

# The role of plant size in the selection of glyphosate resistance in *Sorghum halepense*

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## Abstract

**BACKGROUND:** The effect of plant size (seedlings versus young plants versus adult plants) on the phenotypic level of glyphosate resistance and selection intensity (SI) in *Sorghum halepense* with and without a reduced glyphosate translocation resistance mechanism was evaluated.

**RESULTS:** Resistance parameters [the 50% lethal dose ( $LD_{50}$ ) and the dose required to cause a 50% reduction in plant growth ( $GR_{50}$ )] in adult plants were notably higher than in seedlings regardless of the resistance status. However, under similar plant size increases, populations comprised of glyphosate-resistant (R) individuals showed higher survival and growth when glyphosate treated compared with glyphosate-susceptible (S) plants. An increase in SI was always evident with increasing glyphosate doses. However, the rate of increase in SI was higher under glyphosate selection of young R and S plants, followed by seedlings and adult R and S plants. However, in conditions of R seedlings coexisting with adult S plants under glyphosate treatment ( $1000\text{--}4000\text{ g ha}^{-1}$ ), selection against glyphosate resistance was observed.

**CONCLUSION:** Any increase in size from the seedling stage of R plants translates into an amplification of resistance. Depending on the particular size combinations of spatially coexisting R and S plants, selection for glyphosate resistance may be faster, slower or even not evident.

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Supporting information may be found in the online version of this article.

**Keywords:** agroecosystem; perennial species; fitness; resistance trait

## 1 INTRODUCTION

Since their introduction seven decades ago, herbicides have imposed a strong selection intensity (SI) leading to herbicide resistance evolution in an ever-increasing list of plant species.<sup>1–3</sup> Among other factors, such as population size, gene flow and mutation frequency, the evolutionary rate of herbicide resistance is determined by the interplay between the intensity of herbicide selection and standing genetic variation in weed populations.<sup>4–6</sup> SI (i.e. selection pressure) is a function of the herbicide dose and its relative effect on the fitness ( $W$ ) of herbicide-resistant (R) and -susceptible (S) phenotypes.<sup>7</sup> The higher the SI, the higher the rate of resistance evolution, particularly for resistance mechanisms controlled by major genes.<sup>4</sup>

The effect of a herbicide on plant fitness depends on the amount of active ingredient reaching and inhibiting the herbicide target protein. This is the case for adaptive non-target site herbicide resistance mechanisms, which endow a significant fitness advantage under herbicide treatment by reducing the amount of active ingredient reaching the herbicide target site.<sup>8</sup> Little or no detrimental herbicide effect on plant fitness is expected provided that a significant amount of the active ingredient is blocked from reaching and inhibiting its target protein.

Plant size at the time of treatment with leaf-applied herbicides also determines the amount of active ingredient reaching the herbicide target protein. For a particular herbicide dose, large plants intercept less herbicide per unit biomass compared with smaller

plants.<sup>9</sup> This plant size-based herbicide dilution effect reduces the amount of active ingredient reaching the target protein, which is relatively in excess in large plants compared with small plants. Whether this process has any impact on SI and thus the rate of herbicide resistance evolution will depend on the relative plant size-based herbicide dilution effect in R versus S plants.

If the effect of large plants in the above-mentioned herbicide diluting effect is similar for both R and S individuals, then the expected differential fitness ratio of R versus S plants for a particular herbicide dose (i.e.  $W_R/W_S > 1$ ) will be constant regardless of the plant size ( $W_{\text{large R}}/W_{\text{large S}} = W_{\text{small R}}/W_{\text{small S}}$ ). This result would translate into a herbicide SI that remains constant with changes in plant size and the magnitude of SI will then be only a function of the fitness advantage endowed by the resistance trait in the R phenotype. However, if there were an interaction effect between

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the resistance trait and plant size on the herbicide diluting process, for instance large R plants dilute a herbicide dose more than large S plants, then the expected differential fitness ratio of R and S plants will not be constant with changes in plant size. This would be the case for a resistance mechanism that increases its efficiency as plants become larger. In this case, the herbicide SI at any specific dose would depend on the particular differential fitness ratio of large and small R and S individuals under herbicide selection ( $W_{\text{large R}}/W_{\text{large S}} \neq W_{\text{small R}}/W_{\text{small S}}$ ).

Variations in plant size among conspecifics may result from differences in both age and asymmetric competition.<sup>10</sup> In agroecosystems, spatial coexistence of R and S individuals of different size is often found in perennial weed species under recurrent herbicide selection, and in populations of annual species in which R individuals exhibit a fitness penalty associated with the resistance trait.<sup>11–13</sup> As a result, seedlings and larger plants are likely to coexist in populations that are under recurrent herbicide selection in agroecosystems.

