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Article

# Comparison of Sensitivity to Glyphosate of *Euphorbia davidii* Populations

*Comparação da Sensibilidade ao Glyphosate de Populações de* ***Euphorbia davidii***

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ABSTRACT - *Euphorbia davidii* (Euphorbiaceae) is a summer annual weed present in the Province of Buenos Aires, Argentina. The aim of this study was to compare the sensitivity to glyphosate of different populations of *Euphorbia davidii*. Two techniques were used: a seed bioassay and shikimate determination in glyphosate-treated plants. Germination rates of seeds belonging to the populations from Espartillar, Barrow, Olavarria and Azul exposed to different concentrations of glyphosate were compared. Plants from the same locations treated with glyphosate were evaluated to determine shikimate levels. The most sensitive population was from Espartillar, with a $CE_{50}$ (average effective concentration) of 4.00 g e.a. $L^{-1}$. Based on this finding, the resistance index for each population was 1.76, 2.39 and 2.48 for Azul, Olavarria and Barrow respectively. Regarding glyphosate-treated plants in which shikimate levels were determined, a significant increase in the concentration of this metabolite was only observed in the population from Espartillar. Accumulation of shikimate was not detected in the other groups. These results show differential sensitivity to glyphosate of the evaluated populations.

**Keywords:** herbicide resistance, germination, bioassay, shikimate, EUDA5.

*RESUMO -* ***Euphorbia davidii*** *(Euphorbiaceae) é uma planta infestante de ciclo anual primaveril-estival presente na Província de Buenos Aires, Argentina. O objetivo deste estudo foi comparar a sensibilidade ao glyphosate de diferentes populações de* ***Euphorbia davidii****. Para isso, foram usadas duas técnicas: bioensaio com sementes e determinação dos níveis de chiquimato nas plantas tratadas com glyphosate. Fez-se uma comparação da germinação de sementes de populações provenientes de Espartillar, Barrow, Olavarria e Azul, expostas a diferentes concentrações de glyphosate. Procedeu-se à determinação dos níveis de chiquimato em plantas provenientes das mesmas localidades e tratadas com o herbicida. A população mais sensível foi a de Espartillar, com uma* $CE_{50}$ *(concentração efetiva média) de 4,00 g a.e.* $L^{-1}$*. Com base neste valor, o índice de resistência de cada local de origem foi de 1,76 , 2,39 e 2,48 para Azul, Olavarría e Barrow, respectivamente. Entre as plantas tratadas, em que os valores de chiquimato foram determinados, observou-se apenas aumento significativo na concentração desse metabólito na população de Espartillar. Nas outras populações não foi identificada qualquer acumulação de chiquimato. Os resultados mostram a sensibilidade diferencial ao glyphosate das populações avaliadas.*

**Palavras-chave:** resistência a herbicidas, germinação, bioensaio, chiquimato, EUDA5.

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## INTRODUCTION

*Euphorbia davidii* Subils (Euphorbiaceae) is an invasive species, native to the northern hemisphere (Mexico, United States and Canada), which has recently been introduced in South America (Argentina), Australia and Europe (Geltman, 2012).

It is an spring-summer annual weed that causes significant yield reduction in soybean in central and southeastern sections of Buenos Aires Province, Argentina (Juan et al., 2011). In the last two decades it has been observed an increase in constancy, density and geographical distribution of *E. davidii* in this region.

Studies conducted in the late 1990s, on agricultural lots in the central area of Buenos Aires Province, reported weed densities rates between 20 and 200 plants $m^{-2}$ (Juan et al., 1996), where as some recent researches revealed an increase in population density, ranging from 300 to 900 plants $m^{-2}$ (Juan et al., 2011; Núñez Fré et al., 2014).

