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Endozoochorous seed dispersal of glyphosate-resistant *Lolium multiflorum* by cattle

C. E. Schaedler[1], R. M. Scalcon[2], J. L. C. Viero[2], D. M. Chiapinotto[3], D. B. David[4], F. Q. Rosa[5] and E. B. Azevedo[2]

[1]Bagé Campus, Sul-Rio-Grandense Federal Institute of Education, Science and Technology (IFSul), Bagé, Brazil; [2]Itaqui Campus, Federal University of Pampa, Itaqui, Brazil; [3]Department of Plant Protection, Federal University of Pelotas, Pelotas, Brazil; [4]Department of Agricultural Diagnostics and Research (DDPA), Secretaria da Agricultura, Pecuária e Irrigação – SEAPI, São Gabriel, Brazil and [5]Uruguaiana Campus, Federal University of Pampa, Uruguaiana, Brazil

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Author for correspondence:
C. E. Schaedler, E-mail: carlosschaedler@ifsul.edu.br

Abstract

Lolium multiflorum, one of the most important temperate forage grasses in the world, is used in integrated crop-livestock systems and as a cover crop. However, it is also one of the main weeds in winter crops. The continuous use of glyphosate to manage this species has led to the selection of resistant biotypes (LOLMU-R), making it important to prevent the dispersal of these seeds. This study aimed to assess the recovery and germination of LOLMU-R that have passed through the digestive system of cattle. The experiments were carried out in metabolism cages, using a completely randomized design with six replications. The animals were given 12 112 seeds each, which were recovered from their faeces over a period of 6 days. Germination of the recovered seeds was assessed in a germination chamber and compared against a control (no animal passage). After germination, a glyphosate dose-response curve was constructed. The results obtained showed a total recovery of 1109 seeds (9.1%), with maximum recovery 2 days after ingestion, decreasing to almost zero on day 6. Germination declined linearly as a function of recovery time; however, 4 days after ingestion, germination potential was 18%. The dose-response curve proved the resistance of the recovered seeds. Cattle is a dispersal agent for LOLMU-R seeds, with animals requiring 7 days of quarantine before moving from one infested area to another.

Introduction

Weeds are the main biotic factor responsible for yield losses in agricultural crops and a threat to global food security (Délye *et al*., 2013). Herbicides are the most practical, efficient and least costly method of controlling these species (Harker and O'Donovan, 2013; Heap 2014). However, excessive use leads to the emergence of herbicide-resistant weeds (Bagavathiannan and Davis, 2018).

Herbicide resistance (HR) in weeds is a natural response to selection pressure (Vencill *et al*., 2012). It is the inherent and inheritable ability of some individuals in a population to survive and reproduce after exposure to a lethal dose of the product (Norsworthy *et al*., 2012; Busi *et al*., 2013; Heap, 2014). Herbicides eliminate susceptible plants, allowing resistant individuals to predominate, limiting or precluding chemical management, a major concern in modern agriculture (Burgos *et al*., 2013).

In recent years, glyphosate has become the most widely used herbicide in chemical management (Heap and Duke, 2018). It is a non-selective foliar-applied product used to manage annual and perennial weeds (Kleinman *et al*., 2015) and the only herbicide that inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase on the shikimate pathway (Heap and Duke, 2018), causing plant death (Gomes *et al*., 2014). However, intensive use in no-till systems, genetically modified and cover crops (González-Torralva *et al*., 2012) has increased resistance (Heap and Duke, 2018).

Among the main glyphosate-resistant species worldwide, Italian ryegrass (*Lolium multiflorum* Lam.) biotypes are particularly prominent, occurring on four continents: the Americas, Europa, Asia and Oceania (Heap, 2021). *Lolium multiflorum* is an annual winter grass considered one of the most important temperate forage species in the world (Wang *et al*., 2016). It is widely used as a cover crop or in integrated crop-livestock systems (ICLS) (Sandini *et al*., 2011) that are characterized by the annual rotation of pastures and crops in a no-till system where the main integrated farming is rotation or succession of summer crops with winter annual grazing grasses or successive natural pastures (Moraes *et al*., 2014).

Due to its adaptability to different soil types, water stress tolerance, and easy dispersal and persistence in the soil seed bank, *L. multiflorum* interferes in winter crops, orchards, vineyards, maize, rice and soybean (Vargas *et al*., 2007; Galvan *et al*., 2011; Niinomi *et al*., 2013; Nandula, 2014). The presence of ryegrass reduces wheat yield by 62% (Paula *et al.*, 2011), making it a major weed worldwide (Ge *et al.*, 2012).

