum* L., *Lolium rigidum*, time of application, tolerance.

RESUMO - Glyphosate é um dos principais herbicidas utilizados controle de azevém. O objetivo desta pesquisa foi avaliar como a curva de resposta à dose de glyphosate é afetada por densidade populacional de azevém e estádio de desenvolvimento das plantas. Para atender este objetivo foram realizados dois experimentos em casa de vegetação (um com **Lolium multiflorum** *e outro com* **Lolium rigidum**) *e dois experimentos em campo. Nos experimentos em casa de vegetação, o delineamento experimental foi inteiramente casualizado em esquema fatoral; o fator A foi a densidade de plantas, e o fator B, doses de glyphosate. Em campo, foram realizados dois experimentos em blocos casualizados, em esquema de parcelas subdivididas. As parcelas foram constituídas de doses de glyphosate (0, 40, 80, 120, 160, 250 e 350 g ha^{-1}), e as subparcelas, por três densidades de azevém (8 plantas m^{-2}; 140 plantas m^{-2} com desbaste antes da aplicação; e 140 plantas m^{-2} com desbaste depois da aplicação do herbicida). Em um experimento a aplicação de glyphosate foi realizada aos 28 dias após a emergência (DAE), e em outro, aos 51 DAE. As plantas de azevém em alta densidade necessitaram de uma dose maior do herbicida para se obter o mesmo nível de controle que as plantas sob baixa densidade. Além do efeito da densidade, na aplicação tardia houve necessidade de maior dose de glyphosate para se obter o mesmo nível de controle, em*

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comparação à aplicação em estádio inicial. A eficácia de herbicida foi afetada pela densidade populacional e pelo estádio de desenvolvimento das plantas.

Palavras-chave: *Lolium multiflorum* L., *Lolium rigidum*, época de aplicação, tolerância.

INTRODUCTION

Ryegrass (*Lolium multiflorum* L.) is a monocotyledonous, allogamous plant adapted to different types of climate and soil. This species is characterized by a high seed production, easy dispersion, and high competitive ability for water, light, CO_2, and nutrients (Tironi et al., 2014). It is considered one of the main annual weeds in various agricultural crops such as wheat, pasture, canola, oat, among others (Tironi et al., 2014). Ryegrass, when under competition with wheat plants, may reduce the crop productivity by around 60% depending on the cultivar and weed density (Perez-Jones et al., 2005; Lamego et al., 2013).

Ryegrass plants are considered difficult to control because of their similar characteristics with crops. Among the forms of ryegrass management, chemical control is the most used and efficient (Bond et al., 2014). However, sometimes limited control of this plant is achieved due to the incorrect use of herbicides, such as intensive use of the same compound, doses below the recommended rate, and developed weed growth stage. In Australia, the intensive use of the herbicide diclofop-methyl at low doses has selected resistant ryegrass (*Lolium rigidum* L.) plants (Michael et al., 2010). The selectivity of the herbicides diclofop, clodinafop, and mesosulfuron in crops and their intensive use to control ryegrass have increased the selection of resistant plants (Rauch et al., 2010; Liu et al., 2013). In Brazil, one of the main herbicides used to control ryegrass is the glyphosate. However, its continued use at different stages of plant development, together with other incorrect practices, has led to the selection of resistant biotypes (Sammons and Gaines, 2014).

The effectiveness in weed control by herbicides is influenced by several factors related to the triad: herbicide, target weed, and environment (Cieslik et al., 2014). Factors often overlooked at the time of herbicide use are the plant density and stage of weed growth development. The increased weed density in the area causes a number of changes in their physiology, morphology, and anatomy. Indeed, plants tend to increase their height, change leaf angle, reduce dry mass accumulation, leaf area and tillering and increase apical dominance (Green-Tracewicz et al., 2012; Yang et al., 2014). The plants *Chenopodium album* and *Amaranthus retroflexus* showed a higher size as population increased (Lindsey et al., 2013). Plants from the species *Alopecurus myosuroides* and *Galium aparine* decreased the stem diameter under intraspecific competition (Munier-Jolain et al., 2014). An increased population density of *Alternanthera philoxeroides* and *Ambrosia artemisiifolia* decreased in plant dry mass (Zhou et al., 2012; Leskovsek et al., 2012; Pan et al., 2013).