The presence of this weed is currently reported in Europe and Asia, and its distribution is observed in Russia, Ukraine, Moldova, Romania, Bulgaria, Hungary, Italy, Belgium, Switzerland, Serbia and France (Barina et al., 2013; Purger et al., 2015). Similarly to Argentina's records, an increase in population size and geographical distribution has been observed in some of these countries (Purger et al., 2015).

Globally, little is known about management strategies to control this species (Storrie and Cook, 1996), and, in Argentina, the most recent studies by Juan et al. (2011) have demonstrated the increasing difficulty of controlling *E. davidii* using glyphosate as the only herbicide in transgenic glyphosate-resistant (GM) soybeans. This could be due to an evolution of populations towards resistance.

Glyphosate (N-phosphonomethyl glycine) is currently the most widely used herbicide in the world and its intensity of use in agriculture has significantly increased since the introduction of herbicide resistant crops (Duke and Powles, 2008). Although this has initially facilitated weed management in these crops, it has led to the appearance of multiple cases of resistance, as a result of the selection pressure exerted by the repeated use of this herbicide. Due to the intensity of glyphosate use, it is expected that new resistant biotypes will be developed each year in species that are currently controlled by this product (Singh and Shaner, 1998).

Given this situation, it becomes more and more important to evaluate the sensitivity to glyphosate of problem weed populations, in order to achieve rapid detection and characterization of resistant biotypes, facilitating management and avoiding their dispersal.

Some herbicides such as glyphosate, when applied on leaves, may harm sensitive plants, but their effect is relatively slow; several days must elapse before the symptoms of injury appear (Duke, 1988). As a consequence, rapid methods have been developed to detect the effect of glyphosate (Madsen et al., 1995; Harring et al., 1998), including methodologies based on root absorption (Duke and Hoagland, 1978).

One of the methods for the evaluation of herbicide sensitivity consists in performing seed bioassays, in which the propagules are arranged on an inert substrate impregnated with the herbicide, and the response is determined by measuring some parameters such as survival or germination (Pérez-Jones et al., 2007).

Other methods assess sensitivity to herbicides directly on the site of action (target enzyme). These generally consist of *in vitro* extraction and evaluation of the activity of specific enzymes such as 5-enol piruvil shikimate-3-phosphate synthetase (EPSPs; EC 2.5.1.19) for glyphosate (Singh and Shaner, 1998).

At the biochemical level, the inhibition of the enzyme EPSPS is the primary mode of action for glyphosate (Rubin et al., 1982). This results in blocking the shikimate pathway, causing reduction in the synthesis of aromatic amino acid and proteins, as well as limited growth and premature cell death (Duke, 1988).

The blockage of the shikimate pathway resulting from the inhibition of this enzyme causes the accumulation of shikimic acid, substrate of the EPSPS (Amrhein et al., 1980; Lydon and

Duke, 1988). Therefore, the quantification of shikimic acid has been proposed as a biochemical marker to detect sensitive plants exposed to glyphosate (Anderson et al., 2001; Henry et al., 2007). Significant increases in internal concentrations of shikimate occur even after the application of low doses of glyphosate, which occasionally would not cause phytotoxicity or visible symptoms (Velini et al., 2008).

The measurement of the accumulation of shikimic acid in response to inhibition by glyphosate is a rapid way to quantify and predict the damage induced by this herbicide in sensitive plants (Harring et al., 1998; Pline et al., 2002; Henry et al., 2007), besides, it has been observed that its accumulation, in a certain range, depends on the amount of glyphosate applied (Lydon and Duke, 1988).

The objective of the present study was to compare the sensitivity to glyphosate of different populations of *Euphorbia davidii* from Buenos Aires Province (Argentina).

## MATERIALS AND METHODS

### Plant material used

The *E. davidii* seeds used for both evaluations were collected from isolated populations, obtained from different localities of Buenos Aires Province, Argentina: Espartillar (37°212’403" S - 62°262’103" O); Olavarría (36°57’55.8648" S - 60°11’55.7448" O); Azul (36°49’51.9276" S - 59°52’44.2878" O); and Barrow (38°182’003" S - 60°142’003" O).