Glyphosate resistance has evolved in the perennial  $C_4$  grass *Sorghum halepense* (Johnsongrass) invading cropping systems in Argentina.<sup>14</sup> A reduced glyphosate translocation resistance mechanism which retains much of the applied glyphosate in the treated leaves, preventing its movement to meristematic tissues, has been identified in several resistant *S. halepense* populations infesting soybean crops.<sup>15</sup> A similar resistance trait has also naturally evolved in *S. halepense* infesting crop fields in Arkansas (USA).<sup>16</sup>

The aim of this study was to quantify the effect of plant size on plant survival and growth of glyphosate R and S populations of *S. halepense* under increasing glyphosate doses. Estimations of the intensity of glyphosate selection with changes in plant size of R and S plants were also performed, and the implications for glyphosate resistance evolution are discussed.

## 2 MATERIALS AND METHODS

### 2.1 Plant material

Two glyphosate-resistant *S. halepense* populations ( $R_1$  and  $R_2$ ) were collected from transgenic glyphosate-resistant soybean fields in Salta Province in Argentina (22°39' S, 63°49' W).<sup>14</sup> Glyphosate resistance in these two populations is endowed by a mechanism that reduces glyphosate translocation to growing

tissues.<sup>15</sup> A known glyphosate-susceptible (S) accession collected from the Pampas cropping area (34°35' S, 58°35' W) in the Province of Buenos Aires in Argentina was used as a reference population.<sup>14,15</sup>

### 2.2 Plant size and glyphosate

Plant survival and growth of glyphosate-resistant ( $R_1$  and  $R_2$ ) and glyphosate-susceptible (S) populations under increasing glyphosate doses were evaluated in plants at three different growth stages and thus three different sizes. The plant sizes correlated with three plant stages: seedling, young plant and adult plant. To obtain the corresponding plant size classes, seeds from the three populations were germinated. To overcome seed dormancy, field-collected seeds of the  $R_1$ ,  $R_2$  and S populations were immersed in sodium hypochlorite bleach (chlorine 5.5% w/v) for 8 h, then washed with tap water and germinated in transparent plastic boxes filled with 0.7% (w/v) agar. The boxes were incubated in a chamber set at 12-hourly alternating 30/20 °C with a 12-h photoperiod and radiation of 60  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  provided by fluorescent lamps.

Variations in plant size were generated by the sequential seeding and transplanting of similar-size seedlings at three time intervals in 25-L pots (35 cm diameter  $\times$  28 cm height) (eight seedlings grown per pot) containing a mix of organic soil, sand and peat moss (50%, 25% and 25%, v/v, respectively). A multifactorial experimental design (3  $\times$  3  $\times$  9) was used in which plants of the three different plant sizes (seedling, young plant and adult plant) from the three populations [two glyphosate-resistant (R) and one glyphosate-susceptible (S)] were exposed to increasing glyphosate doses (0, 250, 500, 1000, 2000, 4000, 6000, 8000 and 10 000 g ae ha<sup>-1</sup>). Using three replicates per treatment, a total of 243 experimental units (i.e. pots) were randomly placed in a heated glasshouse at a fluctuating temperature of 25/21 °C (day/night). Plants were regularly irrigated and fertilized with urea (46% nitrogen). Characterization of individual plant size classes at the time of glyphosate treatment was carried out by estimating dry matter, plant height, and the number of vegetative tillers (including the main shoot) and flowering tillers (Table 1). Aboveground biomass of surviving plants after glyphosate treatment was harvested and dried at 70 °C for 72 h and then weighed. Thermal-time (TT) units accumulated from the time of transplanting to glyphosate

**Table 1.** Average biomass (dry matter), height, number of vegetative and reproductive tillers and rhizome biomass characterizing individual plants in the three size classes (seedling versus young plant versus adult plant) from the glyphosate-resistant ( $R_1$  and  $R_2$ ) and -susceptible (S) *Sorghum halepense* populations at the time of glyphosate treatment

Trait	Plant size	Population		
		$R_1$	$R_2$	S
Aboveground biomass (g)	Seedling	0.02 (0.005)	0.02 (0.003)	0.01 (0.005)
	Young plant	0.35 (0.1)	0.40 (0.1)	0.40 (0.1)
	Adult plant	2.40 (0.2)	1.90 (0.1)	2.10 (0.1)
Height (cm)	Seedling	8 (1)	9 (0.5)	9 (0.5)
	Young plant	42 (1)	43 (2)	48 (1)
	Adult plant	74 (8)	71 (1)	78 (3)
Vegetative tillers (number)	Adult plant	1.1 (0.1)	1.2 (0.2)	2.3 (0.2)
Rhizomes (mg)	Adult plant	90 (0.04)	60 (0.04)	150 (0.06)
Flowered tillers (number)	Adult plant	0.20 (0.1)	0.04	0.25

Values in parentheses denote standard error of the mean using 10 plants per population. Vegetative and reproductive tillers and rhizomes were only evident in adult plants.