More developed plants tend to present a higher tolerance for the action of herbicides, requiring higher doses or complementary management practices to attain the same level of control. When glyphosate was sprayed in *Lolium perenne* developed plants, it was observed a reduced efficacy, when compared to applications in small plants (Bond et al., 2014). The objective of this research was to evaluated whether the glyphosate dose-response curve is affected by density of ryegrass plants in two phenological stages.

MATERIAL AND METHODS

Four experiments were carried out, two of them in a greenhouse and the other two under field conditions. In the greenhouse experiments, ryegrass seeds were 4 sowed in 500 mL pots containing a 2:1:1 substrate (50% soil + 25% sand + 25% carbonized rice husk) and 24 g of the NPK formulation 5-20-30 + 10 g of urea (45% N) for each 20 kg of substrate. Two extra seeds were sown per pot in all treatments, being thinned after seedling emergence. The pots were drilled and kept in a tray with a water slide to maintain the plants constantly irrigated by capillarity.

The greenhouse experiments had a completely randomized design with treatments arranged in a two-factorial scheme with four replications. The species *Lolium rigidum* was used in the first experiment. Factor A consisted of a low density (1 plant pot^{-1}), high density with thinning before application (5 plants pot^{-1} before thinning, remaining 1 plant pot^{-1}), and high density without thinning after application (5 plants pot^{-1}). Factor B was composed of seven glyphosate doses (0, 35, 70, 100, 150, 200, and 250 g a.e. ha^{-1}.

The species *Lolium multiflorum* was used in the second experiment. Factor A consisted of a low density (1 plant pot^{-1}), high density with thinning before application (7 plants pot^{-1} before thinning, remaining 1 plant pot^{-1}), and high density with thinning after application (7 plants pot^{-1} before thinning, remaining 1 plant pot^{-1}). Factor B consisted of seven glyphosate doses (0, 43.75, 87.5, 125, 187.5, 250, and 312.5 g a.e. ha^{-1}). The herbicides of both experiments were sprayed at 21 days after emergence (DAE) using a spray chamber equipped with a 110.02 E nozzle and calibrated for a spray solution volume equivalent to 100 L ha^{-1}. To each herbicide solution it was added se sufactant 0.2% Agral® v/v.

At 28 days after application (DAA), plant shoot was collected and its fresh mass was determined for *L. rigidum*. For *L. multiflorum*, the shoot dry mass was determined after drying in a forced air circulation oven at 60 °C for seven days. The variables fresh and dry mass of the shoot were converted to a percentage value in relation to the plants not sprayed with herbicide (% in relation to the untreated control).

Field experiments were conducted from June to September 2015. The soil in the area is classified as a sandy clayey silt textured Ultisol (Argissolo Vermelho-escuro distrófico, Brazilian Soil Classification System), with 34% of clay. The conventional soil tillage was used in the area.

The experimental design was a randomized block design in a slip-plot scheme with four replications. The experimental units were composed of four subplots of 0.25 m^2, totaling 1 m^2 in each plot. The species *Lolium multiflorum* was used. Sowing density was 200 seeds m^{-2}. At 10 days after emergence (DAE), plants were thinned, remaining two densities (8 and 140 plants m^{-2}).

The plots were composed of glyphosate at doses of 0, 40, 80, 120, 160, 250, and 350 g ha^{-1}. The subplots consisted of three density. Density conditions were a low density (8 plants m^{-2}), high density with thinning before application (140 plants m^{-2} before thinning, remaining 8 plants m^{-2}), and high density with thinning after application (140 plants m^{-2} before thinning, remaining 8 plants m^{-2}).