### Seed germination bioassay for detecting sensitivity to glyphosate

Seed germination of different populations of *E. davidii* was evaluated in increasing concentrations of glyphosate. For this purpose, thirty seeds were placed in a Petri dish containing filter paper and a 5 mL aliquot of glyphosate solution (ammonium salt 40.5% SL) in distilled water at a ratio of 0; 2.24; 4.47; 6.72; 8.95; 17.91; 35.82 and 71.64 g e.a. $L^{-1}$ performing 3 repetitions. They were incubated in a growth chamber at 18 °C ± 1 °C, the optimal temperature zone for seed germination of the species (Marchessi et al., 2011), and the number of germinated seeds per treatment were counted after 21 days. By means of the log-logistic model proposed by Streibig et al. (1993), the average effective concentration (EC50) was calculated, i.e. the herbicide concentration required to inhibit 50% of germination in comparison with the control. Based on the EC50, resistance indices (RI) were calculated for the different sources, with the quotient between the EC50 of each locality and the EC50 value of the population with the highest sensitivity (Yanniccari et al., 2012), which the present research considered the population of Espartillar, with a short history of glyphosate application.

### Assay for measuring shikimate as a bioindicator of sensitivity to glyphosate

Ten seeds of each population were planted in 300 $cm^3$ pots containing typical Argiudol soil of loamy-clay and muddy texture, and the plants were kept under greenhouse conditions, with controlled irrigation. One week after the emergence, a manual thinning was performed, leaving four plants in each pot. When they reached the phenological state of initial branching, glyphosate 40.5% SL (ammonium salt) was applied at 0X; 0.5X and 1X corresponding to 0, 540 and 1,080 g e.a. $ha^{-1}$, where X is the recommended dose for the control of this species in the aforementioned phenological state (Juan et al., 2011). These treatments were performed under laboratory conditions using an equipment for pumping $CO_2$ at a constant pressure rate (3 bar), XR8001 nozzles with a flow rate equivalent to 130 L $ha^{-1}$, and three replicates per treatment and population. Within 72 hours after the application, 50 mg fresh weight samples were obtained from the last pair of fully expanded leaves to perform quantification of shikimic acid levels.

### Shikimate extraction

The extraction was performed using the technique described by Singh and Shaner (1998). Immediately after sampling, the plant material was submerged in HCl (0.25N) at a ratio 1:20

(tissue weight/HCl volume). Subsequently, each sample was ground with a mortar and pestle, and then the extract obtained was centrifuged. The supernatant was collected and directly used for the determination of shikimate as described below.

### Spectrophotometric determination of shikimate

The shikimate was determined according to a modification of the method proposed by Gaitonde and Gordon (1958), 0.8 mL of a periodic acid solution 1% was added to an aliquot of the supernatant obtained from each sample (from 10 to 50 µl, depending on the amount of shikimate present in the extract), and incubated at 40 °C for 45 minutes to oxidize shikimic acid. Subsequently, 0.8 mL of NaOH (1N)/$Na_2SO_3$ (0.05M) was added. Finally, the absorbance at 382 nm wavelength was measured using a spectrophotometer (Shimadzu UV-160A). The shikimate concentration of each sample was determined from a shikimate standard curve (99%, Sigma Aldrich).

### Statistical analysis

Using data obtained from the germination bioassays with seeds of each source, non-linear (log-logistic) regression models were adjusted from the dose-response equation (Seefeldt et al., 1995), which relates the germination percentage and the concentration of glyphosate. Statistical software GraphPad Prism v 6.01 was used.

The mathematical expression used for this adjustment is expressed as: $Y = C + ((D\text{-}C) / (1+\exp(b \,.\, \ln(X)\text{-}\ln(CE_{50}+1))))$; where $Y$ represents the germination percentage, $X$ is the concentration of glyphosate expressed in g e.a. $L^{-1}$; $C$ is lower limit; $D$ is the upper limit; $b$ represents the slope and $CE_{50}$ is the average effective concentration, i.e. the concentration rate at which 50% inhibition is obtained.