treatment were estimated for each plant size (Table 1):

$$TT \left( ^\circ\text{C day}^{-1} \right) = \sum_{t=0}^x (T_m - T_b) \quad (1)$$

where  $T_m$  is the mean daily temperature ( $^\circ\text{C}$ ),  $T_b$  is the base temperature ( $15^\circ\text{C}$ )<sup>17</sup> and  $t_0$  and  $t_x$  are dates of sowing (different for each plant size class) and date of glyphosate treatment, respectively. The calculated thermal time was 130, 350 and  $585^\circ\text{C day}^{-1}$  for seedlings, young plants and adult plants, respectively. Thermal time represents, then, the time in thermal units above  $15^\circ\text{C}$  required for R and S individuals to be seedlings, young plants or adult plants at the time of glyphosate treatment.

Glyphosate (potassium salt;  $500\text{ g ae ha}^{-1}$ ) was applied using a  $\text{CO}_2$  pressurized backpack sprayer fitted with a Lurmark TT 11001 nozzle delivering  $100\text{ L ha}^{-1}$  at  $200\text{ kPa}$ . Plant survival, aboveground dry biomass and the number of newly emerged tillers on surviving plants were evaluated 4 weeks after glyphosate treatment.

### 2.3 Effect of plant size on glyphosate resistance level

Variations in plant survival and biomass with increasing glyphosate dose were analyzed using dose–response models with the package *drc*<sup>18</sup> in R.<sup>19</sup> These models are regression models where the independent variable is the glyphosate dose while the dependent variables were either plant survival or biomass (one model for each dependent variable).<sup>18</sup> We fitted the three-parameter log-logistic function (LL.3 in *drc*) given by the expression:

$$y = \frac{d}{1 + \exp\{b[\log(x) - \log(e)]\}} \quad (2)$$

where  $y$  denotes plant survival or biomass attained at herbicide rate  $x$ ,  $d$  is the upper limit, and  $b$  is the slope at  $e$  which accounts for the herbicide dose causing a 50% reduction in survival [50% lethal dose ( $\text{LD}_{50}$ )] or plant biomass [dose causing 50% growth reduction ( $\text{GR}_{50}$ )] between the upper limit  $d$  and the lower limit fixed to 0. The three-parameter log-logistic function (LL.3) was chosen as it showed a better fit than the four-parameter model (LL.4) (Supporting Information Figs S1 and S2).

The models fitted with the function *drm* (in *drc*) included dose, genotype and plant size class as independent variables. To evaluate the effect on different parameters, we compared this full model with models where each of the parameters was fixed (the same for all genotypes and size classes). In the case of survival, the  $F$ -test for the model reduction was not significant for the upper limit. Then, we fitted the final model with a fixed upper limit, and different slope and  $\text{LD}_{50}$  according to the genotype and the size class (Fig. S1). In the case of plant biomass, the final model had different slope,  $\text{GR}_{50}$  and upper limit according to the genotype and the size class (Fig. S2).

In both cases, survival and biomass, a test to check the fit of the model showed non-significant  $P$ -values, indicating that the model provided a good fit (function *modelFit*) (Figs S1 and S2). The function *modelFit*() can be used to perform a lack-of-fit test, comparing the three-parameter log-logistic to the more general one-way analysis of variance (ANOVA) model, which has a parameter for each dose level. Then, we calculated the  $\text{LD}_{50}$  and  $\text{GR}_{50}$  estimated values, the standard error and the 95% confidence intervals (function *ED* in *drc*). We compared the  $\text{LD}_{50}$  and  $\text{GR}_{50}$  for the resistant populations ( $R_1$  and  $R_2$ ) against those for the susceptible population (S) for each plant size class by means of ratios

(*compParm* function in *drc*). The squared correlations between observed and fitted values ( $R^2$ ) were 0.93 and 0.94 for the plant survival and biomass models, respectively (Figs S1 and S2). Full details of regression analyses can be found in Figs S1 and S2.

The level of resistance to a herbicide is often defined as the ratio of the amount of herbicide required to kill or affect growth to a certain level in a herbicide-resistant population to that in a herbicide-susceptible population. The glyphosate resistance index (RI) attained in each plant size class was estimated as the ratio of  $\text{LD}_{50}$  or  $\text{GR}_{50}$  in the  $R_1$  and  $R_2$  populations to  $\text{LD}_{50}$  or  $\text{GR}_{50}$  in the S population (i.e.  $\text{RI} = \text{LD}_{50R}/\text{LD}_{50S}$ ).