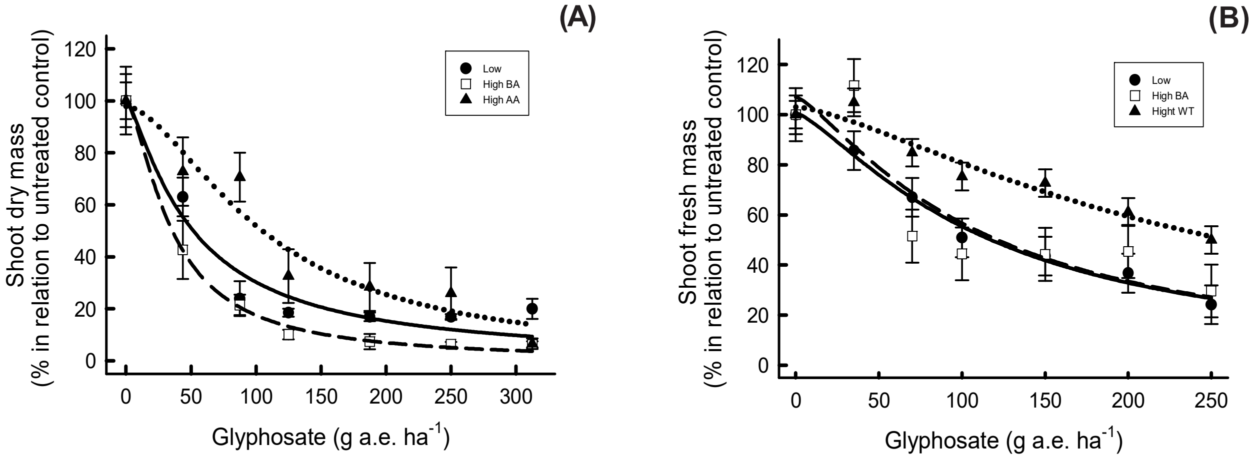
In the first experiment, the herbicide Glizmax® (glyphosate, 480 g L^{-1} ha^{-1}) was sprayed at 28 DAE and at 51 DAE in the second experiment. A CO_2-pressurized backpack sprayer equipped with a boom with 110.02 XR spray nozzles, pressure of 200 kPa, and spray volume equivalent to 160 L ha^{-1}. To all herbicide treatment it was added the adjuvant 0.2% Agral® v/v. During herbicide spraying, the average temperature was 18 and 17 °C and the relative air humidity was 85 to 80% at 28 DAE and 51 DAE, respectively.

At 28 days after application (DAA), the assessment of the control treatment was carried out by assigning scores according to the intensity of symptom presented by the plants. Control scores ranged from 0 (no control) to 100 (total control). Among these extreme values, the others corresponded to the magnitude of the following symptoms: reduction of internodes and leaves, discoloration, chlorosis, and necrosis of leaf tissues and meristems. At 28 DAA, one plant was collected per sub-plot. Size and shoot dry mass were determined in these collected plants. The variables were converted to a percentage value in relation to the plants not sprayed with herbicide (% in relation to the untreated control).

The data were submitted to analysis of variance (ANOVA). Regressions were performed by adjusting linear or non-linear models to the data averaged for each treatment using the software Sigma Plot 12. For the regression analysis, the herbicide dose was considered as an independent factor and the assessed variables as a dependent factor. The herbicide dose that provides a 50% reduction in the assessed variables (D_{50}) was estimated when possible.

RESULTS AND DISCUSSION

In the greenhouse experiments, both species presented a reduction in the shoot dry mass accumulation as glyphosate doses increased (Figure 1), except for high densities of *Lolium rigidum* (Figure 1B) when sprayed at a dose of 35 g a.e. ha^{-1}, not differing from the untreated controls. For the species *Lolium rigidum* (Figure 1B), plants at a high density without thinning (WT) obtained a higher shoot fresh mass in relation to the other densities at doses of 70, 100, 150, and 250 g ha^{-1} of glyphosate.



Vertical bars represent the standard error. Low = low density of ryegrass plants; High BA = high density of ryegrass plants with thinning before herbicide application; High AA = high density of ryegrass plants with thinning after herbicide application; High WT = high density of ryegrass plants without thinning.

Figure 1 **-** Shoot dry mass of *L. multiflorum* (A) and shoot fresh mass of *L. rigidum* (B) (% in relation to the untreated control) as a function of glyphosate doses and plant density. Equations in Table 1.

