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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,