In addition, data obtained from germination bioassay and shikimate assessment were evaluated by analysis of variance using a completely randomized design. The test for homogeneity of variances applied was Levene's (a 95% confidence level), and then the comparison of means was performed using the Fisher LSD test, $p<0.05$, with the statistical software InfoStat v. 2012 (InfoStat Group, FCA, National University of Cordoba, Argentina). In the case of data obtained from the shikimate measurements, comparisons of means were also carried out, grouped by source.

## RESULTS AND DISCUSSION

### Seed germination bioassay for detecting sensitivity to glyphosate

Based on the results obtained from the seed bioassay, it was determined that none of the populations evaluated was sensitive to glyphosate at a concentration of 2.24 g e.a. $L^{-1}$. In fact, the germination percentage of the seeds exposed to this concentration did not differ from the control group in any of the four *E. davidii* populations assessed (Table 1).

The minimum concentration at which a statistically significant response was obtained depended on the populations studied, showing different levels of sensitivity.

The Espartillar population was found to be the most sensitive, displaying a statistically significant response in comparison with the control from the concentration of 4.47 g e.a. $L^{-1}$. In the case of seeds from Azul, an inhibitory effect from the concentration of 6.72 g e.a. $L^{-1}$ was observed, and the populations from Olavarría and Barrow only showed a decrease in germination percentages in concentrations equal to or greater than 8.95 g e.a. $L^{-1}$.

On the other hand, when evaluating the behavior of the different populations at higher concentrations of glyphosate it was found that in order to reach a percentage of germination inhibition above 90%, the minimum concentration necessary for the population of Espartillar was 8.95 g e.a. $L^{-1}$; 17.91 g e.a. $L^{-1}$ for those from Azul and Barrow and 35.82 g e.a. $L^{-1}$ in the case of that from Olavarría.

***Table 1*** **-** Average percentage germination of *Euphorbia davidii* seeds obtained after 21 days with different concentrations of glyphosate, for each population from Espartillar, Azul, Olavarría and Barrow

| Glyphosate (g e.a. $L^{-1}$) | Espartillar | Azul | Olavarría | Barrow |
|---|---|---|---|---|
| 0 | 97.5 a | 40.0 a | 96.7 a | 95.6 a |
| 2.24 | 97.5 a | 38.3 a | 94.4 a | 93.3 a |
| 4.47 | 43.8 b | 38.3 a | 94.4 a | 93.3 a |
| 6.72 | 33.8 b | 28.3 b | 90.0 a | 93.3 a |
| 8.95 | 5.0 c | 11.7 c | 58.9 b | 74.4 b |
| 17.91 | 6.3 c | 0.0 d | 16.7 c | 5.6 c |
| 35.82 | 0.0 c | 0.0 d | 0.0 d | 0.0 c |
| 71.64 | 0.0 c | 0.0 d | 0.0 d | 0.0 c |

The statistical comparisons displayed are only valid for each population. Different letters indicate statistically significant differences at $p<0.05$ according to Fisher's LSD test.

Figure 1 shows the non-linear regression model based on the germination percentage of each population and the concentration of glyphosate. According to the adjusted equations, the average effective concentration was calculated (Table 2). It is observed that the lowest value obtained in this parameter belongs to the population from Espartillar (4.00 g e.a. $L^{-1}$), confirming that it is the most sensitive population. With the seeds from Azul it was found a $CE_{50}$ 75% higher value in comparison with the previous population (7.03 g e.a. $L^{-1}$). $CE_{50}$ values of the two remaining populations (Barrow and Olavarría) were statistically higher than those reported for Azul and Espartillar, but no significant differences were detected between them (Barrow and Olavarría).

