

LOTUS spp: BIOTECHNOLOGICAL STRATEGIES TO IMPROVE THE BIOECONOMY OF LOWLANDS IN THE SALADO RIVER BASIN (ARGENTINA)

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ABSTRACT

The Salado River Basin region is the most important livestock breeding area in Argentina, where the *Lotus* species has been traditionally cultivated as forages. Nearly 60% of their land surface is dominated by salt-affected soils with severe constraints for crop cultivation. In order to cope with that limitation, farmers have utilized species such as non-native *L. tenuis* (ex-*Lotus glaber*), which shows a very good adaptation. As a result, inter-seeding of *L. tenuis* has been proposed as a strategy of choice for improving forage production in marginal areas. The increase in soil quality by these means is achieved by an increment of the organic matter content, improvement of soil fertility as well as microbial biodiversity. Thus, the introduction of *L. tenuis* and/or other *Lotus* genotypes could have enormous benefits for similar constrained lands around the world. We are developing an integrated analysis of the changes that occur in soils under legume production. We will not only analyze the microbial diversity associated, but also soil physical and chemical characteristics and the impact of different legume-microbes association on mitigation of GHG emissions. In addition, we are identifying the main genetic determinants associated with interesting agronomic traits such as plant tolerance against biotic and abiotic stresses and the content of condensed tannins. Our future and present research will build a solid base for the improvement of agronomically-important species and the development of better strategies for the management of constrained lands such as the lowlands in the Argentinean Pampas.

Keywords: *Lotus spp.*, flooding pampas, Salado River basin, condensed tannins, constrained soils.

INTRODUCTION

The *Lotus* genus is included in Fabaceae, which is the third largest family of Angiosperms. This family has a high biological diversity, with approximately 720 genera and more than 18,000 species worldwide. The legume species take profit from root symbiosis with mycorrhizal fungi (Sannazzaro *et al.*, 2004), characterized by their ability to establish symbiotic interactions with nitrogen-fixing bacteria (Estrella *et al.*, 2009). These associations increase their competitiveness, turning them as “pioneer” plants in constrained soil environments. These legumes advantages became an important part of sustainable agricultural systems and others marginal soil area. In particular, the tribe Loteae DC is a monophyletic group composed by four genera. The name *Lotus* was introduced by Linnaeus in 1753 and since then, there were several changes in the species delimited by this name. Recently, developed molecular tools have significantly contributed to restrict the genus to 100–130 species. Most *Lotus* species are native to Europe, Asia, Africa, Australia and some to the Atlantic and Pacific Ocean Islands. Few species were described as native to the New World, but they were later segregated in non-*Lotus* genera. However, *Lotus* species have a worldwide distribution, except in very cold regions and certain tropical areas of Southeast Asia and Central America. This worldwide distribution is partially due to their introduction to non-native areas by human activities and its adaptability to different environmental stresses (Escaray *et al.*, 2010).

Lotus genus crops species in South America

The genus *Lotus* has been historically used in the Southern Cone of South America for pasture improvement. Taking in mind this agronomical target, there are intends to contribute to the sustainability of them in the region through the generation of biotechnological tools to allow the development of better adapted genotypes of the forage species (*Lotus corniculatus* L., *L. tenuis* Waldst. et Kit. (Syn. *L. glaber* Mill.), *L. uliginosus* Schkuhr (Syn. *L. pedunculatus* Cav.) and *L. subbiflorus* Lag.) in agricultural marginal soils.. All of these projects took advantage of the model species *Lotus japonicus* (Regel) to develop new strategies to accelerate breeding of cultivated species. In parallel, there are some research groups working in the identification, characterization and selection of microorganism that could guarantee the optimum N fixation in those soils improving legume nutrition, thereby increasing productivity, quality and sustainability of *Lotus* pastures in marginal soils (Escaray *et al.*, 2012a).

The Salado River Basin and the importance of *Lotus spp.*

The Salado River Basin is located in the center of the Flooding Pampa, a vast area located in the Buenos Aires province, Argentina. It is a very flat region, and constantly affected by periods of flood/drought. About 40% of the soils in this area are composed of humus with a great water retention capacity and excellent quality