Plant tillering was recorded on surviving plants of the  $R_1$ ,  $R_2$  and S populations. The average number of newly emerged tillers produced per plant after glyphosate treatment was analyzed by generalized least squares (gls) linear regression.<sup>19</sup>

### 2.4 Effect of plant size on glyphosate selection intensity (SI)

Whereas the herbicide RI integrates the mean efficiency of a resistance mechanism in protecting plants from herbicide damage (survival or growth) over a wide range of herbicide doses, estimations of herbicide SI are based on plant fitness. Ideally, estimates of SI at particular single herbicide doses should include the size of the progeny produced by the proportion of plants that survived the herbicide treatment.<sup>7,20</sup> Here, we followed Neve and Powles' methodology<sup>21</sup> and estimated the SI at each particular glyphosate dose ( $1000$ ,  $2000$ ,  $3000$  and  $4000\text{ g ha}^{-1}$ ) as the linear combination of two plant traits: the proportion of plants surviving glyphosate treatment and the relative vegetative growth of those surviving individuals compared with the mean biomass production of glyphosate-untreated plants, the latter being a proxy of the effect of the herbicide on plant growth and fecundity. The glyphosate SI was then calculated for each plant size class (seedling, young plant or adult plant) by dividing the combination of the two fitness traits of both R phenotypes (pooled) by that of the S phenotype<sup>5,7,20</sup>:

$$SI = \frac{\text{survival}_R \times \text{growth}_R}{\text{survival}_S \times \text{growth}_S} \quad (3)$$

where 'survival' and 'growth' are, respectively, the frequency of surviving plants and the mean vegetative growth relative to the growth of glyphosate-untreated plants for the  $R_1$ ,  $R_2$  and S populations under glyphosate treatment. The coefficient of SI was estimated for a range of glyphosate doses ( $1000$ – $4000\text{ g ha}^{-1}$ ) normally used to control *S. halepense* populations in field conditions. Mean plant survival and biomass estimates for the calculation of glyphosate SI were derived from the three-parameter log-logistic regression equations (Eqn 2) for this glyphosate dose range.<sup>7,22</sup>

Variations of glyphosate SI with changes in plant size were estimated following two approaches. Firstly, SI was calculated within each of the three plant size classes as if the R and S plants coexisted in space and time at equal frequencies and with the same plant sizes (e.g. R seedlings *versus* S seedlings). Secondly, SI was estimated for three particular ecological scenarios in which R and S plants may coexist in space and time at similar frequencies but with different plant size classes. The case of R plants expressing a moderate fitness penalty associated with the resistance trait and thus coexisting with larger S plants was evaluated (e.g. young R plants coexisting with adult S plants). Additionally, SI was also estimated in the potential case of R seed dispersal into an environment already infested with S plants (e.g. R seedlings coexisting with young or adult S plants).

**Table 2.** Estimates of the regression ( $y = d/1 + \exp\{b[\log(x) - \log(e)]\}$ ) parameters  $LD_{50}$  and  $GR_{50}$  associated with glyphosate-susceptible (S) and -resistant ( $R_1$  and  $R_2$ ) *Sorghum halepense* populations exposed to increasing glyphosate doses at three plant size stages

Size class	Population	$LD_{50}$	Resistance index
Seedling	S	511 (26)	
	$R_1$	937* (63)	1.8 (0.1)
	$R_2$	1285* (111)	2.5 (0.2)
Young plant	S	869 (59)	
	$R_1$	3301* (239)	3.8 (0.3)
	$R_2$	3545* (228)	4.1 (0.3)
Adult plant	S	1213 (108)	
	$R_1$	6303* (556)	5.2 (0.5)
	$R_2$	5918* (411)	4.9 (0.4)

Size class	Population	$GR_{50}$	Resistance index
Seedling	S	441 (189)	
	$R_1$	559 (294)	1.3 (0.8)
	$R_2$	577 (404)	1.3 (0.6)
Young plant	S	815 (84)	
	$R_1$	2645* (584)	3.2 (0.07)
	$R_2$	3390* (430)	4.1 (0.04)
Adult plant	S	1424 (164)	
	$R_1$	10 852* (1183)	7.6 (0.02)
	$R_2$	16 414* (5303)	11.5 (0.03)

Values in parentheses are standard error of the mean. Asterisks indicate significant differences ( $P < 0.01$ ) after pairwise comparisons of  $LD_{50}$  or  $GR_{50}$  between resistant and susceptible populations within each plant size class using a *t*-test (*compParm* function in R software).

Glyphosate SI models considered glyphosate dose, the size class (or the group that resulted from the combination of size classes) and the interaction. Linear models were adjusted using generalized least squares using the *gls* function of the *nlme* package in R.<sup>23</sup> When it was necessary to fulfill the assumptions, the varIdent variance function was included for the size class (or the combinations). Nested models were compared with the AICtab function of the *bbmle* package.<sup>24</sup> The assumptions were verified graphically. The distribution of the normalized residuals was observed as a function of the adjusted values and of the size classes or groups to evaluate homogeneity in the variances. Q-Q graphs of the quantile distribution of the observed residuals were plotted against the theoretical normal distribution to evaluate normality. The inference was made using the Anova function of the *car* package.<sup>25</sup> When there were differences between the size groups, *a posteriori* comparisons of least-squares means were made using the *emmeans* function of the *emmeans* package in which regression slopes for groups were compared using Tukey's test ( $\alpha = 0.05$ )<sup>19</sup> (Figs S3 and S4).