As such, strategies are needed to mitigate the evolution of glyphosate resistance in Italian ryegrass. One of these strategies is preventing seed migration by reducing the dispersal of the resistance gene (Beckie and Harker, 2017; Bagavathiannan and Davis, 2018). This strategy is vital in cases involving glyphosate because the frequency of initial resistance is considered low. In practical terms, the dispersal of resistance is far more important as a source of new infestations than new cases that emerge *in situ* (Heap and Duke, 2018).

Endozoochory is one of the main sources of seed dispersal in ICLS, with ruminants as dispersal agents, enabling the migration of herbicide-resistant weed seeds (Viero *et al.*, 2018). Endozoochory, the dispersal of seeds via ingestion by vertebrate animals and their subsequent elimination in faeces (Alvarez *et al.*, 2016), is an important process for weed species (Chuong *et al.*, 2016). However, seeds ingested by ruminants undergo physical and chemical processes as well as microbial activity, affecting their recovery and germination (Lisboa *et al.*, 2009; Oliveira *et al.*, 2013; Milotić and Hoffmann, 2016; Viero *et al.*, 2018; Wang *et al.*, 2019).

The aim of this study was to assess the recovery and germination of glyphosate-resistant *L. multiflorum* seeds that have passed through the digestive system of cattle.

Materials and methods

The research protocol was reviewed and approved by the Animal Ethics Committee of the Department of Agricultural Diagnostics and Research (DADR).

Lolium multiflorum *seed collection and experimental procedures*

Glyphosate-resistant *L. multiflorum* seeds (LOLMU-R) were collected from a biotype at the EMBRAPA Trigo company (28° 16′S and 52°24′W) in the Passo Fundo city, Rio Grande do Sul state (RS), Brazil. The biotype was previously identified by dose-response curve studies after surviving chemical treatment in apple orchards (Vargas *et al.*, 2004). The recovery and germination experiments were conducted between June and December 2015, in two stages: recovery, carried out at the Center for Forage Research (30°21′S and 54°16′W) in São Gabriel (RS); and germination and resistance assessment at the Itaqui Campus of the Federal University of Pampa (RS) (29°12′S and 56°18′W).

Seed recovery

A completely randomized experiment with six replications was conducted to assess the recovery of LOLMU-R seeds that had passed through the digestive system of cattle. The treatments consisted of the following seed recovery times: 1, 2, 3, 4, 5 and 6 days after ingestion. Six Hereford steers with an average age of 2 years and an average weight of 350 kg were used, with each animal considered one replicate.

The animals were allocated in metabolism cages for 15 days for metabolic assays. Over the first 9 days, the animals were allowed to adapt to the environment and diet. The diet offered was composed of fresh forage from native grassland in sufficient quantity to meet the animal's nutritional requirement. Its botanical composition was mainly constituted by *Axonopus affinis*, *Desmodiun incanum*, *Paspalum notatum* and *Paspalum plicatulum*; and a chemical composition of 409 g/kg of dry matter, 71 g/kg of ash, 92 g/kg of crude protein and 604 g/kg of neutral detergent fibre. On day 9, LOLMU-R seeds were supplied via a flexible feeding tube manually inserted into the glottis, with 25 g of seeds/animal (12 112 seeds), estimated based on the 1000 seed weight of the LOLMU-R biotype.

The steers were fitted with polyvinyl chloride saddles and bags. The bags were changed three times a day to prevent the accumulation of faeces. The samples were homogenized every 24 h and a 10% subsample removed for analysis. These samples were washed under running water in a 1 mm mesh sieve to separate the LOLMU-R seeds. After recovery, the results were converted to 100% of faecal volume. The seeds were counted and pre-dried, then placed in plastic bags and stored at 6°C (±2°C) under 85% relative humidity (RH) until the germination tests.

Germination of the recovered seeds

A completely randomized design was used; with six replications (the seeds recovered from each animal were considered as different replications). The recovered LOLMU-R seeds were deposited onto Germtest® paper soaked in distilled water (equivalent to 2.5 times the weight of the paper), which was rolled up and placed into sealed plastic bags. The rolls were placed into a Biochemical Oxygen Demand (BOD) incubator at a constant temperature of 20°C, under a 12 h photoperiod and 70% RH.

