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Assessing the glyphosate tolerance of *Lotus corniculatus* and *L. tenuis* to perform rhizoremediation strategies in the Humid Pampa (Argentina)

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ABSTRACT

The broad-spectrum herbicide glyphosate (N – phosphonomethylglicine) is the most common pesticide used in the Humid Pampa, the main agricultural region in Argentina. According to agronomical practices and topogeographical characteristics of the region, rhizoremediation arises as a promising technology to mitigate glyphosate impact on health and agroecosystems. *Lotus corniculatus* L. (birdsfoot trefoil) and *Lotus tenuis* Waldst. et Kit. (=*Lotus glaber* Mill., narrowleaf trefoil) were selected to carry out tolerance studies as the starting point of a rhizoremediation process. *L. corniculatus* presented the highest root and foliar tolerance to glyphosate, corresponding to 5.0 mg kg^{-1} and 700 g ha^{-1} respectively. The enzyme enolpyruvylshikimate-3-phosphate synthase (*EPSP synthase*) partial cDNA sequence and whole plant shikimate accumulation assay were performed on *L. corniculatus* in order investigate tolerance mechanisms. No amino acid substitution related to glyphosate tolerance was found on *EPSP synthase* cDNA sequence. The shikimate accumulation study indicates that limited uptake and/or translocation of the herbicide is the most probable tolerance mechanism. Results obtained in this study, plus the productive and adaptive advantages of *L. corniculatus* make it a valuable candidate to develop rhizoremediation strategies.

1. Introduction

The Humid Pampa is the main agricultural region in Argentina and one of the most important land fields in South America, covering approximately 52 million ha mainly dedicated to cropping (Viglizzo et al., 2001). As the result of intensive agriculture practices, more than 300,000 t of pesticides are applied every year, which about 65% are formulations of the broad-spectrum herbicide glyphosate (N – phosphonomethylglicine) (CASAFE, 2013).

The intensive and widespread use of glyphosate on knoll implanted cultivars, combined with topogeographical characteristics of the Humid Pampa region, leads this compound to indirectly reach non-tilled soils and surface and groundwater sources located in the floodplains (Aparicio et al., 2013; Peruzzo et al., 2008).

In the last two decades, several studies were carried out in order to assess the ecological impact and establish the environmental and health risk associated to glyphosate use (Antoniou et al., 2011; Paganelli et al., 2010; Tsui and Chu, 2003; Vera et al., 2012).

The dilemmatic situation between the sustained increasing use of this agrochemical in the agro-industrial sector and the concern about its impact requires an answer that takes both scenarios into account. Ideally, technological alternatives should be found to minimize the agrochemical pollution problems and make agronomic exploitation an environmentally sustainable activity.

Considering the wide territory extension of the Humid Pampa region and the need of preserving the soil texture and quality of the croplands, an in situ, low implementation cost remediation approach comes up as the best option. In this context, rhizoremediation, which is the use of plants and its associate microflora to remove pollutants from the environment, arise as a promising clean-up technology (Glick, 2003; Kuiper et al., 2004; Shukla et al., 2013). Considering glyphosate physicochemical characteristics, its adsorption to clay in phosphorus deficient soils, the lack

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of significant metabolism in green plants and the agronomical implementation context, rhizoremediation stands out from other phytotechnologies (Gerhardt et al., 2009). Among other interesting benefits of this technology, there are the reduction of wind and water erosion, the wide public acceptance and the eventual agronomical profit from the incorporated vegetal species (Conesa et al., 2012; Peuke and Rennenberg, 2005).

When designing a rhizoremediation strategy to mitigate the impact of agrochemical in the agronomic context, the first step is the selection of the vegetal species (Merini et al., 2011). Here, the selected plant species should adequately tolerate glyphosate and, at the same time, represent an agronomic and economic advantage to producers. In this case, some specially adapted legume species arise as candidates since they can be settled in flooded and high salinity areas where standard crops cannot, improving a "cropping and cattle" productive diversification system.

