Differential susceptibility and resistance to glyphosate in annual ryegrass and wavy-leaved fleabane

Flávia Regina da Costa, Humberto Henrique Ansolin and Leonardo Bianco de Carvalho
Department of Agronomy, Santa Catarina State University, 88520-000 Lages SC, Brazil

ABSTRACT

Annual ryegrass (*Lolium multiflorum*) and wavy-leaved fleabane (*Conyza bonariensis*) were tested, aiming to investigate the dose-response of biotypes of both species to glyphosate. Glyphosate herbicide at doses varying from 0 up to 1.440 g e.a. ha⁻¹ was sprayed onto annual ryegrass plants showed four leaves and wavy-leaved fleabane showed three pair of leaves. The fresh weight of shoot was obtained at 21 days after herbicide application. The response of biotypes of *L. multiflorum* and *C. bonariensis* to glyphosate was clearly different. For *L. multiflorum*, the S2, R1, and R3 biotypes supported glyphosate doses 1.5, 3.0, and 8.3 times higher than the biotype S1. For *C. bonariensis*, the S2 and R biotypes supported glyphosate doses 2.0 and 15.5 times higher than the biotype S1. We found a low glyphosate-resistant (R1) and a high glyphosate-resistant (R2) biotypes of *L. multiflorum*, in agricultural regions where other biotypes had been found. In addition, a high glyphosate-resistant (R) biotype of *C. bonariensis* was identified in an agricultural area of Santa Catarina State, Brazil, where weed resistant was not previously found.

Keywords: *Conyza bonariensis*, *Lolium multiflorum*, N-(phosphonomethyl)glycine, dose-response, weed resistance.

*Corresponding author
E-mail: leonardo.carvalho@udesc.br
Phone: +55 49 21019149

INTRODUCTION

Annual ryegrass (*Lolium multiflorum*) and wavy-leaved fleabane (*Conyza bonariensis*) are two important weeds of annual and perennial crops in Brazil and worldwide. Both species were also found as resistant to glyphosate (Heap 2014). Herbicide resistance has been one of the most important challenges in agricultural systems in recent years, with cases of herbicide resistance having increased exponentially in the last three decades (Heap 2014). Strong selection by herbicides has resulted in the widespread evolution of herbicide resistance in populations of agricultural weeds (Jasieniuk et al. 2008). Weed populations evolve resistance in response to repeated treatment with herbicides having the same mechanism of action or metabolic degradation pathway (reviewed in Gressel 2002, Duke and Powles 2008, Powles 2008, Powles and Yu 2010). There were found 434 unique cases (species x site of action) of herbicide resistant weeds globally, with 237 species (138 dicots and 99 monocots) (Heap 2014). Also according to that author, weeds have evolved resistance to 22 of the 25 known herbicide sites of action and to 155 different herbicides, being reported in 82 crops in 65 countries.

The resistance to glyphosate is an important example of herbicide resistance. This herbicide has been used extensively in agriculture worldwide for over 30 yr, and, today, it is the most commercialized herbicide in the world (Duke and Powles 2008). Glyphosate has a unique chemical structure and a molecular target site related to the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) in the shikimate pathway (Duke et al. 2003). Inhibition of this enzyme results in starvation of EPSP and ensuing metabolic products, such as the aromatic acids phenylalanine, tyrosine, and tryptophan that are required for protein synthesis (Herrman and Weaver 1999, Siehl 1997). However, a more rapid and dramatic effect is the accumulation of precursors of the chorismate pathway, most notably shikimate (Amrhein et al. 1980, Lydon and Duke 1988).

The resistant to herbicides can occur at different levels, as well as the susceptibility of plants in the populations. So, the objective of this research was to investigate the dose-response of biotypes of *L. multiflorum* and *C. bonariensis* to glyphosate.

MATERIAL AND METHODS

Seeds of annual ryegrass and wavy-leaved fleabane were collected from urban areas with no glyphosate application in Lages, SC, Brazil, in 2012. In addition, seeds from the species were also collected from

agricultural areas in South of Brazil (Santa Catarina State and Rio Grande do Sul State), in 2012.

Seeds of both species were sowed in 300 mL plastic pots filled with commercial substrate. Plants grew under controlled conditions, in a growth chamber with temperature of 25 °C, humidity of 60%, and photoperiod of 14h delivered by incandescent lights (800 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Glyphosate herbicide was sprayed at doses of 0, 5.625, 11.25, 22.5, 45, 90, 180, 360, 720, and 1.440 g e.a. ha^{-1} , by using a CO_2 -backpack equipped with four flat fan nozzles (TeeJet 80.02 VS), delivering 200 L ha^{-1} . The herbicide application occurred when annual ryegrass plants showed four leaves and wavy-leaved fleabane showed three pair of leaves.