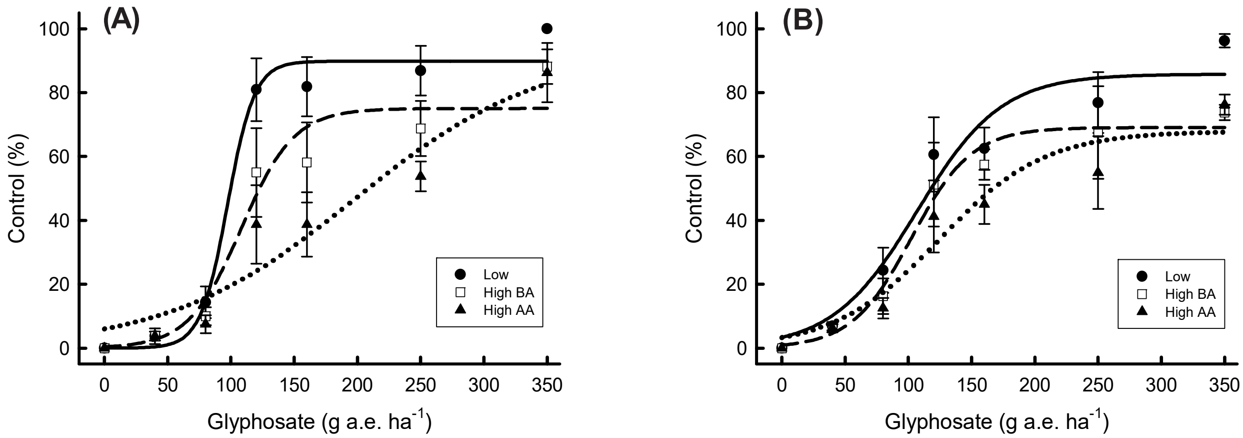
Regarding the species *L. multiflorum*, the values of D_{50} (Table 1) for shoot dry mass were 50, 35, and 107.45 g ha^{-1} of glyphosate for low density (LD), high density with thinning before application (BA), and high density with thinning after application (AA), respectively. These values demonstrate that plants under AA required more than twice the dose to obtain the same control in relation to the plants under LD. A similar effect was observed for *L. rigidum* regarding the variable shoot fresh mass: the value of D_{50} was 116, 109, and 248 g ha^{-1} of glyphosate for LL, BA, and WT, respectively (Table 1).

Table 1 **-** Regression equations used to determine the effects of *L. multiflorum* and *L. rigidum* on three density conditions and glyphosate doses, 2015

Sp(1)	DNS(2)	Equation parameters (SE)(3)		D_{50}(4)	R^2	p(5)	Equation
		a	b				
Shoot dry mass							
L. m.	Low	100.9(9.3)**	1.25(0.35)*	50.89	91.9	<0.01	$y=a/1+(x/D_{50})^b$
L. m.	High BA	100.0(2.1)**	1.50(0.12)*	35.63	99.6	<0.01	$y=a/1+(x/D_{50})^b$
L. m.	High AA	97.8(10.1)**	1.68(0.47)*	107.45	90.0	<0.01	$y=a/1+(x/D_{50})^b$
Shoot fresh mass							
L. r.	Low	100.6(3.3)**	1.33(0.13)**	116.21	98.4	<0.01	$y=a/1+(x/D_{50})^b$
L. r.	High BA	107.0(17.1)**	1.31(0.63)ns	109.46	68.9	<0.05	$y=a/1+(x/D_{50})^b$
L. r.	High WT	103.1(5.2)**	1.48(0.36)*	248.53	91.6	<0.01	$y=a/1+(x/D_{50})^b$

(1) Sp = used species; L.m. = *Lolium multiflorum*; L. = *Lolium rigidum*. (2) DNS = plant density; Low = low density of ryegrass plants; High BA = high density of ryegrass plants with thinning before herbicide application; High AA = high density of ryegrass plants with thinning after herbicide application; High WT = high density of ryegrass plants without thinning. (3) SE = standard error of the estimate; * p<0.05; ** p <0.01; ns = not significant. (4) Dose required to provide 50% reduction of the variable in the plant. (5) Model probability.

Ryegrass control obtained in the field experiment with initial glyphosate spraying (28 DAE) showed a direct relationship between herbicide dose and level of control at all tested densities (Figure 2A). Plants cultivated at a low density presented a lower tolerance to the herbicide when compared to those cultivated at other densities and doses of 120, 160, and 250 g ha^{-1}. A similar result was observed in the control of the experiment with late herbicide application (51 DAE). However, at this assessment time, the densities differed only in the highest glyphosate dose (Figure 2B).



Vertical bars represent the standard error. Low = low density of ryegrass plants; High BA = high density of ryegrass plants with thinning before herbicide application; High AA = high density of ryegrass plants with thinning after herbicide application.

Figure 2 - Control (%) of ryegrass plants as a function of glyphosate doses and densities with spraying at 28 DAE (A) and 51 DAE (B). Equations in Table 2.