In this way, *Lotus corniculatus* L. (birdsfoot trefoil) and *Lotus tenuis* Waldst. et Kit. (=*Lotus glaber* Mill., narrowleaf trefoil) present several productive and adaptive advantages over other pastures. These two foreign species are used in Argentina from the beginnings of twentieth century and they showed an excellent adaptation to the Humid Pampa region condition (Vignolio and Fernandez, 2006; Miñón et al., 1990). As legumes, they present higher digestibility, crude protein content and homogeneity in yield than grasses (Escaray et al., 2012). They also establish nitrogen-fixing symbioses increasing the nitrogen levels in bottoms and have a low phosphate requirement, both desirable characteristics as a result of low nutritional levels of nitrogen and phosphate in the region. They present also long and branched roots as well as high aerenchyma formation, all relevant features for the rhizoremediation systems (Blumenthal and McGraw, 1999). Finally, there is some evidence of tolerance assays performed by Boerboom et al. in the early '90 with *L. corniculatus* cultivars where it exhibits differential tolerance to glyphosate (Boerboom et al., 1990).

The aim of this study was to assess the glyphosate tolerance of two *Lotus* species specially adapted to the unique characteristics of the Humid Pampa region as the starting point of a glyphosate rhizoremediation strategy. Furthermore, the mechanisms of tolerance of *L. corniculatus* were explored and some accurate indicators for monitoring future field assays assessed.

2. Materials and methods

2.1. Plant material

Commercial seeds of *L. corniculatus* (var. Gladiador) and field-collected seeds of *L. tenuis* were tested. In this regard, commercial seed ensure the availability of genetically stable vegetal material, in order to overcome the possible heterogeneity generated by natural outcrossing at field scale. In the same way, field collected *L. tenuis* seeds, provide the substrate for bioprospecting natural selection tolerance events.

Seeds of *M. sativa* (cultivar Express) were used as glyphosate sensitive control.

Seeds were surface sterilized with ethanol 70% (v/v) for 1 min and rinsed with sterile distilled water four times; then sodium hypochlorite 5% (v/v) was added, gently shaked for 30 min and rinsed six times. Seeds of *L. corniculatus* and *L. tenuis* were scarified before surface sterilization by using 200 μm grit size sandpaper.

2.2. Tolerance assays

2.2.1. Semisolid agar media assay

One of the first steps in phytoremediation assays is to assess the tolerance of the candidate vegetal species to the contaminant. If the remediation strategy implies the use of a rhizoremediation technology, performing semisolid agar media assays in flasks results particularly useful. They provide a simple, fast and inexpensive method where germination rate and growing parameters can be evaluated considering the root as the sole organ in contact with pollutant, what is expected to occur in the field. Moreover, the agar medium ensures a maximum bioavailability of nutrients and contaminants, as in Petri plates assays (Merini et al., 2011). In this experiment, *L. corniculatus*, *L. tenuis* and *M. sativa* were tested.

For each plant species, a set of 360 ml glass flasks containing 50 ml of 0.8% (w/v) agar Murashige Skoog (MS) medium (Murashige and Skoog, 1962) with increasing glyphosate concentrations was prepared by adding Roundup Ultramax® (Monsanto – 74.7% of the ammonium salt). Accordingly, the final concentrations were 0.5, 1.0, 5.0, 20.0 and 50.0 mg kg^{-1}, which correspond to a 1 kg ha^{-1} dose (747 g of ammonium salt) spread in 10.0, 5.0, 1.0, 0.25 and 0.01 cm of soil depth respectively (assuming a soil apparent density of 1.5 g ml^{-1}).

In this way, a literature review of studies carried out in the Humid Pampa and other regions where glyphosate was quantified, indicates that levels from 0.5 to 5.0 mg kg^{-1} could be found in soils with different agronomic properties (Aparicio et al., 2013; Peruzzo et al., 2008; Veiga et al., 2001). On the other hand, levels of 20.0 and 50.0 mg kg^{-1} were tested to evaluate the potential of these plant species for further application in heavily polluted areas.

Flasks containing MS medium were screw capped and sterilized by autoclaving. Glyphosate solutions were filtering sterilized (0.20 μm) and then added to flasks under sterile condition.

Five replicates of each concentration level were aseptically sown with ten superficially sterilized seeds, sealed with plastic film and incubated in culture chamber at 24 ± 1 °C, 50 ± 5% relative humidity and 16 h of photoperiod (400 μM cm^{-2} seg^{-1} of light intensity).

The experiment progress was daily recorded and 28 days after treatment (DAT), it was finished and the number of germinations, biomass as fresh weight (Fw), and whole plant shikimic acid concentration were measured. Tolerance index and Exposure index were calculated as follow:

Tolerance index. The Tolerance index (Ti) was calculated as Ti = treated plant biomass/control plant biomass, according to the reported by Tong et al. (2009).

