

MORPHO-ARCHITECTURAL TRAITS THAT ALLOW THE REGENERATION OF *Eustachys retusa* (POACEAE) IN SYSTEMS WITH INTENSIVE GLYPHOSATE APPLICATION¹

Características Morfoarquiteturais que Possibilitam a Regeneração de Eustachys retusa (Poaceae) em Sistemas com Uso Intensivo de Glifosato

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ABSTRACT - *Eustachys retusa* has low sensitivity to glyphosate at the reproductive stage. The purpose of this study was to analyze the effect of glyphosate on adult *E. retusa* plants in order to identify the traits that make possible their regeneration in no-tillage systems. For this purpose, reproductive-stage specimens from wild populations were treated with glyphosate at two different rates (480 and 1,200 g a.i. ha⁻¹) including a non-treated control. Results demonstrated that glyphosate could control *E. retusa* plants at reproductive stage at 1,200 g a.i. ha⁻¹ dose. However, a certain proportion of plants can survive despite having full chlorosis as a consequence of basal bud activation (bud bank) and the presence of reserves in the rhizome. These combinations of morphological traits facilitate the recovery of foliar mass in some plants after the application of the herbicide. This behavior represents a serious problem because *E. retusa* plants retain the ability to regrow. This shows that, *E. retusa* management at reproductive stage must be complemented with other cultural and/or chemical tactics.

Keywords: argentine fingergrass, herbicide, weed, tolerance, morphological changes.

RESUMO - *Eustachys retusa* é uma praga com baixa sensibilidade ao herbicida glifosato na etapa reprodutiva. O objetivo deste estudo foi analisar o efeito desse herbicida sobre as plantas adultas de *E. retusa*, para identificar as características que possibilitam a sua regeneração nos sistemas na semeadura direta. Foram utilizados indivíduos de populações silvestres em etapa reprodutiva e três tratamentos (0, 480 e 1.200 g i.a. ha⁻¹). Os resultados mostraram que o glifosato pode reduzir a população de *E. retusa* na etapa reprodutiva com uma dose de 1.200 g i.a. ha⁻¹. Entretanto, um certo percentual das plantas pode rebrotar após aplicação, como consequência da ativação de gemas basais (banco de gemas) e da presença de reservas no seu rizoma. Essas características morfológicas facilitam a recuperação da massa foliar após a aplicação em plantas com clorose total. Essa resposta representa um sério problema, devido ao fato de que parte da população de plantas adultas de *E. retusa* tratadas pode conservar a capacidade de rebrotar apesar dos efeitos severos do herbicida sobre o broto. Isso demonstra que, no estado reprodutivo, o manejo da praga citada com glifosato deve ser complementado com outras estratégias culturais e/ou químicas.

Palavras-chave: capim-coqueirinho, herbicida, praga, tolerância, mudanças morfológicas.

INTRODUCTION

In South America, the incorporation of genetically modified organisms changed the agricultural model into one dependent on the massive use of herbicides (López et al., 2012).

This has been widely adopted by producers because of the simplicity of using one single herbicide, as well as its effectiveness and application flexibility and the fact that it does not damage crops or soils (Kudsk and Streibig, 2003). However, unlike other control practices,

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herbicides have a high potential for selecting genotypes that persist after treatment by controlling the most vulnerable ones (Baucom and Mauricio, 2004). Thus, they produce qualitative and quantitative changes in the weed community (Christoffoleti et al., 2008), promoting the persistence of weed species such as *Eustachys retusa* (Poaceae).

Eustachys retusa is a species native to South America (Molina and Rùgolo, 2012). It is a perennial, caespitose, C4 species with short rhizomes and compressed tillers (Burkart, 1969). The leaves have flat blades and laterally-folded (Watson and Dallwitz, 1992), fan-shaped sheaths (Burkart, 1969). It has a spring-summer period (Burkart, 1969). It spreads mainly by seeds, and it shows spikelets of hermaphrodite flowers arranged in digitate spikes (Watson and Dallwitz, 1992). It is considered a troublesome weed because of its low sensitivity to glyphosate at the reproductive stage (Puricelli and Faccini, 2005), mainly in the north of the Argentine Pampean region (Dellaferrera et al., 2007). It has been shown that the control of *E. retusa* can only be achieved at the virginal stage (Puricelli and Faccini, 2005).