According to the values of D_{50} (Table 2), plants at a low density (LD) showed the highest values for visual control, followed by the high density with thinning before application (BA) and after application (AA), with values of 101, 137, and 189 g ha^{-1} of glyphosate, respectively, at 28 DAE. For 51 DAE, the values of D_{50} (Table 2) was similar to that of the previous experiment although the plants were more developed, showing values of 117, 143, and 183 g ha^{-1} of glyphosate for LD, BA, and AA, respectively.

The variable plant size (Figure 3) presented a decrease as glyphosate dose increased, except for the dose of 350 g ha^{-1} of glyphosate, in which a stabilization of this reduction occurred in both experiments at 28 and 51 DAE. At 28 DAE, a difference between ryegrass densities was verified at the highest herbicide dose (Figure 3A), with a slight increase of plant size under LD in relation to the other densities. The values of D_{50} were not found in both experiments, except for BA.

An inverse relationship was found between herbicide dose and shoot dry mass accumulation in both experiments (Figure 4). In the experiment with herbicide spray at 28 DAE, a difference between densities was observed only when plants were sprayed with 80 g ha^{-1} of glyphosate (Figure 4A), whereas in the experiment at 51 DAE, BA differed from the other densities at the highest herbicide dose (Figure 4B).

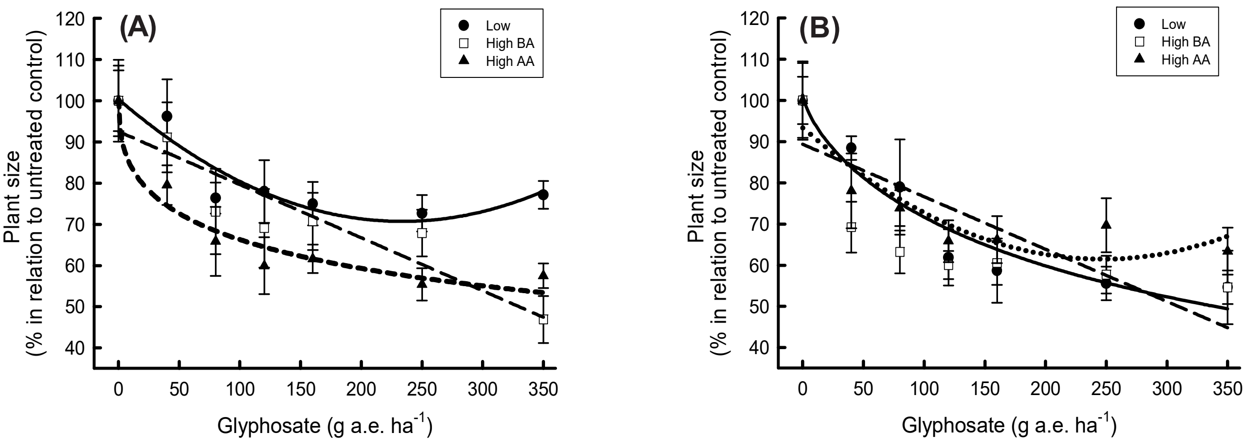
Regarding the values of D_{50} (Table 2) for the variable shoot dry mass at 28 DAE, high-density plants required a higher herbicide dose to obtain the same level of control as the low-density plants. The values of D_{50} (Table 2) were 73, 147, and 112 g ha^{-1} of glyphosate for plants under low density, BA, and AA, respectively. At 51 DAE, the values of D_{50} (Table 2) of plants under a low density and AA were similar, i.e. 177 and 169 g ha^{-1} of glyphosate, respectively. The highest value of D_{50} for this variable was observed under BA (341 g ha^{-1}).

The results obtained for all variables of all experiments showed that an increase in glyphosate doses also increased plant control at all densities in both experiments (Figures 1, 2, 3, and 4). However, the level of control varied according to the density and time of application. At a high density, plants present changes in several morphological and anatomical characteristics that can affect herbicide effectiveness. These changes occur due to a limitation of resources considered

Table 2 - Regression equations used to determine the effects of ryegrass plants on three density conditions and glyphosate doses, 2015