Exposure index. Considering that shikimate concentration in vegetal tissues is an accurate indicator of glyphosate exposure in plants (Singh and Shaner, 1998), an Exposure index (Ei) was calculated as Ei = shikimic acid concentration in treated plant tissues/shikimic acid concentration in control plant tissues on the same logical basis that Ti.

2.2.2. Spray application assay

Although the strategy is designed to rhizoremediate the glyphosate residues in soil and in view of the implementation context, the foliar application was considered since it is the typical agronomic use of the herbicide. To set the spray application assay, seeds of *L. corniculatus*, *L. tenuis* and *M. sativa* were superficially sterilized and sown in Petri dishes over filter paper embedded in half strength Hoagland's solution (Hoagland and Arnon, 1950). Five days after the radicle emerged, 25 seedlings of each species with similar phenological stage were manually transplanted to nurseries containing fifty 100 cm^3 capacity pots filled with a sand–perlite mixture (1:1).

Pots were flood irrigated with half strength Hoagland's solution to field capacity. Plants were grown in culture chamber at 24 ± 1 °C, 50 ± 5% relative humidity and 16 h of photoperiod (400 μM cm^{-2} seg^{-1} of light intensity).

Plants at 5–7 leaves were sprayed with glyphosate (74.7% of the ammonium salt) (Roundup Ultramax®, Monsanto) at product doses of 0; 700; 1400; 2800 and 5600 g ha^{-1}. Five nurseries were used,

one for each dose. The sprayer was equipped with a flat fan nozzle with an output volume equivalent to 200 L ha^{-1}. The doses between 1400 and 2800 g ha^{-1} correspond to typical post-emergence agronomic applications of this product in several crops, according to manufacturer. A dose of 700 g ha^{-1} represents a 70% value of the minimal recommended application, and could be assumed as an application drift. A dose of 5600 g ha^{-1} represents a product splashing due by machinery, which is frequent in lot edges.

Fourteen days after treatment, eight samples of *L. corniculatus* and *L. tenuis* and *M. sativa* were harvested from each glyphosate level and whole plant biomass (Fw) was recorded.

2.3. Shikimic acid accumulation in whole plant and chlorophyll measurement assays

Shikimate concentration in vegetal tissues has been used as a reliable indicator of glyphosate impact (Henry et al., 2007; Pline et al., 2002a; Singh and Shaner, 1998). Glyphosate exerts its effects through inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPS, EC 2.5.1.19). This enzyme catalyzes the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to shikimate-3-phosphate, a key step in the synthesis of aromatic amino acids, hormones and other relevant metabolites (Maeda and Dudareva, 2012; Schmid and Amrhein, 1995). As a consequence of this enzyme inhibition, shikimate concentration increases in plant tissues.

L. corniculatus plants were grown in sand–perlite mixture (1:1) in culture chamber at 24 ± 1 °C, 50 ± 5% relative humidity and 16 h of photoperiod (400 μM cm^{-2} seg^{-1} of light intensity). Plants at 5–7 leaves stage were sprayed with glyphosate at 400 and 1400 g ha^{-1} doses as described previously. Eight plants per treatment were harvested at 0, 1, 3, 5, 7, 14 and 21 DAT. Chlorophyll and shikimic acid concentrations were also measured.

Chlorophyll. Chlorophyll content was recorded using a portable chlorophyll meter (SPAD 502, Minolta, Konica Minolta Sensing, Inc.). The value of chlorophyll per plant was the average of ten measurements taken on three complete developed leaves with eight replicates per treatment.

Shikimate. Shikimate extraction and analysis was carried out according to the method proposed by Cromartie and Polge (2000) with minimal modifications. Briefly, whole plants were frozen with liquid nitrogen and mortar grounded. After grinding, 100 mg of tissue were weighted into 1.5 ml Eppendorf tubes and 1 ml of 0.25 M HCl was added. Samples were homogenized in vortex and immediately stored at −20 °C. After thawed at room temperature, tubes were centrifuged at 25,000 × *g* for 15 min and a 250 μl of supernatant were mixed with 250 μl 0.25% periodic acid/0.25% sodium (meta) periodate solution. Reaction mixture was incubated at 37 °C for 30 min. Then, 500 μl of NaOH 0.6 N/Na_2SO_3 0.22 M solution were added and absorbance was measured at 382 nm within 10 min. Measures were interpolated in a shikimic acid (Sigma–Aldrich, Buenos Aires, Argentina) standard curve and concentrations calculated.