### 3 RESULTS

#### 3.1 Plant survival and growth under glyphosate selection

*Sorghum halepense* survival and growth under glyphosate selection varied depending on the particular population (S versus  $R_1$  and  $R_2$ ), the glyphosate dose and the size of the plants. Within each plant size class (e.g. seedling versus seedling), individuals from both the  $R_1$  and  $R_2$  populations exhibited significantly higher survival and aboveground biomass, as determined by  $LD_{50}$  and

$GR_{50}$ , respectively (with the exception that  $GR_{50}$  values at the seedling stage were similar between the R and S populations), when exposed to the wide range of glyphosate doses compared with plants from the S population (Table 2 and Fig. 1). Regression  $LD_{50}$  and  $GR_{50}$  estimates increased with increases in the size of the treated plants regardless of the population (S,  $R_1$  or  $R_2$ ) (Table 2 and Fig. 1). However, differences in glyphosate resistance levels among populations were not proportionally constant with increases in plant size of the glyphosate-treated plants (Table 2). Thus, a significant increase in the glyphosate RI, accounting for both plant survival and growth, from seedlings to adult plants was observed. At the seedling stage, the estimated RI for plant survival and growth was, on average, 2 and 1.3, respectively, whereas at the adult stage, the estimated RI was 5 and 9.5, respectively (Table 2).

The emergence of new tillers in response to glyphosate treatment was not observed in seedlings or young plants that survived glyphosate treatment (data not shown). However, a positive correlation between the number of newly emerged tillers in plants surviving glyphosate treatment and glyphosate dose was observed in adult plants (Fig. 2). This response was evident in adult individuals from the  $R_1$  and  $R_2$  populations but not in adult plants from the S population that survived the glyphosate treatment (Fig. 2).

#### 3.2 Plant size and glyphosate selection intensity

The assessment of plant survival and growth responses to increasing glyphosate doses for the *S. halepense*  $R_1$ ,  $R_2$  and S populations enabled the estimation of the glyphosate SI associated with each of the three plant size classes (seedling, young plant and adult plant).

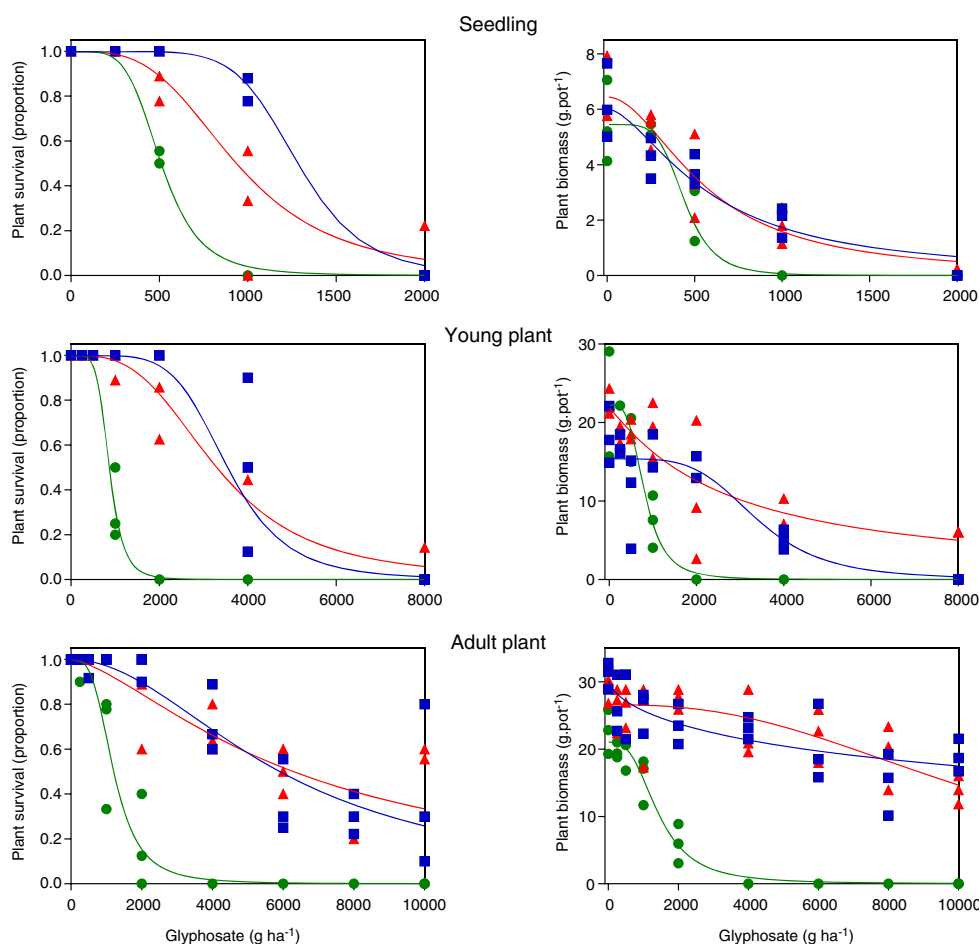
For an ecological scenario in which glyphosate R and S plants coexist in a similar proportion and are of the same size class (e.g. seedling versus seedling), glyphosate SI increased linearly ( $P < 0.001$ ) with increasing glyphosate dose regardless of the plant size (Fig. 3a and S3). However, as the glyphosate dose increased to 4000 g ha<sup>-1</sup>, the greatest increase in SI corresponded to the selection of R and S young plants followed by seedlings and adult plants (Fig. 3a and S3).