DAE(1)	DNS(2)	Equation parameters (SE)(3)			D_{50}(4)	R^2	p(5)	Equation
		a	b	c				
				Control				
28	Low	89.8(3.9)**	10.6(2.9)*	97.3**	99.73	97.6	<0.01	$y=a/1+exp(-(x-c)/b)$
28	High BA	75.0(6.8)**	20.7(9.1)^{ns}	108.1**	122.5	91.7	<0.01	$y=a/1+exp(-(x-c)/b)$
28	High AA	93.2(25.2)**	73.8(32.5)^{ns}	197.6*	208.3	88.4	<0.01	$y=a/1+exp(-(x-c)/b)$
51	Low	85.7(7.0)**	33.3(10.5)*	106.8**	118.1	93.2	<0.01	$y=a/1+exp(-(x-c)/b)$
51	High BA	69.1(3.3)**	24.2(5.1)**	102.4**	122.6	97.4	<0.01	$y=a/1+exp(-(x-c)/b)$
51	High AA	68.1(7.4)**	42.5(15.9)*	124.6**	208.4	90.7	<0.01	$y=a/1+exp(-(x-c)/b)$
				Plant size				
28	Low	100.3(3.7)**	-0.25(0.05)**	0.001*	-	82.9	<0.05	$y=a+b*x+c*x^2$
28	High BA	92.5(4.5)**	-0.12(0.02)**	-	329.6	81.3	<0.01	$y=a+b*x$
28	High AA	100.3(4.2)**	0.43(0.11)*	-	-	92.9	<0.01	$y=a/1+(x/D_{50})^b$
51	Low	101.3(5.8)**	0.74(0.19)*	-	-	89.17	<0.01	$y=a/1+(x/D_{50})^b$
51	High BA	89.3(5.9)**	-0.12(0.03)*	-	-	70.0	<0.05	$y=a+b*x$
51	High AA	93.3(5.4)**	-0.25(0.07)*	0.001*	-	71.57	<0.05	$y=a+b*x+c*x^2$
				Shoot dry mass				
28	Low	101.51(10.4)**	0.89(0.30)*	-	73.8	86.2	<0.01	$y=a/1+(x/D_{50})^b$
28	High BA	102.54(7.41)**	1.24(0.27)*	-	148.7	92.2	<0.01	$y=a/1+(x/D_{50})^b$
28	High AA	102.43(8.27)**	1.15(0.27)*	-	112.7	91.4	<0.01	$y=a/1+(x/D_{50})^b$
51	Low	103.73(7.25)**	1.60(0.37)*	-	177.9	92.2	<0.01	$y=a/1+(x/D_{50})^b$
51	High BA	100.76(7.54)**	0.47(0.20)*	-	341.8	82.1	<0.05	$y=a/1+(x/D_{50})^b$
51	High AA	99.78(5.57)**	0.73(0.15)**	-	168.9	93.4	<0.01	$y=a/1+(x/D_{50})^b$

(1) DAE = days after emergence. (2) DNS = plant density; Low = low density of ryegrass plants; High BA = high density of ryegrass plants with thinning before herbicide application; High AA = high density of ryegrass plants with thinning after herbicide application. (3) SE = standard error of the estimate; * p<0.05; ** p <0.01; ns = not significant. (4) Dose required to provide 50% reduction of the variable in the plant. (5) Model probability.

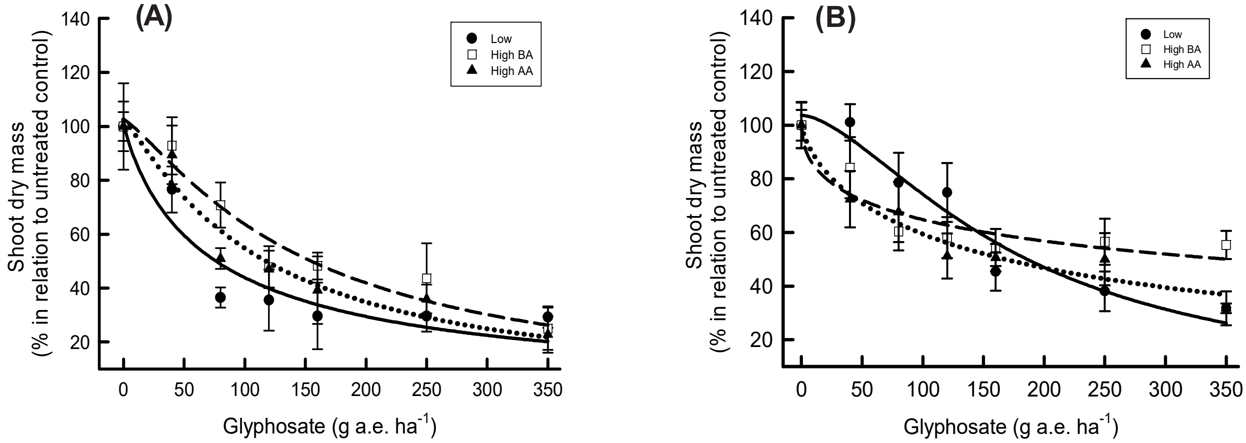


Vertical bars represent the standard error. Low = low density of ryegrass plants; High BA = high density of ryegrass plants with thinning before herbicide application; High AA = high density of ryegrass plants with thinning after herbicide application.