2.4. Visual scoring

As part of the standard process of assessing herbicide effectiveness on weeds and its effects on crops, agronomists include the visual scoring assessment in their decision charts. In this way, although scoring has been standardized in protocols as those from the European and Mediterranean Plant Protection Organization (EPPO, 1997), most manufacturer companies made their own scoring protocols based on the professional experience of using their products and the application context (personal communication). Accordingly a visual scoring chart was specifically designed to assess the impact of glyphosate spraying on the *Lotus* spp. by combining specific qualitative phytotoxic signs described for legumes with a semi-quantitative scale from herbicide sensitive crops. The scoring was established according to Table 1.

Table 1
Phytotoxicity chart used during experiments.

Phytotoxic symptom	Assigned score
Yellowing	up to 5
Chlorosis	up to 5
Necrosis	up to 20
Defoliation/extended necrosis	up to 70

Plants scored over 20 point were classified as “Irreversibly damaged”.

2.5. EPSP synthase cDNA sequence analyses

Three plants of *L. corniculatus* were grown in culture chamber at 21 ± 1 °C, light intensity of 100 μmol m^{-2} s^{-1} and 16 h of photoperiod and daily watered with half strength Hoagland’s solution. Plants at 5–7 leaves stage were sprayed with glyphosate at 1000 g ha^{-1} dose in order to induce EPSP synthase mRNA expression and then harvested 48 h after treatment. For RNA extraction, aerial parts were harvested, frozen in liquid nitrogen, and immediately stored at −70 °C. Total RNA was isolated from 70 to 90 mg of vegetal tissue using a ZR Plant RNA MiniPrep kit (Zimo Research Corporation, Irvine, CA, USA) according to the manufacturer’s recommendation. Once isolated the RNA, a digestion with DNAse I (Ambion, Austin, TX, USA) was performed to degrade any possible contamination with genomic DNA.

First strand complementary DNA (cDNA) synthesis was carried out by using 2.5 μg of previously isolated total RNA and a Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV RT) (Invitrogen, Carlsbad, CA, USA) in combination with oligo (dT) 12–18 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Primers (primer forward: 5′-ACGGCTATTCGGGTGTGTTT-3′; primer reverse: 5′-TCCAAAGCGCTCCATCAACT-3′) were designed based on the sequence of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC number 2.5.1.19) of taxonomically closely related species *Lotus japonicus* (GenBank accession number AP009741.1) and *Medicago truncatula* cDNA (GenBank accession number XM_003605091.1) in order to amplify a 719 bp fragment of the *EPSP synthase* gene where the mutation site described to confer resistance to glyphosate in *Lolium* spp. and other species is located (Baerson et al., 2002; Yu et al., 2007). Polymerase chain reaction (PCR) was conducted in a reaction volume of 25 μl. The reaction mixture contained 1× PCR buffer, 2 mM of $MgCl_2$, 0.2 μM of each primer, 0.2 mM of deoxynucleotides, 1 unit of Taq DNA polymerase (Thermo Scientific, MA, USA) and 1.5 μl of template cDNA. Cycling conditions were: 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 56 °C and 30 s at 72 °C, followed by a final extension step of 10 min at 72 °C.

An aliquot of PCR product was loaded in a 1.5% agarose gel and electrophoresed to confirm the amplicon size and quality, and the remaining solution was referred to Macrogen DNA sequencing service.

Sequence analyses were conducted using the software MEGA version 5.2.

2.6. Statistical analysis

Data were analyzed using one-way analysis of variance and post hoc comparisons were performed with Dunnett honestly significant difference tests. The software used for the statistical analysis was GraphPad Prism Statistics version 5.01

Table 2
Biomass, shikimate concentration in whole plant, Tolerance and Exposure index determined for *L. corniculatus*, *L. tenuis* and *M. sativa* 28 DAT. Means within a column followed by an asterisk are significantly different compared to 0.0 mg kg^{-1} concentration (control) ($\alpha = 0.05$). Mean values ± SD ($n = 9$).