The glyphosate doses represented the experimental treatments (10 doses), replicated six times.

The fresh weight of plant shoot was weighted (semi-analytical balance – 0.00001 g) at 21 days after the herbicide application. Data were submitted to regression analysis, according to the non-linear, log-logistic equation:

$$y = \min + (\max - \min) / [1 + (x^{\text{Hillslope}}/\text{EC}_{50})]$$

where: min and max are the minimum and the maximum value of fresh weight, EC_{50} is the inflexion point of the curve, representing the dose required to reduce plant fresh weight by 50%, and Hillslope is the slope of the curve at EC_{50} .

Statistical analysis was performed by using SigmaPlot® (Systat, versão 10.0, EUA), that uses Kolmogorov-Smirnov test to check the residual normality and the Spearman Rank correlation test between absolute values of residues and absolute values of dependent variables to check the constancy of variances.

RESULTS

The response of biotypes of *L. multiflorum* and *C. bonariensis* to glyphosate was clearly different (Figure 1). In general, *C. bonariensis* biotypes supported higher glyphosate doses than the biotypes of *L. multiflorum*. In addition, some biotypes supported the glyphosate exposure at low doses with no fresh weight reduction, mainly the R biotypes. On the other hand, glyphosate doses higher than 100 g a.e. ha^{-1} caused more fresh weight reduction of the S biotypes than the R biotypes, for both species. Moreover, the highest dose of glyphosate used in this experiment reduced the fresh weight by 89%, 88%, 86%, 79% for the biotypes of *L. multiflorum* (S1, S2, R1, and R2, respectively), and by 90%, 70%, and 30% for the biotypes of *C. bonariensis* (S1, S2 and R, respectively).

Figure 1. Curves of dose-response to glyphosate of biotypes of annual ryegrass (*Lolium multiflorum*) and wavy-leaved fleabane (*Conyza bonariensis*). Vertical lines indicate the standard error of mean. S and R indicate the biotype showed susceptibility or resistance to glyphosate, respectively.

Table 1. Equation parameters, partial ANOVA, complementary analysis (CVT and NT), and factor of resistance (FR) of biotypes of annual ryegrass (*Lolium multiflorum*).

Biotype	Equation parameters ^{1/}				ANOVA ^{2/}			CVT ^{3/}	NT ^{4/}	FR ^{5/}
	min	max	EC50	Hillslope	R ²	F	P			
S1	10.7	113.3	35	1.605	0.94	45.57	< 0.001	0.101	0.581	1.0
S2	6.1	99.1	52	0.960	0.89	26.02	< 0.001	0.682	0.542	1.5
R1	12.1	106.1	104	2.574	0.99	532.75	< 0.001	0.086	0.752	3.0
R2	21.1	114.6	290	2.871	0.94	44.09	< 0.001	0.076	0.999	8.3

^{1/} Regression equation: $y = \min + (\max - \min) / [1 + (x^{\text{Hillslope}}/\text{EC50})]$, where min and max are the minimum and the maximum value of fresh weight, EC50 is the inflexion point of the curve, representing the dose required to reduce plant fresh weight by 50%, and Hillslope is the slope of the curve at EC50. ^{2/} ANOVA: R², F, and P represent the value of the coefficient of determination of the curve, the value of F (F test) and the value of P (significance of F test), respectively. ^{3/} CVT is the value of significance for constant variance test. ^{4/} NT is the value of significance for residual normality test. ^{5/} FR = EC50(S2, R1, or R2) / EC50(S1).

Table 1. Equation parameters, partial ANOVA, complementary analysis (CVT and NT), and factor of resistance (FR) of biotypes of wavy-leaved fleabane (*Conyza bonariensis*).

Biotype	Equation parameters ^{1/}				ANOVA ^{2/}			CVT ^{3/}	NT ^{4/}	FR ^{5/}
	min	max	EC50	Hillslope	R ²	F	P			
S1	14.6	93.1	105	2.32	0.96	70.14	< 0.001	0.14	0.94	1.0
S2	15.7	99.7	211	0.87	0.95	58.16	< 0.001	0.95	0.97	2.0
R ^{6/}	8.3	94.2	1,623	8.72	0.78	10.87	< 0.05	0.23	0.96	15.5

^{1/} Regression equation: $y = \min + (\max - \min) / [1 + (x^{\text{Hillslope}}/\text{EC50})]$, where min and max are the minimum and the maximum value of fresh weight, EC50 is the inflexion point of the curve, representing the dose required to reduce plant fresh weight by 50%, and Hillslope is the slope of the curve at EC50. ^{2/} ANOVA: R², F, and P represent the value of the coefficient of determination of the curve, the value of F (F test) and the value of P (significance of F test), respectively. ^{3/} CVT is the value of significance for constant variance test. ^{4/} NT is the value of significance for residual normality test. ^{5/} FR = EC50(S2 or R) / EC50(S1). ^{6/} Although the regression analysis was consistent, the EC50 of the R biotype was found at doses higher than the ones tested in the experiment.