Figure 3 - Plant size (% in relation to the untreated control) of ryegrass plants as a function of glyphosate doses and densities with spraying at 28 DAE (A) and 51 DAE (B). Equations in Table 2.

as essential to plant growth and development, causing competition (Rigoli et al., 2009), and effects of initialism (Vidal et al., 2012).

In greenhouse experiments with both species (*L. multiflorum* and *L. rigidum*), high-density plants with thinning after application or without thinning demonstrated that a higher herbicide dose was required to obtain the same level of control of low-density plants. This can be verified by



Vertical bars represent the standard error. Low = low density of ryegrass plants; High BA = high density of ryegrass plants with thinning before herbicide application; High AA = high density of ryegrass plants with thinning after herbicide application.

Figure 4 **-** Shoot dry mass (% in relation to the untreated control) of ryegrass plants as a function of glyphosate doses and densities with spraying at 28 DAE (A) and 51 DAE (B). Equations in Table 2.

comparing the variable D_{50} (Table 1), with values of 50 and 116 g ha^{-1} of glyphosate at a low density and 107 and 248 g ha^{-1} of glyphosate under AA and WT for *L. multiflorum* and *L. rigidum*, respectively. Similar effects were obtained in the field experiments. The same level of D_{50} in plants under BA was obtained with around twice the dose in relation to those under a low density when considering the variable shoot dry mass.

Several studies from the literature have shown that increasing the plant density affects herbicide effectiveness (Lati et al., 2012; Moon et al., 2014; Streibig et al., 2014). The increase in the density of *Echinochloa crus-galli* from 12 to 48 plants m^{-2} required an increase in the flucetosulfuron dose from 8.7 to 20.1 g ha^{-1} to obtain the same degree of control (Moon et al., 2014). Similarly, the species *Eleocharis kuroguwa* and *Cyperus rotundus* required higher doses of the herbicides azimsulfuron and trifloxysulfuron, respectively, at the highest plant densities (Lati et al., 2012; Moon et al., 2014). The increase in barley density from 75 to 450 plants m^{-2} required an increase of about four times the required glyphosate dose to obtain the same level of control (Streibig et al., 2014).

Among the possible explanations for the lower effectiveness of the glyphosate in plants under a high density is the decreased herbicide interception by the plant. High-density weeds have a lower canopy area and, also, there is a reduced leaf angle, decreasing the interception and herbicide effectiveness when compared to low weed densities (Lati et al., 2012). Due to the reduced leaf area exposed to the post-emergence sprayed product, the herbicide deposition, absorption and efficacy is reduced (Stopps et al., 2013). The decreased leaf angle of *Abuthilon theophrasti* reduced the effect of glyphosate (Mohr et al., 2007).

This study has shown that plant development stage can affect the efficacy of glyphosate. The value of D_{50} for shoot dry mass of ryegrass plants at 28 DAE was, on average, 111 g ha^{-1} of the herbicide, whereas at 51 DAE, the average was 229 g ha^{-1}. Other studies also demonstrate a similar effect. Ryegrass plants sprayed with glyphosate showed that the control at the two-leaf stage was 67% higher when compared to plants treated at the tillering stage with the same herbicide dose (Dors et al., 2010). Plants of *Eleusine indica* at the two-leaf stage sprayed with glyphosate obtained a control 66% higher than that observed on plants treated at the tillering stage (Ulguim et al., 2013).

Studies with susceptible populations of *Lolium rigidum* have shown that low glyphosate doses resulted in herbicide resistance populations (Busi and Powles, 2009). The results of our research demonstrated that the ryegrass growth stage and, also, the plant population density are important variables to take into account at the time of application in order to avoid weed control failures. Likewise, these variables may favor the development of glyphosate resistance populations of ryegrass.

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