Linear increases ( $P < 0.01$ ) (Fig. 3b) in the selective advantage of glyphosate resistance under increasing glyphosate doses were also evident for those particular ecological scenarios in which young R plants may coexist with adult S individuals (i.e. the fitness cost scenario) and R seedlings may coexist with young S plants (i.e. the resistance dispersal scenario) (Fig. 3b and S4). Interestingly, in an environment where R seedlings may coexist with adult S plants, selection against glyphosate resistance was observed, as indicated by the significant linear decrease (i.e. negative slope) of SI with increasing glyphosate dose from 1000 to 4000 g ha<sup>-1</sup> (Figs 3b and S4).

## 4 DISCUSSION

#### 4.1 Glyphosate resistance increases with plant size

The most commonly identified glyphosate resistance mechanism in plants is attributable to reduced glyphosate translocation.<sup>8</sup> Glyphosate resistance produced by the reduced glyphosate translocation resistance mechanism has been shown to be expressed at early plant stages in *Lolium rigidum* and *L. multiflorum* (i.e. expressed in germinating seeds)<sup>26,27</sup> but not in *Conyza canadensis* (i.e. not expressed in seedlings).<sup>28</sup> In the latter example, glyphosate resistance is strictly dependent on the growth stage, given that it is evident only when 'resistant' plants are glyphosate-treated at the rosette stage and not when they are



**Figure 1.** Effect of increasing glyphosate dose on plant survival (left panel) and biomass (right panel) of plants of different size (seedling, young plant or adult plant) from the glyphosate-susceptible [S (●)] and glyphosate-resistant [ $R_1$  (▲) and  $R_2$  (■)] *Sorghum halepense* populations. Symbols are actual data ( $n = 3$ ), while lines are the logistic models fitted to each population.

treated at the seedling stage.<sup>28</sup> In the present study, compared with glyphosate S seedlings, the higher survival of *S. halepense* R seedlings after glyphosate treatment suggests that the reduced glyphosate translocation resistance mechanism in *S. halepense* is fully functional at the seedling stage (Fig. 1).

Variations in the size of herbaceous plants have been often associated with variations in resource competitive ability,<sup>10,29,30</sup> resistance and tolerance to herbivory<sup>31,32</sup> and water stress tolerance.<sup>33,34</sup> Our study shows that variations in plant size also correlate with quantitative variations in glyphosate resistance at the whole-plant level.

As indicated by the  $LD_{50}$  and  $GR_{50}$  resistance parameters, significantly more glyphosate was required to have the same impact on survival and growth as plant size increased, particularly in glyphosate-resistant plants (Table 2). Although not directly estimated in our study, this suggests that a size-driven 'herbicide dilution' process occurs when larger plants are exposed to glyphosate compared with smaller plants. Larger plants are likely to intercept more active ingredient as a consequence of having a higher leaf area index, but concurrent higher increments in biomass are expected to reduce the ratio of herbicide ingredient : biomass unit.<sup>9</sup>