Quantifying traits in weeds is important to understand how species explore and exploit the environment (Kropff and van Laar, 1992), and it helps to gain better insight into species behavior. By integrating quantitative and qualitative characters, it is possible to understand how a combination of traits determines competitive advantages and disadvantages of the species studied (Klimešová et al., 2008). A thorough knowledge of weed biology and behavior when exposed to herbicide application is essential to understand the reasons for its tolerance and to implement a program of sustainable management (Panigo et al., 2012). Our hypothesis is that *E. retusa* has morphological and architectural characteristics that play a fundamental role in the glyphosate tolerance. In view of these, and due to the fact that studies on *E. retusa* as a weed are rare, we aim to (i) assess quantitative and qualitative traits after glyphosate application, and (ii) identify the morpho-architectural traits of *E. retusa* that contribute to its tolerance in systems with intensive glyphosate application.

MATERIALS AND METHODS

Plant material and growth conditions

The seeds for this research were collected in Esperanza (-31°27'10.4184" S - 60°54'33.48" W), in the province of Santa Fe, Argentina, in areas with no history of herbicide application. Since this species did not tolerate the radiation conditions of the growth chamber, the trials were performed in a greenhouse under natural radiation conditions, and temperature was recorded. The orientation of the greenhouse was South East-North West. The average maximum and minimum temperatures inside the greenhouse in spring, summer and autumn during the trial were 29-15, 32-19 and 24-12 °C, respectively.

The germination was performed on moistened filter paper at 25 °C. The seeds were superficially disinfected with sodium hypochlorite (Panigo et al., 2012). Between 10 and 20 days after planting, uniform seedlings were transplanted in 5 L plastic pots. The plants were irrigated daily during the seedling stage with half-strength Hoagland's solution (Hoagland and Arnon, 1938). Then, plants were irrigated with demineralized water in order to keep them under suitable water conditions.

Herbicide treatments

Glyphosate (Estrella® as isopropylamine salt, 48% w/v) was applied evenly to the plant surface using a spray volume of 200 L ha⁻¹, delivered at a constant pressure of 275 kPa with a flat-fan nozzle. The application was made with a backpack. The herbicide treatment was applied at plants in reproductive stage, when at least one of their axes had flowered at 135 DATr (Days After Transplanting). The herbicide treatments included two doses of glyphosate: 480 g a.i. ha⁻¹ and 1,200 g a.i. ha⁻¹ and an untreated control. The selection of doses was based both on the recommendations of Guide of Phytosanitary Products for Argentina (CASAFE, 2007) and the study about their susceptibility by Puricelli and Faccini (2005). In pretest, a high degree of sensitivity of the species at the virginal stage to both doses was confirmed.



Response to herbicide application

Forty five plants per dose were glyphosate-treated. Thirty out of the 45 plants were used for aerial biomass and photosynthetic pigment determination and the remaining 15 were used to measure qualitative and quantitative traits.

(a) Qualitative traits

An architectural analysis was used to examine the morpho-architectural variations of the shoot system in untreated and treated plants. Different morphological traits such as shoot position, type, and amount of axillary shoots produced were recorded every 30 days. Schemes of the complete branching system structure of all the plants were also made.

Visual shoot damage was assessed daily for 60 days from the time of application. The treated plants were visually examined for the appearance of symptoms and compared with control plants.

(b) Quantitative traits

The following traits were assessed at 75, 105, 165 and 195 DATr (since the beginning of the virginal stage to 60 days after treatment):

- (1) Living Leaves Proportion (LLP) was calculated as:

$$\text{LLP} = (\text{No. Living Leaves} / \text{Total No. Leaves}) * 100$$

- (2) Leaf Area (LA): was estimated as the product of the length (mm) x width (mm) in each leaf, according to the methodology described by Lauri and Terouanne (1991). The linear regression equation used to calculate LA in *E. retusa* was as follows:

$$y = 0.7718x + 0.0639; \quad (R^2 = 0.9911)$$

where y is the LA and x is the product of leaf length and leaf width.

- (3) Number of total branches (NTB) and proportion of branches per order (PBO): the quantity of the branches per order was recorded, the NTB per plant was calculated as the number of all order branches accumulated in each sampling date. The position of each branch indicates its order

number. The principal axis was regarded as the first-order branch that develops second-order branches, which in turn can carry third-order branches and so on, in a hierarchical manner.

Then, chlorophyll (Chl) in fully expanded penultimate leaves was extracted from each treatment according to the methodology proposed by Arnon (1949). Five plants were randomly selected at 24, 48 and 168 hours after application (HAA).

Aerial biomass was determined and evaluated at 75, 165 and 195 DATr in ten plants per treatment. The plant shoots were harvested and then, they were cleaned with deionized water. The biomass of each plant was recorded until constant weight was achieved.