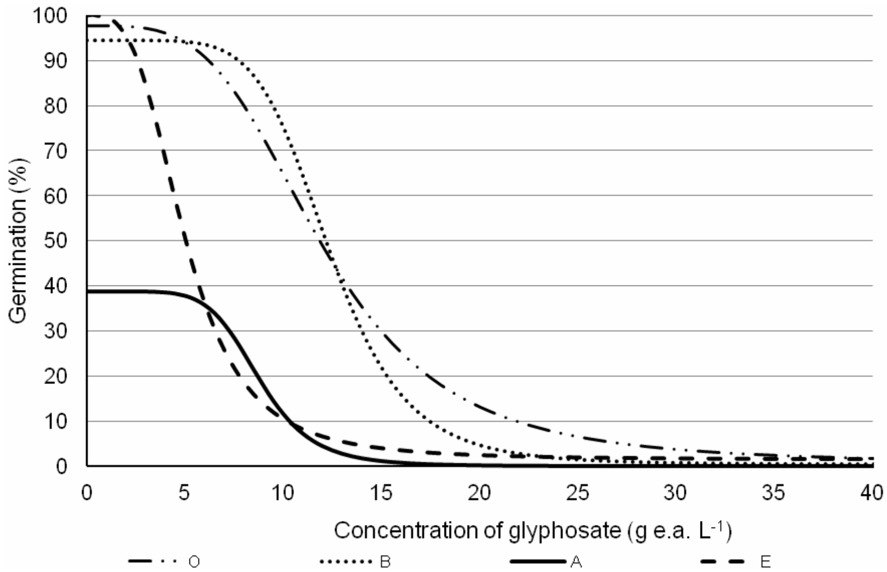


***Figure 1*** **-** Adjustment slopes obtained from the log-logistic model for the different concentrations of glyphosate evaluated and the germination percentage of *Euphorbia davidii* seeds from Espartillar (E), Azul (A), Olavarría (O) and Barrow (B), $r^2$ values obtained were 0.912; 0.940; 0.966 and 0.995 respectively.

Espartillar is a primary livestock-producing locality situated in the southwest section of Buenos Aires Province, where 58% of the soils have livestock-agricultural aptitude, which allow a rotation with a relatively short agricultural stage, after a long-term perennial pastures; 28% of the soils have agricultural-livestock aptitude and the remaining 14% have a purely livestock-rearing capacity (Capello and Fortunato, 2013).

Assuming that the population from Espartillar was the least exposed to selection pressure due to the agricultural history of the

***Table 2*** **-** Average effective concentration ($CE_{50}$) obtained from the adjustment equations of the log-logistic model for the germination percentage of *Euphorbia davidii* seeds from Espartillar, Azul, Olavarría and Barrow

| Population | $CE_{50}$ (g e.a. $L^{-1}$) | Confidence interval | |
|---|---|---|---|
| | | Lower limit | Upper limit |
| Espartillar | 4.00 c | 3.05 | 5.23 |
| Azul | 7.03 b | 6.37 | 7.77 |
| Olavarría | 9.57 a | 8.27 | 11.08 |
| Barrow | 9.93 a | 9.28 | 10.65 |

Lower and upper limits of the confidence interval p>0.95 are included.

area and the use of the herbicide glyphosate, it is logical that plants coming from that area represent a sensitive population. Also, it could be assumed that plants from this region exhibit the original susceptibility to glyphosate of this species.

Resistance indexes (IR) obtained by comparing $CE_{50}$ values f the locations of Azul, Olavarría and Barrow with respect to the $CE_{50}$ value of the susceptible population (Espartillar) were the following: 1.76; 2.39 and 2.48 respectively.

Compared with different cases of resistance to glyphosate observed in other species, the IR values obtained are relatively low (<5). IR values found may indicate that there is a genetic variability between the populations in terms of sensitivity to glyphosate. The increase in the frequency of applications and use of glyphosate in the region has been influencing and may be still subjecting *E. davidii* populations to a growing selection pressure. This would favor an increase in the frequency of less herbicide sensitive individuals less and would force the population to evolve to a higher IR.