Glyphosate dose (mg kg^{-1})	*L. corniculatus*				*L. tenuis*				*M. sativa*			
	Biomass (mg plant)	Tolerance index (Ti)	Shikimate (μg g fresh wt^{-1})	Exposure index (Ei)	Biomass (mg plant)	Tolerance index (Ti)	Shikimate (μg g fresh wt^{-1})	Exposure index (Ei)	Biomass (mg plant)	Tolerance index (Ti)	Shikimate (μg g fresh wt^{-1})	Exposure index (Ei)
0.00	43 ± 17	1.00	60 ± 17	1.00	31 ± 15	1.00	70 ± 18	1.00	90 ± 52	1.00	41 ± 15	1.00
0.5	51 ± 22	1.18	61 ± 24	1.02	44 ± 23*	1.39	101 ± 73	1.43	49 ± 41*	0.55	66 ± 38	1.62
1.0	57 ± 33	1.32	87 ± 32	1.45	48 ± 25	1.53	153 ± 142	2.17	34 ± 22*	0.38	101 ± 51	2.48
5.0	47 ± 23	1.11	278 ± 140	4.63	25 ± 11	0.80	627 ± 305	8.91	23 ± 11*	0.26	531 ± 289	13.08
20.0	27 ± 11	0.63	678 ± 292*	11.30	20 ± 7	0.64	2262 ± 1553*	32.16	19 ± 6*	0.21	996 ± 344*	24.55
50.0	25 ± 7	0.58	1290 ± 445*	21.50	14 ± 7*	0.45	8357 ± 3924*	118.82	22 ± 7*	0.24	2117 ± 1369*	52.16

3. Results and discussion

3.1. Semisolid agar media assay

All the vegetal species assessed showed no significant differences in germination rates at the proposed doses (data not shown). These results are possibly due to the fact that glyphosate concentrations were not high enough to nonspecifically inhibit seed germination. Some authors observed germination suppression at dose ranges varying from 10 to 160 mg L^{-1} (Perez-Jones et al., 2007) and from 12.5 to 400 mg L^{-1} (Perez and Kogan, 2003; Yanniccari et al., 2012a), but such doses are too high to represent the levels of soils chronically polluted with glyphosate in the agronomic ecosystem. Regarding to plant growth, 28 days after sowing, significant differences were observed at the different concentration levels (Table 2 and Fig. 1). *M. sativa*, as expected, showed the most sensitive response to glyphosate with a Tolerance index (Ti) of 0.55. In contrast, *L. tenuis* and *L. corniculatus* increase their biomass to reach a Ti of 1.53 and 1.11 at 1.0 mg kg^{-1} and 5.0 mg kg^{-1} of glyphosate respectively. This paradox effect is probably due to the phenomenon of *hormesis*, previously reported by other authors who worked with plants tolerance to glyphosate (Cedergreen and Olesen, 2010; Petersen et al., 2007; Pline et al., 2002b). In this way, the fact that *L. corniculatus* and *L. tenuis* positively respond to sub agronomic glyphosate doses by increasing its biomass could be a desired characteristic considering the proposed remediation strategy. It is remarkable that *L. corniculatus* increases a 32% and *L. tenuis* 53% of the total biomass at the dose of 1.0 mg kg^{-1}.

Visual scoring of the three plant species showed a correlation with biomass measurement (Fig. 1). *L. corniculatus* presented minimal foliar changes (leaf area reduction) at 5.0 mg kg^{-1} of glyphosate, while at lower concentrations showed no differences with control (Table 2). On the other hand, *L. tenuis* showed shoot and root shortening and reduction in foliar area at the same dose (Fig. 1). Finally, *M. sativa* was able to germinate in the presence

Fig. 1. Response of *L. corniculatus*, *L. tenuis* and *M. sativa* to different glyphosate concentrations in semisolid agar medium at 28 days after treatment.

of glyphosate at 1.0 mg kg^{-1} but showed manifest signs of toxicity (root and shoot shortening accompanied with severe chlorosis and defoliation).

In order to interpret the tolerance assay in terms of plant endurance when implanted under field conditions, biomass differences were validated by visual scoring. In this way, *L. corniculatus* biomass showed no statistically differences at 20.0 mg kg^{-1} and 50.0 mg kg^{-1} of glyphosate when compared with control, while *L. tenuis* showed no differences at 20.0 mg kg^{-1}. However, visual scoring revealed marked toxicity effects in *L. tenuis* at those levels and, in this case, the increment in branching as a consequence of glyphosate toxicity (Dinelli et al., 2006) masked biomass differences which could lead to over estimation in "field effective" plant tolerance.