Statistical analysis

The effect of glyphosate concentrations and time after transplant on LLP, LA and NTB were analyzed by fitting generalized linear mixed models (GLMM). As the measurements were not destructive and they were repeated in time, a random term accounting for within subject variability was included. The LLP was modeled as a binomial response using the logit link function. The NTB was modeled as a Poisson response using the log link function. Finally, the LA was regarded as Gaussian response. The main effects and interactions of the fixed factors were assessed through analysis of variance and specific contrasts were tested using the phia package. These contrasts were made per date and considered significant if $p < 0.05$. The PBO was compared using chi-squared tests for contingency tables ($p < 0,05$) since the variable observed is qualitative.

Glyphosate effects on Chl at each harvesting point and aerial biomass were compared by two-way analysis of variance (ANOVA) followed by LSD test at $p = 0.05$. Data variance was visually inspected by plotting residuals to confirm homogeneity of variance before the statistical analysis. Both variables were analyzed as a completely randomized design. All the statistical analyses were carried out using the R software (R Core Team, 2014).



The experiment was conducted twice, and the data were combined for analyses, as there was no time by treatment interaction. The replication of each treatment was placed randomly inside the greenhouse and rearranged daily.

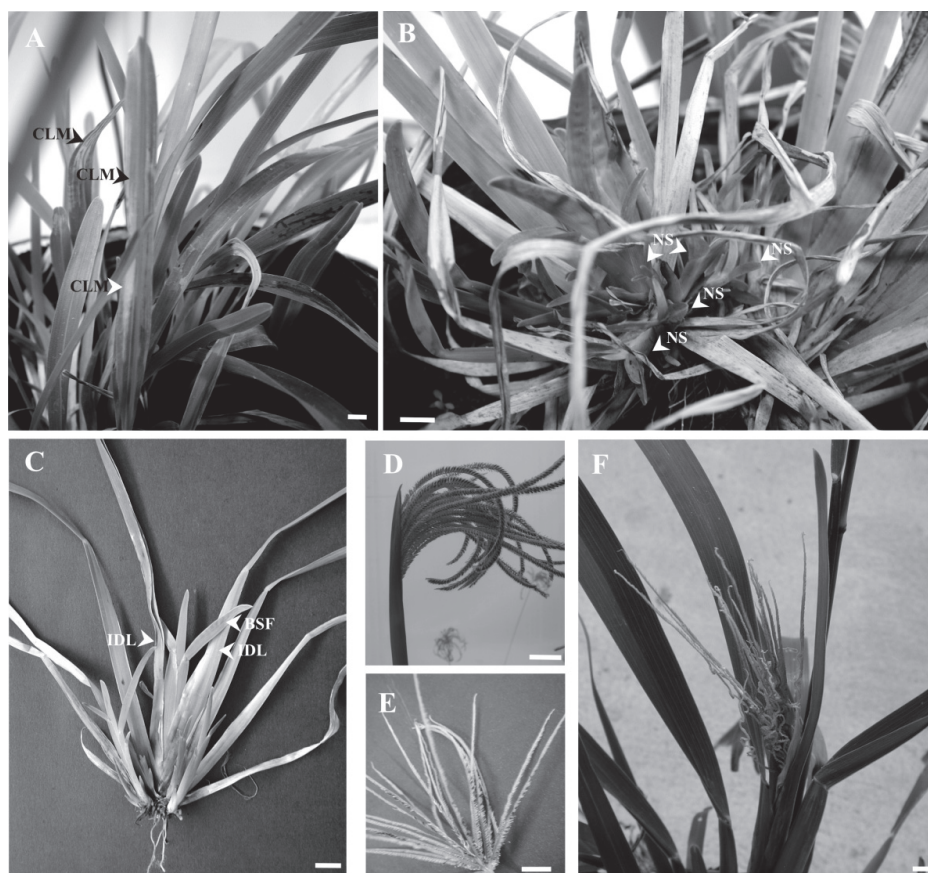
RESULTS AND DISCUSSION

Qualitative traits

Shoot visual damage

Shoot visual damage was evident after application though less apparent in plants treated at the lower concentration of herbicide. As from 5 days after treatment (140 DATr), the shoots treated with 1,200 g a.i. ha⁻¹ showed chlorotic mottles in intercostal areas (striated appearance), in

localized areas or throughout the blade surface (Figure 1A). These chlorotic mottles evolved to total chlorosis of the aerial portion of the shoot system at 150 DATr. However, at 165 DATr a 40% of these specimens were found to have survived and produced new shoots from basal buds located over their rhizome (Figure 1B). The damage caused by the low glyphosate dose was less severe and occurred later (150 DATr). The most prominent characteristic observed at this dose was the presence in shoots of 4-5 consecutive dead leaves in regions of living leaves between 155 and 175 DATr. This group of dead leaves was located just below the newly-formed leaves, which were also affected and had different shape, were less developed and had half-blades folded over themselves (Figure 1C). However, in 195 DATr treated plants the last developed leaves had normal blade and sheath length.