for growing many important crops. It is, therefore, considered one of the richest agricultural regions in Argentina. Most of the wheat, corn and soybean production comes from this area and it is, of course, essential for the economy of the country, where the agricultural activities are among the major economic resources. However, the economic development of the area has lagged because of the presence of thousands of hectares of poor quality salt-affected soils, representing about 60% of the total area. These soils are very conspicuous in Argentina, placing this country in third place among those with the broadest surface dominated by them. In fact, approximately 11.58 Mha (equivalent to 11% of the total area) of saline soils are estimated to occur under a sub humid-humid climate in this country. It has been largely demonstrated that saline soils hinder plant growth due to their high pH, low structural stability and low water and nutrient availability. In this sense, the distribution of natural grasslands in the Salado River Basin is determined by alkalinity levels and flooding frequency. All plant communities consist mainly of grasses and plants belonging to the family *Compositae*, whereas native legumes are largely absent. The grassland community growing on sodic soils is composed by low value foraging grasses, such as *Distichlis scoparia*, *D. spicata*, *Paspalum vaginatum*, *Sporobolus pyramidatus* and the alga *Nostoc*. Although they comprehend a considerable fraction of the forage available to livestock, their net primary productivities and palatability are clearly lower than those of communities growing in more fertile soils. In addition, these species are associated to low soil organic matter (SOM) contents, which may show wide variations among different Great Soil Groups. As will be described later, high SOM values are preferred since they are associated to reduce rates of gas emission to the atmosphere.

The economical practices of the Salado River Basin have suffered notable changes in the last decades. This is mainly due to the advance of agriculture at the expenses of grazing pastures, a phenomenon that has constrained livestock to marginal areas such as the case of native grasslands growing on sodic soils. Therefore, these soils are currently subjected to increasing use-pressures to improve forage offer. In this trend, many attempts to replace grassland communities by exotic species have been made with relative success. For instance, one of the species that has gained considerable importance is the naturally adapted legume *L. tenuis*. The tolerance of this species to alkalinity and long term flooding periods has allowed a wide and fast spread over the Salado River Basin. *L. tenuis* can also grow under low extractable phosphorus (P) concentrations, as it usually happens in most sodic soils, because its association with mycorrhiza enables the acquisition of P by plant roots (Sannazzaro *et al* 2004, Echeverría *et al* 2013). In addition, the introduction of *L. tenuis* represents a substantial benefit for the region since it is an important source of N for the soil (Castagno *et al* 2014). Even though *L. tenuis* has high nutritional value, similar to other forager legumes, it is unable to accumulate adequate levels of condensed tannins in the foliage and this deficiency is one of the principal aims for legume breeders in Argentina (Escaray *et al* 2014).

Abiotic stresses

Alkaline stress refers to the presence of alkaline salts (Na_2CO_3 or NaHCO_3) in the soil, whereas saline stress is related only to neutral salts such as NaCl or Na_2SO_4 . Although saline stress has been addressed in numerous studies, alkalinity rather than salinity is the main constraint for cropping activity (Paz *et al* 2012). Studies on abiotic stress in *L. tenuis* have been focused principally on NaCl tolerance (Sánchez *et al* 2005; Sannazzaro *et al* 2006, 2007; Echeverria *et al* 2008; Uchiya *et al*, 2016), whereas less studies has addressed its tolerance level to alkaline and mixed salt–alkaline stresses. Alkaline salts have a more severe effect on plant growth than neutral salts. When soil salinity is high and/or the pH is 8.5 or above, a number of micronutrients such as P, Fe, and Zn become deficient, triggering differential gene expression (Paz *et al*, 2014b). Both saline and alkaline conditions frequently coexist in nature, with the proportion of neutral salt to alkaline salt varying in different soils. However, little is known about the effects of alkaline and salt–alkaline stresses on plants. In this sense, root architecture has been directly related to plant productivity because in no-till systems it can provide clues about resource cost, transport, and exploration efficiency, especially under limiting edaphic conditions. Salinity affects root developmental processes in different ways. Evident differences in the morphology of the root system as a function of salt stress treatment were found in *L. tenuis* (Echeverria *et al*, 2008). Neutral and alkaline salts produced a similar detrimental effect on *L. tenuis* growth, whereas the effect of their combination was synergistic (Paz *et al* 2012). The pattern of morphological changes in *L. tenuis* root architecture after the alkaline treatment (in the absence of NaCl) was similar to that found in the mixed salt–alkaline treatment and different from that observed in neutral salt. A unique root morphological response to the mixed salt-alkaline stress was the reduction in the ratio between xylem vessels and root cross-sectional areas (Paz *et al* 2014a).