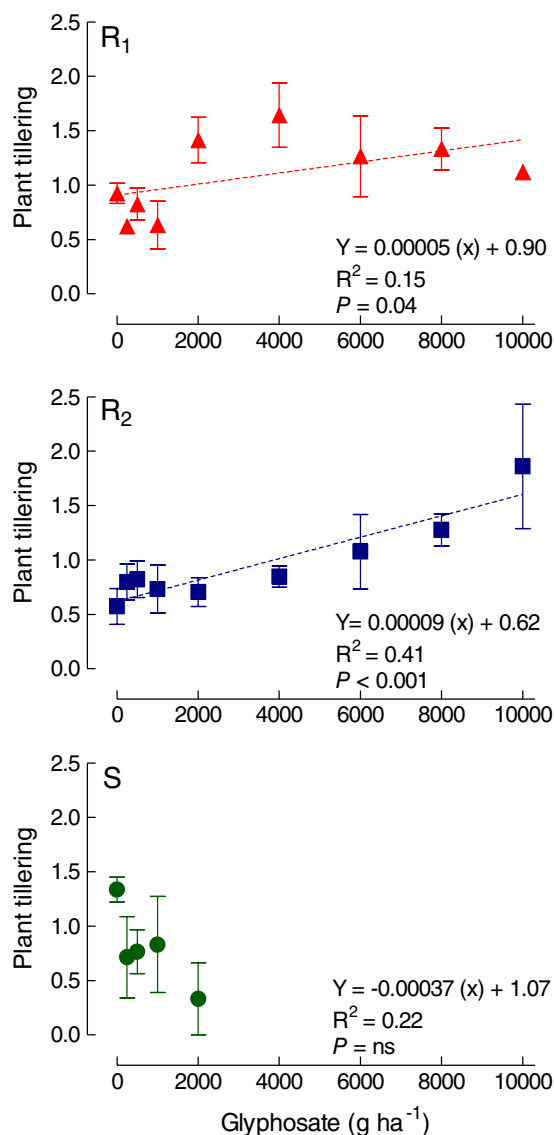
The greater increase in plant survival and growth observed in adult plants compared with seedlings was not proportional in glyphosate R compared with S plants under glyphosate treatment.

This resulted in a higher glyphosate resistance factor (Table 2) when R and S plants were glyphosate-treated at the adult stage compared with the seedling and young plant stages. It is speculated that another factor contributing to glyphosate resistance has a greater effect as R plants become larger. We hypothesize that, as glyphosate R plants become larger, the efficiency of the resistance mechanism leading to reduced glyphosate translocation increases.

#### 4.2 Intensity of glyphosate selection (SI) depends on glyphosate dose and plant size

Quantification of the effect of herbicides on plant fitness is required to understand the strength and direction of selection for resistance evolution. Regardless of the resistance level attained by a genotype under a wide range of herbicide doses (RI), the herbicide selective advantage of an R genotype over an S genotype (SI) must be assessed under the recommended herbicide field rates. Based on the combination of survival and growth traits, results presented here have shown that the glyphosate SI in *S. halepense* is a function of the dynamic combination of both glyphosate dose and the size of spatially coexisting R and S individuals.

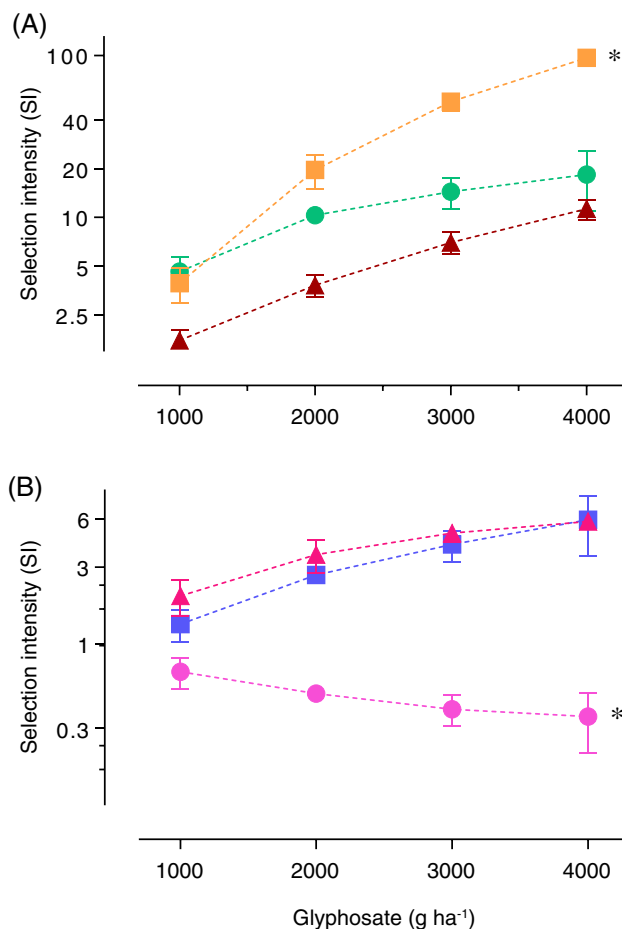
At early plant successional or invasion phases in agroecosystems, individuals are more likely to exhibit similar plant size and developmental stage. Within this ecological scenario in which



**Figure 2.** Average number of newly emerged tillers (plant tillering) per plant surviving the glyphosate effect in glyphosate-susceptible [S (●)] and glyphosate-resistant [R<sub>1</sub> (▲) and R<sub>2</sub> (■)] *Sorghum halepense* populations. Symbols are actual data (mean ± standard error; n = 3), while lines are significant linear regression models fitted to each population. ns, not significant linear regression.

glyphosate-resistant and -susceptible *S. halepense* plants are of similar size, estimates of glyphosate SI increased linearly over a range of increasing glyphosate recommended field doses (1000–4000 g ha<sup>-1</sup>), an indication that glyphosate resistance would evolve under higher glyphosate doses (Figs 3a and S3). In addition, for a given glyphosate dose > 1000 g ha<sup>-1</sup>, the intensity of glyphosate selection also showed higher estimates for R and S young plants than for seedlings and adult plants.