Scale bar = 1 cm.

Figure 1 - Effects of the herbicide on shoot and inflorescences of *Eustachys retusa*: (A) chlorotic mottles (CLM); (B) new shoots (NS), (C) interval dead leaves (IDL) and blades folded over themselves (BSF); (D) inflorescence in the flag leaf, (E) spikelet development failure, (F) sterile inflorescence axes: total spikelet development failure.

The branches of different order observed on all the treated surviving plants resulted in four traumatic branching types. Two types of branches showed the death of the apical meristem and they differed by a total or partial cessation of growth. The third type had smaller-size blades since the axes were produced immediately after application from regrowth shoots or they had several consecutive dry leaves (Figure 1B, C). The fourth type was characterized by a lack of elongation of the apical portion of the shoot, which resulted in the inflorescence being confined within the flag leaf (Figure 1D).

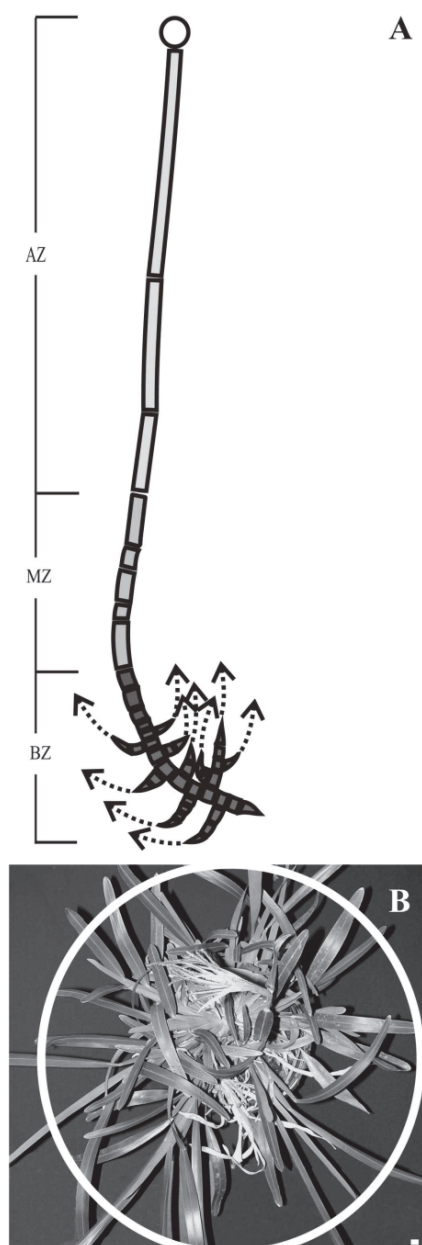
The inflorescences that emerged after application also suffered several alterations. In the plants subjected to lower doses, the observations were as follows: (1) lack of inflorescence development due to the death of the apical meristem, (2) lack of spikelet development at certain segments of the inflorescence (Figure 1E), and (3) total spikelet development failure due to sterile inflorescence axes (Figure 1F). All of these modifications had an indirect impact on the normal production of seeds, but did not compromise inflorescence number, which was similar in all treatment plants (data not shown).

All observed effects on the structure, leaf senescence of a defined segment along the axis, development of new leaves with shorter blades, failure in inflorescence spikelet formation, apical meristem death in certain axes, and lack of internode elongation were due to the fact that these zones were the main target of resources at the time of application. Glyphosate is transported mainly by phloem and it works both on the application site and on target regions of photoassimilates (Shaner, 2009). The presence of leaves and inflorescences deformed following the glyphosate application has also been observed in other species (Panigo et al., 2012).

Morpho-architectural variations of the shoot system

The structure of *E. retusa* showed morphologically equivalent and homogeneous plumular and axillary axes. Based on structural patterns, three distinct zones were

recognized in these axes (Figure 2A). These zones were delimited according to internode length and axillary meristem activity. The basal zone is rhizomatous, composed of 13-15 short internodes (Figure 2A) and most of its axillary buds developed. The middle zone has longer internodes, with axillary productions only in its proximal portion (close to the basal



Scale bar = 1 cm.