According to biomass quantification and visual assessment during the semisolid tolerance assays, *L. corniculatus* tolerated a 5.0 mg kg^{-1} glyphosate dose, whilst *L. tenuis* tolerated a 1.0 mg kg^{-1} glyphosate dose. Those tolerance limit values were obtained in a maximum bioavailability condition ensured by agar medium. It is important to indicate that soil bioavailability is lower than agar, therefore the limit values informed for both species can be considered conservatives regarding to the different soils textures and compositions found in the Humid Pampa region.

In this experiment, shikimate concentration in whole plant tissue increases in the three species at all levels tested, except for *L. corniculatus* at 0.5 mg kg^{-1} (Table 2). In this trend, *L. corniculatus* exhibits the lowest shikimate concentration compared to *L. tenuis* and *M. sativa* at all doses tested. As it was expected, *M. sativa* showed the most sensitive response to glyphosate. Regardless of the three species were affected by glyphosate, the relationship between shikimate concentration, biomass production and visual scoring was differentially expressed depending on the plant species. As an example, an Exposure index (Ei) of 2.48 in *M. sativa* was associated to significant effects in fresh weight and visual injury, while an Ei of 2.17 in *L. tenuis* produces no changes on the same variables. Moreover, an Ei of 4.63 in *L. corniculatus* was only evidenced by a slight leaf size reduction. In this way, the lower shikimate concentration observed in *L. corniculatus* at all glyphosate levels could be associated to a difference in the uptake and/or translocation from the root or to a better metabolic dealing of the shikimate in this species. As a consequence of these observations and considering the unique response of each species to the same exposition level, special care should be taken when shikimate concentration is employed to assess glyphosate impact in future phytoremediation assays.

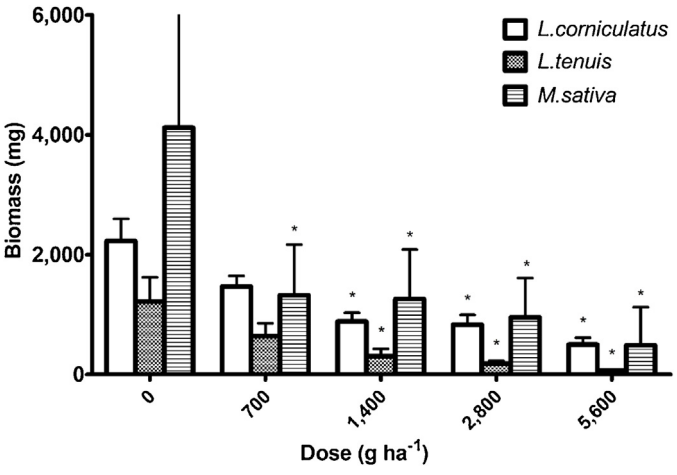

Fig. 2. Effect of glyphosate in spray application on *M. sativa*, *L. corniculatus* and *L. tenuis* biomass at 14 DAT. Asterisk marked bars are significantly different compared to 0 g ha^{-1} application (control) as determined by Dunnett test ($\alpha = 0.05$). Vertical bars represent ±SD ($n = 8$).

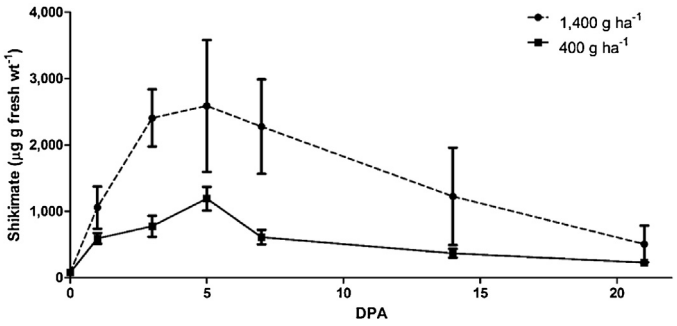

Fig. 3. Shikimate accumulation (µg g fresh wt^{-1}) in *L. corniculatus* whole plant at doses of 400 and 1400 g ha^{-1}. Vertical bars represent ±SD ($n = 7$).