For germination, only seeds recovered on the 2nd, 3rd and 4th days were considered, given the small number retrieved on the remaining assessment days. A germination test was also performed in the control treatment (no gut passage), using 50 seeds per repetition. At 14 days after sowing, the germination percentage (% in relation to the control) was determined considering normal seedlings with a root and shoot, in accordance with Regulations for Seed Analysis (Brasil, 2009).

Dose-response curve

In order to establish the resistance of the LOLMU-R seeds recovered after animal ingestion, a greenhouse experiment was carried out using a completely randomized design with four replications. After germination assessment, 100 seedlings were transplanted into 0.2 litres plastic pots (one plant per pot) filled with commercial substrate and maintained at field capacity. When the plants reached phenological stage 23 (BBCH, 1997), the maximum recommended dose (1080 g a.e/ha) of glyphosate [Roundup Original®, 360 grams of acid equivalent per litre – (g a.e/l)] was applied.

Twenty days after treatment, the tillers were transplanted to new experimental units (0.2 litres) – two tillers per pot, totalling 28 pots. The treatments were applied when the plants obtained from the tillers reached stage 23 (BBCH, 1997), with the following glyphosate doses: 0, 1080, 2160, 4320, 8640, 17 280 and 34 560 g a.e./ha. Application was performed using a CO_2 pressurized sprayer positioned 0.5 m from the target, equipped with a boom containing flat spray tip nozzles (XR 110.015) spaced 0.5 m apart, at a working pressure of 250 kPa. The average temperature, RH and wind speed during application were 24°C, 69% and 6.5 km/h, respectively.

At 28 days after application, population control (%) was visually assessed. The control was assessed using a percentage scale where a score of zero (0%) was established for no control and 100% for plant death (Burril *et al.*, 1976). The remaining plants were cut at ground level and dried in a forced-air oven (60°C) until constant weight, when shoot dry weight (SDW) was determined.

Statistical analysis

The data obtained were analysed using R software (R Core Team, 2021). The recovered seeds and dose-response curve data were fitted via the three or four-parameter logistic model (3 or 4PL) (Ritz *et al.*, 2015), using the *drc* package (Eqns (1) and (2), respectively).

$$Y = \frac{d}{1 + \exp\{b(\log(x) - \log(\mathrm{D50}))\}} \quad (1)$$

$$Y = c + \frac{d - c}{1 + \exp\{b(\log(x) - \log(\mathrm{D50}))\}} \quad (2)$$

where Y is the resulting response value for dependent variables, either number of recovered seeds, control or SDW; d is the upper limit, defined by the maximum response from the number of recovered seeds or from non-treated plants (control or SDW); c is the lower limit, determined by the response levels from a high dose of herbicide – if $c = 0$, then the four-parameter model (Eqn (2)) reduces to the three-parameter model (Eqn (1)), with the lower limit being zero; D_{50} is the time or dose that causes 50% of recovery seed, control or SDW reduction; and b the slope of the curve at D_{50} (Ritz *et al.*, 2015).

The regression model was submitted to lack of fit testing using the modelFit function and when not significant ($P \geq 0.05$) indicates that the data are well described by the selected model. The parameters and their standard errors were estimated using the *drm* function, with P values ($P \leq 0.05$) dictating whether the parameters were significant. D_{50} was estimated using the ED function, with a 95% confidence interval. Data on SDW and control were converted into a percentage in relation to the control treatment.

Germination of the recovered seeds data was fitted via linear regression (Eqn (3)), using *lm* function (Kniss and Streibig, 2019).

$$Y = a + b * x \quad (3)$$

where Y corresponds to germination (%); a is the intercept of regression line, the predicted value when $x = 0$; b is the slope of the regression line; x = days after ingestion.

The regression model was submitted to lack of fit testing, treating the independent variable as a factor variable and comparing with the fitted linear model using the ANOVA function, when not significant ($P \geq 0.05$), regression analysis describes data appropriately. The *lm* function was used to analyse whether parameters were significant ($P \leq 0.05$), to estimate the standard errors and to determine the adjusted r^2 (Kniss and Streibig, 2019).