The dose required to reduce the fresh weight of *L. multiflorum* by 50% was 35, 52, 104, and 290 g a.e. ha⁻¹, respectively for the S1, S2, R1, and R2 biotypes (Table 1). Thus, the factor of resistance found was 1.0, 1.5, 3.0, and 8.3 for the S1, S2, R1, and R2 biotypes, indicating that S2, R1, and R3 supported glyphosate doses 1.5, 3.0, and 8.3 times higher than the biotype S1.

The dose required to reduce the fresh weight of *C. bonariensis* by 50% was 105, 211, and

1,623 g a.e. ha⁻¹, respectively for the S1, S2, and R biotypes (Table 2). Thus, the factor of resistance found was 1.0, 2.0, and 15.5 for the S1, S2, and R biotypes, indicating that S2 and R supported glyphosate doses 2.0 and 15.5 times higher than the biotype S1.

We assumed that the adjusted regression curves were adequate, since all regressions were significant ($P < 0.05$), all variances were constant ($P > 0.05$), and all residues were normally distributed ($P > 0.05$).

DISCUSSION

For *L. multiflorum*, the S1 and S2 biotypes were found as susceptible to glyphosate, since the FR was lower than 2.0 (Table 1). On the other hand, the R1 biotype showed lower susceptibility to glyphosate than S1 and S2, and the FR of 3.0 indicates that biotype can show resistance to glyphosate at low level. The FR higher than 8.0 of the R2 biotype indicates that biotype showed resistant to glyphosate, supporting doses higher than eight times greater than a highly susceptible biotype.

For *C. bonariensis*, the S1 and S2 biotypes were found as susceptible to glyphosate, since the FR was up to 2.0 (Table 2). On the other hand, the R biotype showed lower susceptibility to glyphosate than S1 and S2, and the FR of 15.5 indicates that biotype show resistance to glyphosate, supporting doses higher than fifteen times greater than a highly susceptible biotype.

In Brazil, first cases of resistance to glyphosate reported for biotypes of *L. multiflorum* (Roman et al. 2004, Vargas et al. 2004) and *C. bonariensis* (Vargas et al. 2007, Lamego and Vidal 2008), in both annual and perennial crops. To date, new resistant biotypes have been found (Heap 2014), indicating that the problem of weed resistance, especially with glyphosate, is increasing, so that efforts must be done to prevent the evolution of new weed resistant populations.

In our study, new populations suspected to be resistant to glyphosate were tested. We found a biotype of *C. bonariensis* from Campos Novos, SC, Brazil, showing resistance to glyphosate at high level (FR = 15.5). In addition, we also found two biotypes of *L. multiflorum* resistant to glyphosate in agricultural areas where other resistant biotypes had been found. The presence of new biotypes in agricultural areas indicates that the resistant populations are spreading faster than we are solving the issue, and then the problem of weed resistance is increasing.

It is essential to know whether or not a lack of weed control after glyphosate application results from herbicide resistance or other reasons (Duke and Powles 2008; Powles 2008). For example, when using herbicides at a sublethal dose, some plants are affected but survive, and therefore, there can be a rapid resistance evolution (Neve and Powles 2005a,b). In the case of *L. multiflorum*, plants cannot be killed using glyphosate when the herbicide is applied after the plant affiliates or attains the flowering stage. In the case of *C. bonariensis*, the aboveground parts of well-developed plants can be killed, but the plant can re-grow by basal meristems. On the other hand, seedlings can be controlled successfully. The use of glyphosate on advanced growth-stage plants may

have exacerbated the development of glyphosate resistance in sourgrass.

Using the identification of herbicide-resistant weeds as a first step in resistance management demands an efficient and effective screening test; only after obtaining an accurate diagnosis can the nature, distribution and abundance of resistant weed populations be monitored (Perez and Kogan 2003). In addition, understanding the factors influencing the evolution of glyphosate resistance will help delay the appearance of resistance (Preston et al. 2009). For *C. bonariensis* and *L. multiflorum*, the main factors can be cited as intensive use of glyphosate and the lack of other weed management practices. Orchards and fallow situations are at high risk of glyphosate resistance evolution. In addition for *L. multiflorum*, the fact of being a plant used as pasture, the commerce of not-certified seeds can increase the problem of spreading resistant biotypes. Thus, changing management practices to include other weed control tactics will help delay the evolution of glyphosate resistance in this species, whereas the use of glyphosate as the only control method will exacerbate glyphosate resistance.