Figure 2 - Morpho-architectural characteristics of *Eustachys retusa*. (A) Structural area: basal zone (BZ), middle zone (MZ), apical zone (AZ); (B) crown-like tuft.



zone). Alternating internode length was a frequent trait found in this zone. The apical zone included the last three internodes, which were the longest along the axis and did not develop buds in their leaf axils. As a result, axillary production was completely inhibited. *Eustachys retusa* plants form a crown-like, circular, caespitose tuft (Figure 2B) with all axillary axes produced by iterative innovation from the plant base, showing an overall growth strategy matching Tomlinson's architectural model (Hallé et al., 1978).

The regrowth shoots generated after application showed the same components with the same architectural traits (model and zones) as described above. However, the emergence of new shoots after application increased the degree of branching (Figure 2C). This increase in branch order and number due to the development of axillary buds, originally inhibited in no-treated plants, has been reported in other perennial species that have survived the application of glyphosate (Panigo et al., 2012; Dellaferrera et al., 2015). Treated plants redirected resources to the production of new shoots, which allows them to recover photosynthetically active foliar mass loss. Viable bud and reserve availability is critical to recover biomass after a disturbance (Busso et al., 1990). Regrowth shoots from inhibited buds located at the base of *E. retusa* tillers made it possible to recover the active photosynthetic area, thus modifying the normal development of the branching system after herbicide application. While the branching system is genetically determined, it is characterized by its plasticity (Shimizu-Sato and Mori, 2001). Plasticity allows plants to adapt to specific environmental requirements, minimizing stress (Bradshaw, 2006).

Quantitative traits

Leaves

As shown in Figure 3A, after application, LLP decreased in the treated plants and this was found to be positively correlated with the dosage of the herbicide. At 165 DATr, LLP was significantly reduced only in the plants treated with 1,200 g a.i. ha⁻¹. At the end of the trial, this difference was more pronounced and

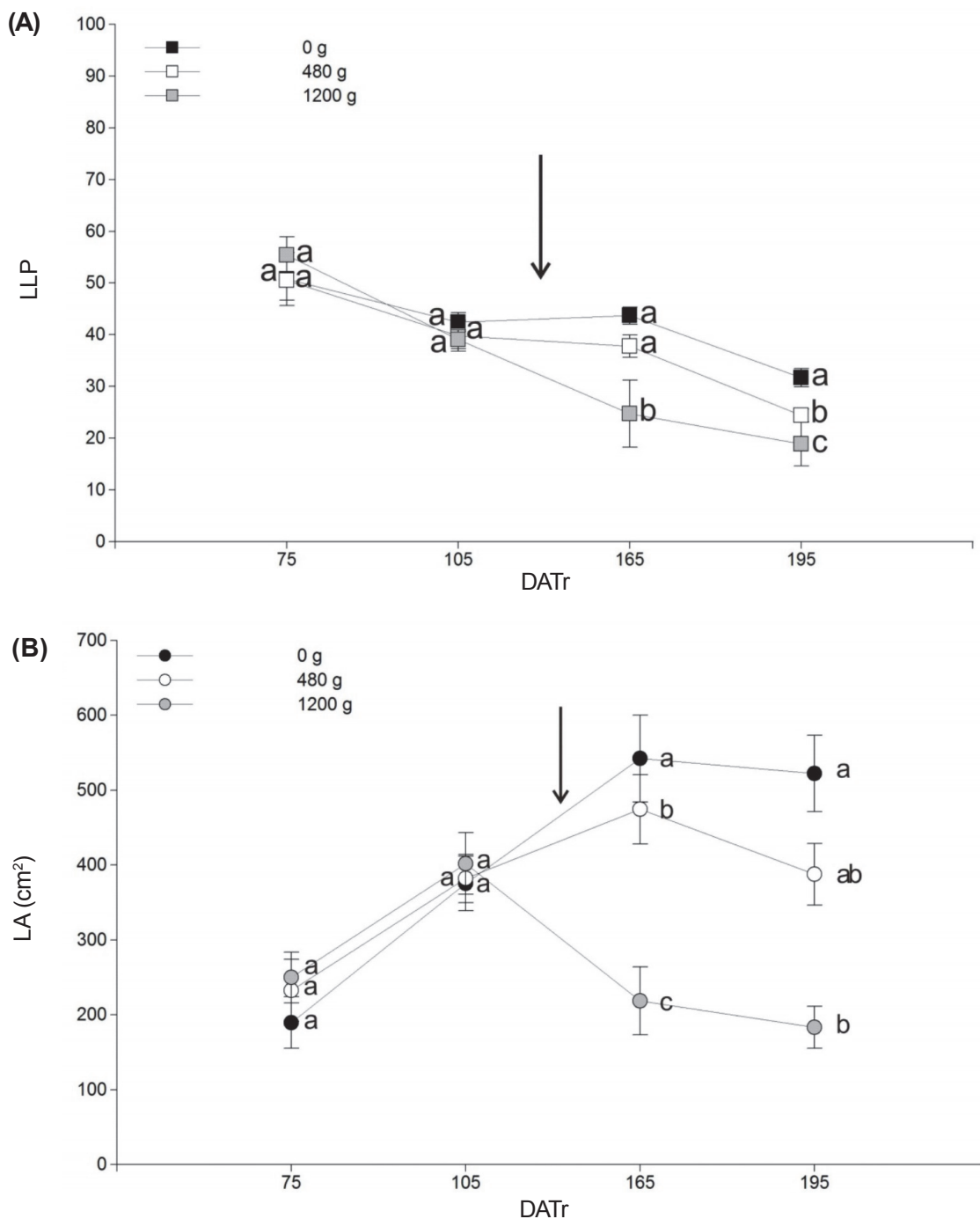
there were statistical differences ($p < 0,01$) in the LLP of all the treatments. Treated plants showed a lower LLP with respect to the control and in the ones treated with the higher dose LLP was significantly reduced.