Results observed in this assay indicate that *L. corniculatus* is the best candidate to be implanted in agronomic soils chronically polluted with glyphosate under field condition. In addition, the chance of using commercial seeds instead of plantlets represents a meaningful agro-technological advantage and substantially reduces implementation costs. Besides, *hormesis* observed on this species during plants growth produces extra benefits on biomass production, improving the possibilities of implantation, which represents an ecological advantage in the competition to other weed species (Díaz et al., 2005).

Finally, it is important to emphasize that multiple parameters should be combined when performing a tolerance assay of the candidate rhizoremediation species, especially when they are meant to endurance field condition. In this assay, the results of biomass, visual assessment and shikimate concentration in plant tissue complemented each other, allowing a thoughtful interpretation of the experiment.

3.2. Spray application assay

L. corniculatus and *L. tenuis* showed no statistical differences in their fresh weight between control and 700 g ha^{-1} of sprayed glyphosate but a significant difference was observed at that dose for *M. sativa* (Fig. 2). For all other application doses, the three species differed significantly from control.

Even though *M. sativa* is the species that gain the largest biomass under control conditions, a sub lethal application of sprayed glyphosate (700 g ha^{-1}) reduced its total weight in 68%, while *L. corniculatus* and *L. tenuis* were reduced in 34% and 47% respectively.

Coherent results were obtained in both tolerance assays, aiming *L. corniculatus* as the most tolerant and the best strategy fitting

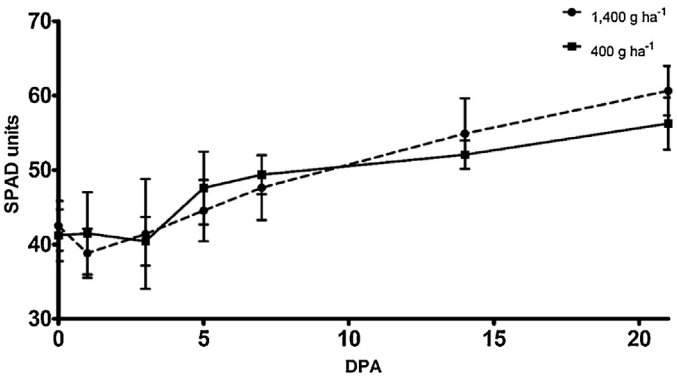

Fig. 4. Chlorophyll content (SPAD units) in *L. corniculatus* leaves at doses of 400 and 1400 g ha^{-1}. Vertical bars represent ±SD ($n = 7$).

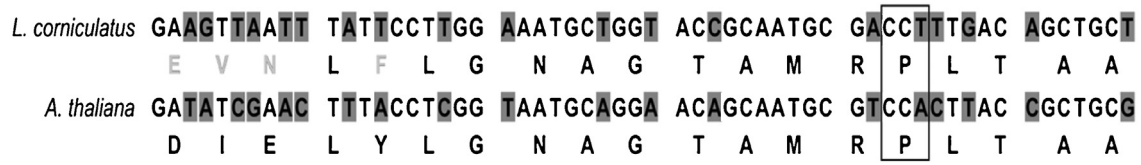

Fig. 5. Partial alignment of the EPSP synthase cDNA and deduced amino acid sequence of *L. corniculatus* and *A. thaliana*. The boxed codon shows the amino acid position number 106 based on *A. thaliana* sequence.

species. Both experiments adequately describe the response of the plant species to glyphosate, which is necessary to perform a rhizoremediation strategy.

3.3. Shikimate accumulation and chlorophyll measurement

L. corniculatus was selected to perform a whole-plant shikimate accumulation and chlorophyll assay. Although it does not represent a conclusive assessment on the molecular basis of glyphosate tolerance, measurement of shikimate levels after glyphosate spraying allows understanding the nature of the tolerance mechanisms (Feng et al., 2004). Furthermore, these inexpensive and easy to carry out determinations could be useful in the future for monitoring the glyphosate impact on plants while field rhizoremediation experiments are performed.