Table 1. Estimated parameters (b, d and D_{50} – with standard error and P value) and lack of fit by the non-linear regression equation[a], based on the glyphosate-resistant *Lolium multiflorum* seeds recovered from cattle faeces as a function of time (days after ingestion)

Parameters	Value	Standard error	*P* value
b	6.60	1.95	0.0024
d	820.12	105.98	2.539×10^{-8}
D_{50}	2.74	0.19	3.818×10^{-14}
Residual standard Error	76.80	–	–
Degrees of freedom	27	–	–
Lack of fit	–	–	0.9507

[a] $Y = d/(1 + \exp[b(\log(x) - \log(D_{50}))])$.

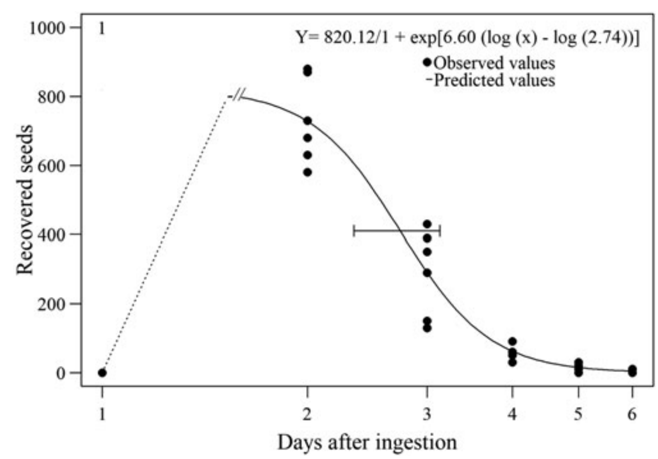

Fig. 1. Observed and predicted values for the number of glyphosate-resistant *Lolium multiflorum* seeds recovered from cattle faeces as a function of time (days after ingestion). Horizontal bar represents the 95% confidence interval to obtain 50% recovery.

Table 2. Estimated parameters (a and b – with standard error and P value) and lack of fit by the linear regression equation[a], based on the germination of glyphosate-resistant *Lolium multiflorum* seeds as a function of recovery time after passage through the digestive system of cattle, 14 days after sowing

Parameters	Value	Standard error	*P* value
a	66.71	2.68	2.00×10^{-16}
b	−11.68	0.99	6.29×10^{-11}
Residual standard error	7.22	–	–
Degrees of freedom	22	–	–
Lack of fit	–	–	0.0667

[a] $Y = a + b \times$ days after ingestion.

Results

Seed recovery

The number of glyphosate-resistant *L. multiflorum* (LOLMU-R) seeds recovered showed a sigmoidal behaviour from the 2nd day after ingestion by the steers (lack of fit, $P \geq 0.05$). The average time to recover 50% of the seeds (D_{50}) was 2.7 days (Table 1). Of

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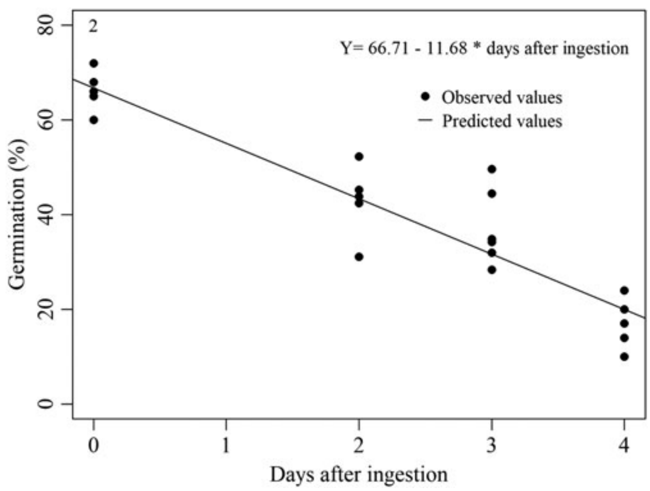

Fig. 2. Observed and predicted values for the germination of glyphosate-resistant *Lolium multiflorum* seeds as a function of recovery time after passage through the digestive system of cattle (compared with the control treatment, no digestive system passage), 14 days after sowing. $r^2 = 0.85$.

the 12 112 seeds supplied per animal, 1109 (9.1%) were recovered during the assessment period, with 1027 retrieved in the first 3 days (92.6% of the total). However, during the first day after ingestion, no seed was recovered. Maximum recovery (728 seeds or 65%) occurred 2 days after ingestion, which declined to 3.33 seeds (or 0.3%) on day 6 (Fig. 1).