Under relatively high glyphosate doses (> 1000 g ha<sup>-1</sup>), young R plants seem to have a greater selective advantage compared with R seedlings and adult plants (Fig. 3a). The explanation for this may be that, as R and S plants become larger (i.e. reach the adult stage), there is a lower glyphosate selective effect for R plants, as S plants also show a size-based fitness increase under these herbicide doses (Fig. 3). At the adult stage, the differential fitness advantage of R over S plants at, for instance, 4000 g ha<sup>-1</sup> is reduced as adult S



**Figure 3.** Estimates of glyphosate selection intensity in *Sorghum halepense* populations under increasing glyphosate doses. Populations are comprised of equally frequent glyphosate-resistant (R) and glyphosate-susceptible (S) plants of (A) the same size [seedlings (●), young plants (■) or adult plants (▲)], and (B) different sizes [young R and adult S plants (▲) (e.g. fitness cost scenario); R seedlings and young S plants (■) (e.g. dispersal of resistant seeds); or R seedlings and adult S plants (●) (e.g. dispersal of resistant seeds)]. Selection intensity estimations include survival and growth of individuals of both glyphosate-resistant R<sub>1</sub> and R<sub>2</sub> populations (pooled) as compared with individuals of the glyphosate-susceptible (S) population according to Eqn 3 (see main text). Symbols are mean ± standard error. Asterisks (\*) denote a significant difference in the slope of the particular linear regression compared with the other regression slopes after Tukey's test (see Figs S3 and S4).

plants exhibit higher fitness (survival 15%; growth 21%) compared with young S plants (survival 2.5%; growth 7%) (Fig. 1). Conversely, at the seedling stage, the glyphosate resistance mechanism is probably not efficient enough to protect R seedlings from damage at high glyphosate doses (Table 2) and therefore the differential fitness advantage of R seedlings over S seedlings is reduced.

These results highlight that the intensity of glyphosate selection is influenced by both the size of plants and the glyphosate dose, suggesting a dependence of the evolutionary rate of glyphosate resistance on these two factors.

In a number of other ecological conditions, glyphosate R and S plants of dissimilar size may coexist: for example, if there is dispersal of glyphosate-resistant seed into new cultivated areas in which, for instance, glyphosate R seedlings coexist with established young or adult S plants. In the former case (R seedlings coexisting with young S plants), selection of glyphosate resistance would be

evident in the field (Fig. 3b). However, in the latter case (R seedlings coexisting with adult S plants), as shown by a negative linear slope (Fig. S4), estimates of glyphosate SI indicate that there would be no selection advantage for glyphosate R seedlings under glyphosate treatment (Fig. 3b). This outcome suggests that, all other factors being equal, glyphosate resistance would not evolve under these particular ecological conditions.

Another ecological environment in which glyphosate R and S *S. halepense* plants of dissimilar size may coexist is when fitness costs are associated with glyphosate resistance alleles.<sup>12,35</sup> Provided that herbicide resistance fitness costs originate from reduced relative growth rates (RGRs), coexistence of young R plants with adult S plants in the field is expected.<sup>11</sup> Despite this fitness cost, estimates of glyphosate SI indicate a clear selective advantage for resistance under a wide range of glyphosate field doses (Figs 3b and S4).

### 4.3 Modeling resistance evolution

Predictive models for resistance evolution seldom assume that coefficients of SI are dependent of variations in size among R and S individuals.<sup>36</sup> Our results suggest that the implementation of more accurate resistance models is possible using a covariant parameter affecting the SI according to the size class structure exhibited by R and S individuals. Herbicide failures in controlling weeds in agroecosystems are usually followed by herbicide applications using higher herbicide doses. Results presented here suggest that the increase in herbicide field doses may exacerbate the SI for resistance provided that resistant plants are large enough. Results also show that management of herbicide resistance should consider not only the presence/absence of herbicide resistance traits within populations but also the variability associated with the distribution of plant size classes within the herbicide-treated populations.

Use of higher glyphosate doses will increase the SI for resistance as long as coexisting glyphosate R and S plants have similar plant sizes or glyphosate R plants have a size corresponding to the young plant stage or seedling stage but coexist only with young S plants. In contrast, no selective advantage for glyphosate resistance would be expected when increments of glyphosate doses occur in *S. halepense* populations comprised of glyphosate R seedlings coexisting with adult glyphosate S plants.

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## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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