When plants were exposed to 400 g ha^{-1} of glyphosate showed similar shikimate concentration values at 1, 3 and 5 days post-application than the reported by Perez-Jones and Dinelli (Dinelli et al., 2006; Perez-Jones et al., 2007) in tolerant species (Fig. 3). Shikimate concentration levels are consistent with those of plants which have acquired a high innate tolerance to glyphosate by limited uptake and/or translocation of the herbicide, reducing its accumulation in meristematic tissues (Feng et al., 2004). These tolerance mechanisms are desirable in future remediation strategies, where an optimal root physiological status is required to ensure the continuous elongation and preservation of the degrading bacteria within the rhizosphere environment.

As it was also reported by different authors whose analyzed glyphosate tolerance in several vegetal species, the maximum increment in shikimate concentration was reached between 4 and 7 DAT. From that day, shikimate concentration decreases but never returns to baseline level, probably due to the time required to complete the *EPSP synthase* pool turnover exceeded the experiment time. At the end of the experiment *L. corniculatus* plants survived to 400 g ha^{-1} application.

Shikimate concentration in vegetal tissue evidence glyphosate impact before visual effects can be observed. Moreover, if baseline shikimate concentration values are known, the applied glyphosate dose could be roughly estimated.

Regarding to the 1400 g ha^{-1} application, the same profile of 400 g ha^{-1} curve was obtained at, but with higher shikimate concentration values (Fig. 3). From the 5 DAT, shikimate concentration also decreases, but *L. corniculatus* plants died at this application dose.

As it was discussed previously, it is important to consider not only biochemical parameters to monitor plant tolerance but also visual score, since both assessments complement each other. Shikimate concentration in vegetal tissues results more informative at the proximity of application.

Although chlorophyll measurement was sensitive to glyphosate application and presented an unusual profile when compared to literature (Gao et al., 2014; Yanniccari et al., 2012b), no significant differences were observed between doses.

The results obtained from total chlorophyll measurement showed a concentration decrease at 1 DAT (1400 g ha^{-1}) or 3 DAT (400 g ha^{-1}) and then a sustained increment until 21 DAT at both doses (Fig. 4). This profile in chlorophyll concentration is possible caused by an initial stress state associated to glyphosate application followed by its specific effects on meristematic tissue, where inhibition on the new leaves development induce a chlorophyll accumulation in lower leaves. Nutrient re-mobilization from lower to upper leaves is a common process in plant development possibly disrupted in this case by glyphosate action on meristematic tissues (Avice et al., 1996).

In this context, although shikimate measurement was more informative than total chlorophyll content, the correlation of both parameters improves the analysis of the plant physiological changes after glyphosate application.

3.4. EPSP synthase partial cDNA sequence analyses

The most frequently reported glyphosate tolerance mechanism on weed species is a single nucleotide polymorphism (SNP) in the 106 amino acid of the enzyme enolpyruvylshikimate-3-phosphate synthase (*EPSP synthase*), resulting in a Proline substitution for a Serine, Alanine or Threonine (Christoffers and Varanasi, 2010). A substitution at this site changes the orientation of two other amino acids at the glyphosate binding site in prokaryotes, is proposed to cause a slight narrowing of the glyphosate binding site (Healy-Fried et al., 2007).

We found no substitution in the Pro 106 on *EPSP synthase* cDNA sequence for *L. corniculatus*, which indicates that its glyphosate tolerance cannot be explained based on enzyme activity changes (Fig. 5). Differences observed in the predicted amino acid sequence between *Arabidopsis thaliana* and *L. corniculatus* are not involved in glyphosate tolerance (Christoffers and Varanasi, 2010).

Results are consistent with those obtained in shikimate accumulation and spray application assays, suggesting that tolerance mechanisms are related to a differential uptake and/or translocation.

The partial cDNA sequence from *L. corniculatus* was deposited in the GenBank under accession number KM076642.

4. Conclusions

It was demonstrated that *L. corniculatus* tolerates agronomic-related doses of glyphosate, in both agar and spray application assays. The tolerance mechanism is not caused by the reported single nucleotide polymorphism in the 106 amino in the *EPSP synthase* acid but probably due to a differential uptake and/or translocation, sequestration or increased metabolic detoxification.

It is important complementing biometric, biochemical and visual scoring parameters, since they offer valuable information of the vegetal species at different moments throughout the experiment.

The glyphosate tolerance observed, plus its productive and adaptive advantages in the Humid Pampa region, put forward *L. corniculatus* as an excellent candidate vegetal species to perform rhizoremediation assays in this agroecosystem.

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