The effects of glyphosate on LA were markedly significant in the plants treated with 1,200 g a.i. ha⁻¹ (Figure 3B). These plants showed a significantly smaller LA than the rest of treatment, post application of herbicide. In the plants treated with 480 g a.i. ha⁻¹, the LA was also smaller than in the control plants. At 195 DATr the LA decrease was dose-dependent as for the LLP (Figure 3A). Towards the end of the sampling, a reduction in LLP and LA was observed in all the treatment plants, including the control plants. Climatic conditions (autumn) and the phenological stages of plants (after fruiting) may have been the determining factors of such behavior.

In spite of the fact that LLP and LA decreased significantly with respect to the untreated plants, both treatment plants developed new branches. Investing in this structures indirectly favors carbon gain at whole plant level and the expansion of the branch system (and the crown) (Suzuki, 2000), both important features to ensure persistence.

Branches

The average NTB accumulated per plant, as well as the accumulative proportion of second and higher order branches, is shown in Figure 4. The NTB (Figure 4A) only showed significant differences between sampling dates. After herbicide application, the plants treated with 480 g a.i. ha⁻¹ exhibited an increase in the NTB, as compared with the rest of the treatments. The control plants and the plants treated with 1,200 g a.i. ha⁻¹ had similar values at both dates. When exposed to abiotic stress conditions, many plant species reorient their growth and exhibit a broad range of morphogenic responses, which means a mixture of growth inhibition and activation (Potters et al., 2007). That is, at both doses, plants developed new branches after application, but they were smaller and less vigorous, as reflected in their reduced LA (which combines smaller leaves with lower LLP).