On the other hand, although all populations are adjusted to the same model, one can analyze separately the parameters that determine each adjustment equation.

As shown in Table 3, although the adjustment equations displayed different slope values, it was not possible to statistically establish significant differences between them, due to the variability of the observed data; which is evidenced in the overlap between the confidence intervals (p>0.95). Considering that it is not possible to differentiate the adjustment slopes, it can be affirmed that no differences were observed in the sensitivity levels during seed germination regarding an increase in glyphosate concentration in the medium, in the linear phase of the adjusted model. Differences in sensitivity observed between populations ($CE_{50}$) would only imply a horizontal displacement of the dose-response curve, without changing the shape of the same. Therefore, an increase in one unit of each concentration of glyphosate evaluated would result in an equivalent change in the response of all populations.

***Table 3*** **-** Slopes (b) obtained from the adjustment equations of the log-logistic model for the germination percentage of *Euphorbia davidii* seeds from Espartillar, Azul, Olavarría and Barrow

| Population | Slope (b) | Confidence interval | |
|---|---|---|---|
| | | Lower limit | Upper limit |
| Espartillar | -3.31 | -5.56 | -1.06 |
| Azul | -6.52 | -10.34 | -2.70 |
| Olavarría | -3.71 | -5.28 | -2.14 |
| Barrow | -6.35 | -8.09 | -4.62 |

Lower and upper limits of the confidence interval p>0.95 are included.

## Assay for measuring shikimate as a bioindicator of sensitivity to glyphosate

The concentration of shikimate recorded in control plants of the studied populations (without application of glyphosate herbicide) ranged from 22000 to 37000 $\mu g\ g^{-1}$ of fresh weight for the different populations evaluated, and no differences between control groups of the different localities were observed from the statistical point of view. Variation coefficients for the shikimate measurements in the control treatments were the following: 77.2; 42.3; 96.4; and 34.1% for the populations of Espartillar, Barrow, Azul and Olavarría respectively, which shows a marked variability in the basal shikimate levels of the different populations (Table 4).

It is worth noting that in the population from Espartillar, the concentration of shikimate determined in plants treated with glyphosate was twice as high as that of the control group when the lowest dose of herbicide (0.5X) was applied, and three times higher when the recommended dose for the herbicide control of this species (1X) was used. This confirms that the aforementioned population shows a high sensitivity to the herbicide, consistent with the results of seed bioassays in which germination was inhibited by a low concentration of glyphosate.

Between populations from Azul, Olavarría and Barrow, no statistically significant differences were detected in the shikimate concentration recorded for glyphosate herbicide rates of 0X, 0.5X and 1X.

***Table 4*** **-** Concentration of shikimate in *Euphorbia davidii* leaf (mg of shikimate $g^{-1}$ of fresh weight) determined 72 after application of different doses of glyphosate (0, 540 and 1,080 g e.a. $ha^{-1}$). Average values, standard errors and variation coefficient (VC) are expressed

| Population | Dose ( g e.a. $ha^{-1}$) | [Shikimate] average | Standard deviation | VC (%) | Overall | Analysis per locality |
|---|---|---|---|---|---|---|
| Espartillar | 0 | 22433 | 17321 | 77.2 | AB | A |
| Espartillar | 540 | 46118 | 23163 | 50.2 | DE | AB |
| Espartillar | 1080 | 62952 | 37747 | 60.0 | E | B |
| Azul | 0 | 24341 | 23464 | 96.4 | ABCD | A |
| Azul | 540 | 12046 | 10460 | 86.8 | A | A |
| Azul | 1080 | 9808 | 5693 | 58.1 | A | A |
| Olavarría | 0 | 37463 | 12763 | 34.1 | BCD | A |
| Olavarría | 540 | 45645 | 14088 | 30.9 | CDE | A |
| Olavarría | 1080 | 36043 | 22734 | 63.1 | BCD | A |
| Barrow | 0 | 25257 | 10674 | 42.3 | ABCD | A |
| Barrow | 540 | 23637 | 10624 | 44.9 | ABC | A |
| Barrow | 1080 | 26288 | 22028 | 83.8 | ABCD | A |

Different letters indicate statistically significant differences between averages ($p<0.05$).