Germination of the recovered seeds

The LOLMU-R seed germination showed a linear reduction in response to recovery time (days) after ingestion by cattle (lack of fit, $P \geq 0.05$). The model indicated a reduction around 12% in germination for each day after passage through the digestive system, compared to the control treatment (no digestive system passage) that showed a germination of 67% (Table 2). However, to the end of assessment period or 4 days after ingestion, the germination potential was 18% (or 27% when compared to the control treatment) (Fig. 2).

Dose-response curve

The dose-response curve showed a sigmoidal behaviour (lack of fit, $P \geq 0.05$), where control increased and SDW decreased in resistant *L. multiflorum* as a function of larger glyphosate doses. However, the doses that obtained 50% control (C_{50}) and 50% SDW reduction were 7610 and 5057 g a.e./ha, corresponding to 7 and 5*x*, respectively (Table 3). Thus, LOLMU-R survived the maximum recommended dose (1080 g a.e./ha = 1*x*) (Fig. 3 (*a*) and (*b*)).

Discussion

Endozoochory involves the capture and ingestion of seeds, which are influenced by digestive action as they pass through the digestive system and then eliminated in the faeces (Fazelian *et al.*, 2014). Seeds can be destroyed by mastication and rumination (Alvarez *et al.*, 2016), which may result in more than 80% seed loss (Wang *et al.*, 2017). As such, the recovery rate is directly related to seed characteristics (shape, size and presence or not of an integument), the species that ingests the seeds, diet quality and total retention time in the gastrointestinal tract (Deminicis *et al.*, 2009; Fazelian *et al.*, 2014; Wang *et al.*, 2019).

In general, small round seeds are less likely to be damaged during chewing (Brochet *et al.*, 2010; Picard *et al.*, 2015). *Lolium multiflorum* seeds are compact and medium-sized for a forage grass

Table 3. Estimated parameters (*b*, *d*, *c* and D_{50} – with standard error and *P* value) and lack of fit by the non-linear regression equation[a or b], based on the control and shoot dry weight (both in % relative to the control) of *Lolium multiflorum* plants after seed passage through the digestive system of cattle, 28 days after glyphosate application

Parameters control[a]	Value	Standard error	*P* value
b	−1.51	0.33	1.254×10^{-4}
d	113.76	13.48	8.860×10^{-9}
D_{50}	7610.44	1806.23	2.859×10^{-4}
Residual standard error	14.23	–	–
Degrees of freedom	25	–	–
Lack of fit	–	–	0.0588
Parameters shoot dry weight[b]	Value	Standard error	*P* value
b	2.97	0.73	0.0004
c	27.76	3.29	1.213×10^{-08}
d	99.98	2.79	2.200×10^{-16}
e	5057.92	411.57	7.621×10^{-12}
Residual standard error	7.62	–	–
Degrees of freedom	24	–	–
Lack of fit	–	–	0.5776

[a] $Y = d/(1 + \exp[b(\log(x) - \log(D_{50}))]$.
[b] $Y = c + \{d - c1 + \exp[b(\log(x) - \log(D_{50}))]\}$.

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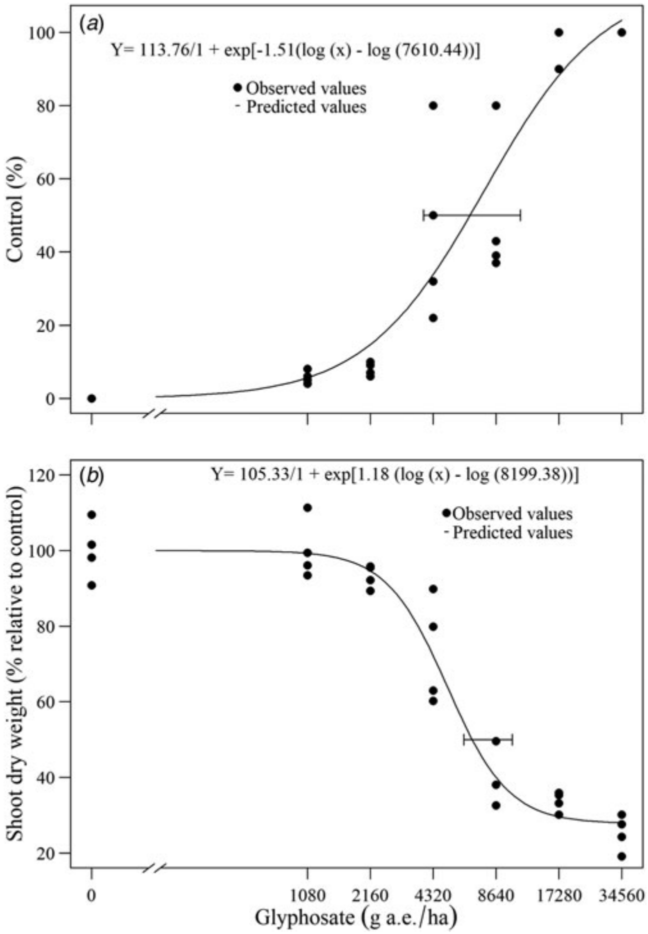