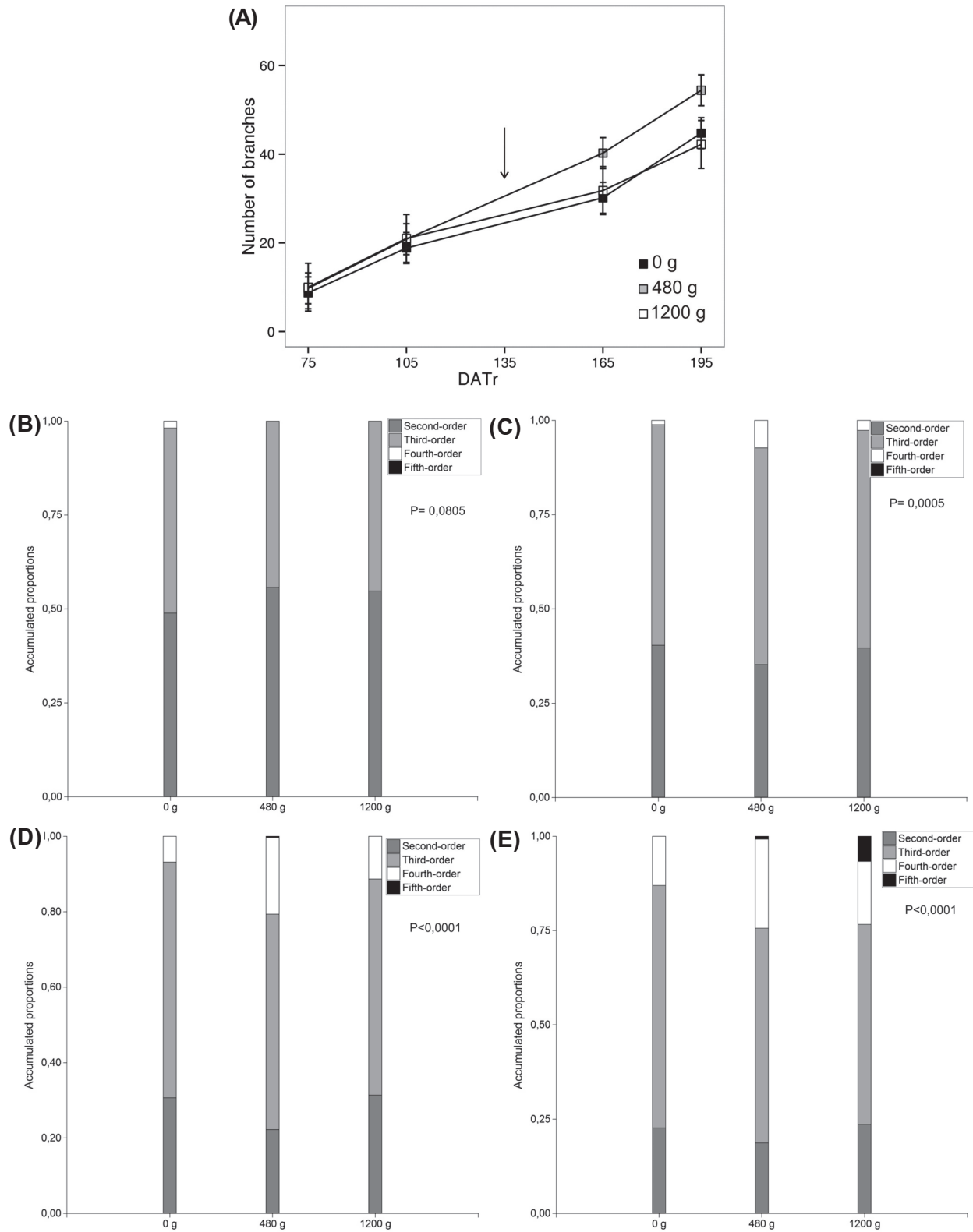
Key: DATr: days after transplanting, g: g a.i. ha⁻¹. Arrows indicates time of application.

Figure 3 - Herbicide effect on quantitative leaf traits in *Eustachys retusa*: (A) Living Leaves Percentage (LLP); (B) leaf area (LA).

The PBO (Figures 4B, C, D and F) was significantly different between treatments at 105, 175, 195 DATr. At 105 DATr the control plants were the first to develop third-order branches, but they did not surpass this order,

as it could be seen in the plants subjected to 480 and 1,200 g a.i. ha⁻¹ of glyphosate at the end of the trial. On the other hand, the plants exposed to the herbicide had a different, dose-dependent response. Those plants exposed to





Key: g: g a.i. ha⁻¹; DAT: Days after transplanting. P values ≤0.05 denote significant differences. Arrows indicates time of application.

Figure 4 - Branches in *Eustachys retusa* under different glyphosate treatments. (A) The accumulative numbers of total branches (NTB); (B-E) accumulative proportion of branches by order (PBO): (B) 75 DAT, (C) 105 DAT, (C) 165 DAT, (E) 195 DAT.



the lower dose did not only show a higher number of branches, but were also the first ones to develop fourth-order branches. The plants treated with the higher dose developed a higher proportion of fourth-order branches than the others as a consequence of the severe injuries observed in shoots of lower orders. Therefore, the glyphosate doses applied were positively correlated with an increase in the complexity of the branching system as in others glyphosate tolerant species (Panigo et al., 2012; Dellaferrera et al., 2015).

Chlorophyll

In *E. retusa* plants, total Chl concentration was dose-dependent since it diminished as the dosage increased (Figure 5A). At 24 HAA, the plants treated with the higher dose reached the lowest value. At this time, control plants had approximately 40-50% more total Chl than the treated ones. Then, all the treatments increased their Chl content over time, and no major statistical differences were found between treatments at 48 and 168 HAA.