In these populations, the lack of accumulation of shikimate evidences the low sensitivity to the herbicide. Despite this, in some circumstances, it was possible to observe accumulation of shikimate in both susceptible and glyphosate resistant populations, however a differential pattern of shikimate accumulation has been observed over time (Mueller et al., 2003). It is possible that there is a pattern of differential accumulation of shikimate over the post-application time among the three *E. davidii* populations that did not respond to glyphosate application (Azul, Olavarría and Barrow); this finding can be associated with different sensitivity levels to the herbicide, which shall be evaluated in future studies, since results of the present research correspond to evaluations performed 72 hours after herbicide application.

The concentrations of shikimate observed in the control treatments of all *E. davidii* populations tested were much higher than those observed in other weed species. Yanniccari et al. (2012) an average of 500 µg of shikimate $g^{-1}$ of fresh weight in susceptible *Lolium perenne* plants and 1000 to 2000 µg $g^{-1}$ of fresh weight in glyphosate-resistant biotypes, which is at least ten times lower than that recorded in *E. davidii.*

In general, endogenous shikimate levels in plants are very low, ranging from 40 to 60 µg $g^{-1}$ of fresh weight (Yoshida et al., 1975), but it is known that the basal level of shikimate is variable according to the species studied. Mueller et al. (2008) found average basal levels of 100 µg of shikimate $g^{-1}$ of fresh weight in populations of nine species evaluated, and concentrations between 1000 and 6000 µg of shikimate $g^{-1}$ of fresh weight in glyphosate-treated plants.

Dogramaci et al. (2015) reported that the concentration of shikimate in *Euphorbia* populations without glyphosate application was 50000 µg of shikimate $g^{-1}$ of fresh weight, while they detected increases up to 120000 µg of shikimate $g^{-1}$ of fresh weight in glyphosate-treated plants.

Accordingly, it is presumed that high concentrations of shikimate could be associated with species of the genus *Euphorbia* and, thus, the low response in the accumulation of this metabolite after a treatment with glyphosate stands out. In some sensitive species belonging to other genera that were treated with the herbicide, the level of shikimate increases up to ten times in comparison with untreated plants, whereas in the sensitive population of the present study, it only increased three times.

On the other hand, Harring et al. (1998) found contrasts in the accumulation of shikimate for different glyphosate formulations, which should be taken into account when comparing results obtained from different evaluations.

Whatever the mechanism or mechanisms involved in the low sensitivity to glyphosate observed in the populations from Azul, Olavarría and Barrow, the impact of this phenomenon on field conditions is similar: a greater difficulty to control and, instinctively, an increase in the normal doses of glyphosate herbicide used by technicians and producers, which in turn leads to a higher selection pressure (Juan et al., 2011).

According to the results presented in this paper and considering that previous findings indicate contrasts in the levels of sensitivity to glyphosate between populations of different species of the same genus as *Euphorbia heterophylla* (Vidal et al., 2007), it is important to watch closely for the risk of evolution io a higher level of resistance in the region assessed.

In this study, the two assays evaluating the sensitivity to glyphosate of different populations showed consistent results indicating that plants obtained from seeds collected in Espartillar were more sensitive to glyphosate than those collected in other locations.

Although the IR calculated is not too high (<5), it indicates a level of differential sensitivity between populations that should be taken into account, since it is an invasive weed that was rarely found in the central area of Buenos Aires Province.

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