Moreover, we characterized phenotypically the response to alkaline stress of the most widely used *L. japonicus* ecotypes, Gifu B-129 and MG-20, and analyzed global transcriptome of plants subjected to 10 mM NaHCO_3 during 21 days, by using the Affymetrix *Lotus japonicus* GeneChipH (Babuín *et al*, 2014). Plant growth assessment, gas exchange parameters, chlorophyll a fluorescence transient (OJIP) analysis and metal accumulation supported the notion that MG-20 plants displayed a higher tolerance level to alkaline stress than Gifu B-129. Overall, 407 and 459 probe sets were regulated in MG-20 and Gifu B-129, respectively. The number of probe sets differentially expressed in roots was higher than that of shoots, regardless the ecotype. Gifu B-129 and MG-20 also differed in their gene regulation related to Fe/Zn homeostatic cellular condition, synthesis of stress response compounds, protein-degradation, damage repair and root senescence, as well as in glycolysis, gluconeogenesis and TCA. In addition, there were differences between both ecotypes in the expression patterns of putative transcription factors that could determine distinct arrangements of flavonoid and isoflavonoid compounds (Babuín *et al*, 2014).

Likewise, we evaluated cold acclimation of the genus by studying *L. japonicus* (Gifu B-129 ecotype) over a stress period of 24 h. High-throughput RNA sequencing was used to identify and classify 1077 differentially expressed genes, of which 713 were up-regulated and 364 were down-regulated. Up-regulated genes were principally related to lipid, cell wall, phenylpropanoid, sugar, and proline regulation, while down-regulated genes affected the photosynthetic process and chloroplast development. Together, a total of 41 cold-inducible transcription factors were identified, including members of the AP2/ERF, NAC, MYB, and WRKY families; two of them were described as putative novel transcription factors. Finally, DREB1/CBFs were described with respect to their cold stress expression profiles. This is the first transcriptome profiling of the model legume *L. japonicus* under cold stress. Data obtained may be useful in identifying candidate genes for breeding modified species of forage legumes that more readily acclimate to low temperatures (Calzadilla *et al*, 2016a, 2016b).

Biotic stress

Legume production is also hampered in many areas by biotic stress originated by pathogenic microorganisms. This factor is the cause of considerable losses every year. Therefore, a deeper understanding of the defense responses deployed by legume plants against pathogens is a crucial step in the development of tolerant cultivars and the establishment of effective disease control strategies. However, there is a rather lack of information on the defense responses that control attacking pathogens in legumes. Even worse, the resistance mechanisms identified in plants belonging to other families cannot be fully extrapolated to legume species, probably due to differences in genomic organization. We examined the interaction between *L. japonicus* (Gifu B-129 and Miyakojima MG-20) and *P. syringae* pv. tomato DC3000, a strain that cause bacterial speck in tomato and *Arabidopsis* (Bordenave *et al* 2013). Our analysis demonstrated contrasting phenotypic differences in the two ecotypes during the response to the bacteria. On these grounds, we next performed a transcriptomic analysis aimed to identify the genes associated with such differential response. We were able to recognize a large number of transcripts differentially expressed, many of them showing high homology to well-known defense genes in other plant species. Importantly, some of the genes associated to virulence in this strain diverge from those described in other legume-parasitic races of this species, as pv. *glycinea*, pv. *phaseolicola* and pv. *pisi*. Thus, these partners could be developed into a useful model pathosystem to study the most general defense mechanisms deployed in this legume against non-pathogenic microorganisms (Bordenave *et al* 2013).

Microorganism symbiotic associations

Like most legumes, *L. tenuis* has the ability to establish mutualistic symbiotic relationships with soil N-fixing bacteria collectively known as rhizobia, thereby rendering this plant more competitive than non-legumes in soils with low N content. Taking into account the relatively recent naturalization of this legume in

Argentina (less than 80 years), it is not surprising that the selection of efficient strains and the development of high quality inoculants for *L. tenuis* is still incipient. However, in many cases *L. tenuis* seeds are inoculated before sowing, by using rhizobia formulations based on strains whose taxonomic identity has not been established. These strains were originally isolated from soils different to those of the Salado River Basin and selected for their ability to symbiotically fix N in environments not necessarily similar to those typical of this region. It is well known that, in the soil, the strains used for inoculation are subjected to the effects of numerous biotic and abiotic factors which could decrease the symbiotic efficiency. In the particular case of the Salado River Basin, the adaptation and survival of rhizobia, as well as their symbiotic efficiency can be affected by soil salinity. Thus, when efficient strains are introduced in environments different to those from which they were isolated, they can be outcompeted by well adapted native rhizobia. In this way, it is reasonable to hypothesize that rhizobia isolates from the Salado River Basin should be better adapted to the soil conditions of this region than introduced strains. In this sense, the use of inoculants based on efficient native strains could be an affordable and sustainable resource to improve the yield of *L. tenuis* in this region. As a consequence, we evaluated inoculants isolated from soils of the region of Salado River Basin (Estrella *et al.* 2009; Sannazzaro *et al.*, 2011).