Chlorophyll a/b ratio is often used to characterize the developmental stage of the photosynthetic apparatus, and while this is stable in fully green leaves of higher plants (approximately 3); it is highly dependent on the plants' physiological state (Kouoíl et al., 1999). In this research, this ratio was stable except for the plants treated with 1,200 g a.i. ha⁻¹ after 168 HAA because the ratio was close to one (Figure 5B) due to an increase in the concentration of Chl b (data not shown). An increase in Chl b biosynthesis entails an increase in light-harvesting efficiency of a photosystem (Tanaka et al., 2001). Therefore,

the relative proportions of absorbed light energy and also the energy flow of photons through the leaf are altered (Tremblay, 2012). That is, the photosynthetic process becomes unproductive after 168 HAA, because it increases the absorption of light energy or concentration of Chl b, which cannot be transformed into chemical due to the reduced concentration of Chl a. As a result of this, plants treated with 1,200 g a.i. ha⁻¹, showed chlorosis of the aerial portion of the shoot system at 150 DATr.

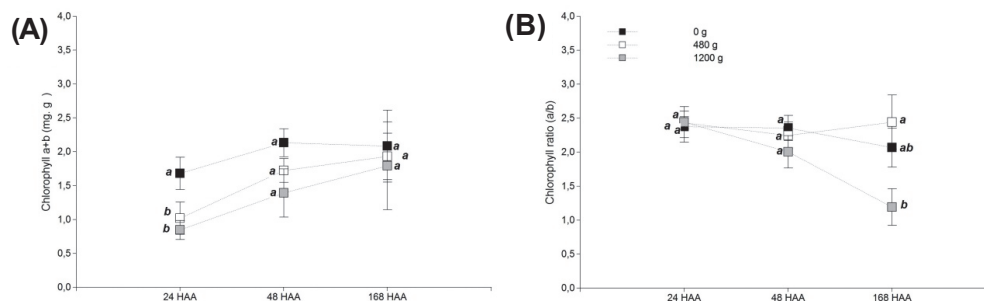
Biomass

Glyphosate phytotoxicity is also reflected in growth variations (Mateos Naranjo and Perez-Martín, 2013). In *E. retusa*, the glyphosate treatment reduced the biomass of the treated plants, but the herbicide only had a significant effect on the plants treated at the higher dose (Table 1). The latter accumulated between 55 and 68% less biomass than the control plants at 165 and 195 DATr, respectively. Meanwhile, the plants treated with 480 g a.i. ha⁻¹ reduced their biomass only between 10 and 20% with respect to the control plants on the same dates.

Table 1 - Effect of glyphosate on aerial biomass (g) of *Eustachys retusa* at different days after transplanting (DATr)

	Means ± SE (g)		
	75 DATr	165 DATr	195 DATr
Control	4.62 ± 2.30 A	22.05 ± 1.88 B	48.47 ± 3.61 B
480 g a.i. ha ⁻¹	3.87 ± 2.30 A	19.28 ± 1.88 B	37.34 ± 3.61 B
1,200 g a.i. ha ⁻¹	4.58 ± 2.30 A	10.36 ± 1.88 A	15.37 ± 3.61 A

Means within a column followed by the same letter are not significantly different at the 0.05 level of significance according to Fisher's LSD.



Key: HAA: hours after application; g: g a.i. ha⁻¹. Means with a common letter were not significantly different ($p \leq 0.05$).

Figure 5 - Effects of treatments with glyphosate on: (A) total chlorophyll contents, and (B) chlorophyll a/b ratio.



The herbicide affected *E. retusa* plants, causing various symptoms and effects on growth that involved organ and meristem death. Such effects were highly dependent on dosage. At 1,200 g a.i. ha⁻¹ doses, *E. retusa* plants were only partially controlled. At lower doses, no control was exerted.

The application of architectural analysis in weed research is still relatively rare. However, the results of the present study indicate that a “morfo-arquitectural approach” has proved to be a relevant tool to recognize *E. retusa* adaptations and strategies in the agro-ecosystem with intensive glyphosate application. That's is, after reproductive stage, this weed survives in fields exposed to intensive glyphosate applications without affecting its potential capacity for regrowth, thanks to presence of a rhizome with viable bud bank and reserves. Thus, this capacity for active colonization provides it with certain adaptive advantages in ecosystems where disturbance is frequent. Consequently, *E. retusa* management at reproductive stage must be complemented with other cultural and/or chemical tactics.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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