CONCLUSION

Dose-response experiments allow us to conclude that we found a low glyphosate-resistant and a high glyphosate-resistant biotypes of *L. multiflorum*, in agricultural regions where other biotypes had been found. In addition, a high glyphosate-resistant biotype of *C. bonariensis* was identified in an agricultural area of Santa Catarina State, Brazil, where weed resistant was not previously found.

Acknowledgements

The authors thank to CAPES from Government of Brazil for the Post-graduate scholarship of the first author.

References

- Amrhein N, Deus B, Gehrke P, Steinrücken HC. 1980. The site of the inhibition of the shikimate pathway by glyphosate: II. Interference of glyphosate with chorismate formation in vivo and in vitro. *Plant Physiol* 66: 830–834.
- Duke SO, Baerson SR, Rimando AM. 2003. Herbicides: glyphosate. pp. 708–869. In: Plimmer JR, Gammon DW, Ragsdale NN (Eds.). *Encyclopedia of Agrochemicals*. New York: John Wiley.
- Duke SO, Powles SB. 2008. Glyphosate: a once in a century herbicide. *Pest Manag Sci* 64: 319–325.
- Gressel J. 2002. Evolution of resistance to herbicides. pp. 78–121. In: Gressel J (Ed). *Molecular Biology of Weed Control*. New York: Taylor & Francis.
- Heap IM. 2014. International Survey of Herbicide-Resistant weeds. <http://www.weedscience.org>. Accessed: June 6, 2014.
- Herrman KM, Weaver LM. 1999. The shikimate pathway. *Annu Rev Plant Physiol Plant Mol Biol* 50: 473–503.

- Jasieniuk M, Ahmad R, Sherwood AM, Firestone JL, Perez-Jones A, Lanini WT, Mallory-Smith C, Stednick Z. 2008. Glyphosate-resistant Italian ryegrass (*Lolium multiflorum*) in California: distribution, response to glyphosate, and molecular evidence for an altered target enzyme. *Weed Sci* 56: 496–502.
- Lamego FP, Vidal RA. 2008. Resistance to glyphosate in *Conyza bonariensis* and *Conyza canadensis* biotypes in Rio Grande do Sul, Brazil. *Planta Daninha* 26: 467–471.
- Lydon J, Duke SO. 1988. Glyphosate induction of elevated levels of hydroxybenzoic acids in higher plants. *J Agric Food Chem* 36: 813–818.
- Neve P, Powles SB. 2005a. Recurrent selection with reduced herbicide rates results in the rapid evolution of herbicide resistance in *Lolium rigidum*. *Theor Appl Genet* 110: 1154–1166.
- Neve P, Powles SB. 2005b. High survival frequencies at low herbicide use rates in populations of *Lolium rigidum* result in rapid evolution of herbicide resistance. *Heredity* 95: 485–492.
- Perez A, Kogan M. 2003. Glyphosate-resistant *Lolium multiflorum* in Chilean orchards. *Weed Res* 43: 12–19.
- Powles SB. 2008. Review. Evolved glyphosate-resistant weeds around the world: lessons to be learnt. *Pest Manag Sci* 64: 360–365.
- Powles SB, Yu Q. 2010. Evolution in action: plants resistant to herbicides. *Annu Rev Plant Biol* 61: 317–347.
- Preston C, Wakelin AM, Dolman FC, Bostamam Y, Boutsalis P. 2009. A decade of glyphosate-resistant *Lolium* around the world: mechanisms, genes, fitness, and agronomic management. *Weed Sci* 57: 435–441.
- Roman ES, Vargas L, Rizzardi MA, Mattei RW. 2004. Resistance of Italian ryegrass (*Lolium multiflorum*) to glyphosate. *Planta Daninha* 22: 301–306.
- Siehl DL. 1997. Inhibitors of EPSPS synthase, glutamine synthetase and histidine synthesis. pp. 37–67. In: Roe RN, Burton JD, Kuhr RJ (Eds). *Herbicide activity: toxicology, biochemistry and molecular biology*. Amsterdam: IOS Press.
- Vargas L, Bianchi MA, Rizzardi MA, Agostinetto D, Dal Magro T. 2007. *Conyza bonariensis* biotypes resistant to the glyphosate in Southern Brazil. *Planta Daninha* 25: 573–578.
- Vargas L, Roman ES, Rizzardi MA, Silva VC. 2004. Identification of glyphosate-resistant ryegrass (*Lolium multiflorum*) biotypes in apple orchards. *Planta Daninha* 22: 617–622.