Moreover, low P soils levels is also an important issue in the Salado River Basin, and symbiotic associations could diminished its detrimental effect. We isolated and characterized phosphate-solubilizing strains from a constrained environment in the region improve the implantation and persistence of *L. tenuis*. The most efficient isolate was identified as *Pantoea eucalypti*(M9I strain), a novel species in terms of plant growth-promoting rhizobacteria (Castagno *et al.*, 2011). Inoculation of *L. japonicus* with this strain, alters the root, resulting in a herringbone pattern of root branching. Additional features include improvement in Fe²⁺ transport to the shoots, acidification of the hydroponic solution of the plant cultures, and an accompanying increase in the efficiency of the PSII parameters. As a whole, *P. eucalypti* M9I showed a beneficial effect on the Fe acquisition, suggesting its potential use as an inoculant for legume crops cultivated in alkaline soils (Campestre *et al.*, 2016). Interestingly, in others studies developed in collaboration with our lab, Actinobacteria also showed promoting growth activity on *Mesorhizobium loti* symbiosis with *L. tenuis* (Solans *et al.* 2015).

Condensed tannins

Proanthocyanidins (PAs) are secondary metabolites that strongly affect plant quality traits. The concentration and the structure of these metabolites influence the palatability and nutritional value of forage legumes. Hence, modulating PAs in the leaves of forage legumes is of paramount relevance for forage breeders worldwide (Escaray *et al.*, 2012b). The lack of genetic variation in the leaf PA trait within the most important forage species and the difficulties in engineering this pathway via the ectopic expression of regulatory genes, prompted us to pursue alternative

strategies to enhance this trait in forage legumes of agronomic interest. The *Lotus* genus includes forage species which accumulate PAs in edible organs and can thus be used as potential donor parents in breeding programs. Taking in mind this, we recovered a wild, diploid and PA-rich population of *L. corniculatus* and crossed it with an argentinean naturalized germplasm of *L. tenuis*. The last species does not accumulate PAs in the herbage. The resulting interspecific hybrid displayed several traits of outstanding agronomic relevance such as rhizome production, PA levels in edible tissues sufficient to prevent ruminal bloating (around 5 mg of PAs/g DW) and adaptability to marginal lands. This material represents the first example of the introgression of the condensed tannins trait in forage legumes to levels known to provide nutritional and health benefits to ruminants (Escaray *et al.*, 2014). Our studies suggest that the hybrids and their progeny are an invaluable tool to gain a leap forward in our understanding of the genetic control of PA biosynthesis and tolerance to stresses in legumes.



Figure 1.A and B. Cattle grazing paddock of *L. tenuis* in the Salado River Basin, Argentina.C. Condensed tannins content in different accessions of *Lotus* spp and hybrid *L. tenuis* x *L. corniculatus*. Adapted from Escaray *et al.* 2014.D. Alkalinity stress response of *L. japonicus* ecotypes after 21 d. Adapted from Babuin *et al.* 2014.

CONCLUSION AND PERSPECTIVES

Actually, our lab is increasing the knowledge on soil microbial diversity in the rhizosphere of *Lotus* species plants growing in the Salado River Basin (Nieva *et al.*, 2016),and evaluating its relationship with soil C sequestration (Nieva *et al.*

Manuscript in preparation). Moreover, farm productivity of *L. tenuis* and the implementation of the different technological protocols designed is being also analyzed (Bailleres *et al.* Manuscript in preparation). Simultaneously we are evaluating the phenotype of different accessions of *L. japonicus* during the attack of the phytopathogenic fungi and bacteria and identifying the main traits associated to plant tolerance looking to improve it in forage crops of outstanding economical relevance for our region. Potential genes involved in the tolerance to biotic and abiotic stresses in *Lotus* model and crops species are under evaluation by High-throughput RNA sequencing and trans-genesis approaches (Espasandin *et al* 2010, 2014). Others lab aims include the better understanding of the molecular basis of condensed tannins biosynthesis in *Lotus* spp and the role of these secondary metabolites on plant stress responses and GHG mitigation. Moreover, we continue exploring the interspecific hybridization as a strategy to generate superior *Lotus* genotypes.

The evaluation under “cow farm conditions” of the incidence of *L. tenuis*, *L. corniculatus* and their interspecific hybrids on the generation of GHG by ruminants and the design and evaluation of a Spatial Distribution Model (SDM) for *L. tenuis* in the Flooding Pampa, and estimate potential GHG emission rates from sodic grasslands on a regional scale complete our actual aims and plan work targets¹.

Finally, we are interested in creating an international working group to coordinate the future transfer of the obtained knowledge to constrained lands around the globe, and the potential of the genus *Lotus sp* in the soil restoration and reclamation activities.

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