Fig. 3. Dose-response curve for *Lolium multiflorum* plants after seed passage through the digestive system of cattle, 28 days after glyphosate application. (*a*) Observed and predicted population control values (% in relation to the control treatment). Horizontal bar representing the 95% confidence interval to obtain 50% control (C_{50}). (*b*) Observed and predicted shoot dry weight values (% in relation to the control treatment). Horizontal bar representing the 95% confidence interval to obtain 50% shoot dry weight reduction (GR_{50}).

(Fontaneli, 2009), justifying the 9.1% recovery rate recorded in the present study and similar to the 12% of *Brachiaria decumbens* seeds recovered from ruminants (Simão Neto *et al.*, 1987).

It should be noted that the highest recovery rate was obtained 2 days after ingestion. Viero *et al.* (2018) assessed the recovery of weedy rice (*Oryza sativa* L.) and barnyardgrass (*Echinochloa crus-galli* L.) seeds and found that they passed through the gut of the cattle on the first day after ingestion, with zero recovery. However, maximum recovery was obtained 2 days after ingestion, exhibiting sigmoid behaviour and declining to zero (Viero *et al.*, 2018), similar to the results of the present study.

The processes involved in endozoochory affect the physical and physiological characteristics of seeds, which are exposed to physical processes, microbial activity in the rumen, chemical action in the abomasum, temperature and internal pH (Oliveira *et al.*, 2013; Fazelian *et al.*, 2014). These processes influence seed viability and, consequently, germination (Blackshaw and Rode, 1991; Gardener *et al.*, 1993; Wang *et al.*, 2019).

Studies that simulated the mastication, body temperature corporal and digestive fluids of ruminants observed inhibitory effects on the germination of most species assessed (Milotić and Hoffmann, 2016).

In weedy rice and barnyardgrass, germination declined in seeds that had passed through the gut of cattle (Viero *et al.*, 2018). This phenomenon may be directly related to the loss of seed viability as a function of retention time in the rumen (Lisboa *et al.*, 2009). Some seeds tolerate a certain amount of time in the rumen, followed by a rapid decline in viability (Blackshaw and Rode, 1991). These previous results corroborate those found here, whereby the germination of LOLMU-R seed decreased as a function of time after ingestion, that is, recovery time. Ruminants, especially cattle, are considered the main vectors of endozoochory, particularly in the dispersal of species from the family Poaceae (Fazelian *et al.*, 2014). In this respect, according to Nakao and Cardoso (2010), cattle are considered a legitimate dispersal agent of glyphosate-resistant *L. multiflorum* seeds.

The occurrence of glyphosate-resistant *L. multiforum* impacts production systems in which the species is used as a winter cover crop (no-till), plant cover (orchards and vineyards) or forage (ICLS) (Peterson *et al.*, 2018). A previous study confirmed the resistance of this species, demonstrating that glyphosate doses above the recommended maximum were ineffective (Vargas *et al.*, 2004). HR can be confirmed by studying dose-response curves (Burgos *et al.*, 2013). Thus, the results presented here prove resistance in LOLMU-R, where the control dose is greater than the recommended maximum.

Conclusions

Cattle are a legitimate dispersal agent of glyphosate-resistant *L. multiflorum* seeds. This may worsen the evolution of resistance, since in these cases, dispersal is far more important as a source of new infestations. Thus, in order to mitigate the evolution of glyphosate resistance, a quarantine period of at least 7 days is recommended before animals move from one infested area to another.

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Conflicts of interest. None.

Ethical standards. The research protocol was reviewed and approved by the Animal Ethics Committee of the Department of Agricultural Diagnostics and Research (DDPA).

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