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Front cover: The photograph on the front cover exhibit stained leaf tannin cells of *Lotus corniculatus* L. The comparison of *Lotus corniculatus* and *Lotus tenuis* Mill (= *Lotus glaber* Waldst. & Kit) and their hybrids is reported by Dr. Francisco J. Escaray *et al.* (pp.69-71).

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Lotus Newsletter (2008) Volume 38 (2), 37.

Abstract, Workshop held at Chascomus, 17-19 November 2008

Diversity of rhizobia nodulating *Lotus* spp.

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Despite the genus *Lotus* includes more than 100 plant species present in diverse environments Worlwide, little is known about the diversity of rhizobia that can nodulate this group of legumes, which is essential to understand both the host symbiotic status and the actual needs for inoculation of those species of agricultural importance like forage species. It is generally accepted that *Lotus* spp. can be nodulated by intermediate growth rate bacteria of the genus *Mesorhizobium*, mainly *M. loti*, or by slow growth rhizobia related to genus *Bradyrhizobium*, depending on the *Lotus* species considered. Nevertheless, the knowledge on the *Lotus* symbiotic bacteria is rather limited and the existing information is fragmentary, compared to what is known of certain Lotus host species, particularly those of agronomic value like *L. tenuis* or *L. corniculatus*, or with a scientific research value like *L. japonicus*.

Within the EU INCO project LOTASSA (www.lotassa.org), we have carried out studies on the genetic diversity and taxonomic adscription of bacteria nodulating several *Lotus* in diverse countries and environments. Here we present some preliminary results on isolates from Spain, Portugal, Argentina and Uruguay. Hundreds of bacterial isolates were obtained from nodules of forage species like *L. tenuis*, *L. corniculatus* y *L. uliginosus*, besides several *Lotus* species native or endemic to the Canary Islands, some of them in danger of extinction. The results suggest a great diversity among *Lotus*-symbiotic bacteria, which could be distributed among several bacterial genera: *Mesorhizobium*, *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Aminobacter*. Particularly interesting is the finding that among all *Lotus* species screened and geographical locations sampled, the species *M. loti*, which has been catalogued as the type species for *Lotus* rhizobia, was rarely found, what points out the need of further studies on the genetic diversity of this particular group of rhizobia.

Lotus Newsletter (2008) Volume 38 (2), 38-39.

Abstract, Workshop held at Chascomus, 17-19 November 2008

Mesorhizobium loti- Lotus spp. interaction: basic and applied research

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Rhizobia-legume interactions lead to the establishment of a symbiosis in which atmospheric-nitrogen fixation occurs. This symbiosis allows the plant to grow in soils with limited nitrogen source. Nodule formation requires a sequence of highly regulated and coordinated events which began with the signal exchange between the host plant and the bacterial symbiont. In our country, the improvement of *Lotus glaber* cultivars have had a great influence on the expansion of cultivated areas to the marginal ones, such as the Basin of Salado River in Buenos Aires. *Mesorhizobium loti* strains inoculated at field must to compete with the indigenous populations of rhizobia that could nodulate but generally will conduce to a poor nitrogen fixation process. Nodulation competitiveness between bacteria could occur in any step of the process from root colonization to the release into the nodule cells. A knowledge of the molecular bases that are involved in the *M. loti*-Lotus interaction and of which are the molecules, components or systems which presence improve or negatively affect this process is necessary if our aim is in an early future create and use organisms more competitive and efficient for nodulation and nitrogen fixation.

Our objective is to explore the symbiosis between *M. loti* and *Lotus* spp. combining the characterization of different bacterial mutants with the study of their nodulation phenotype and the effect of this mutation on the plant transcriptional profile during the interaction. We had analyzed the effect of the mutation of lipopolysaccharide and the cyclic glucan biosynthesis of the bacterium, in the process of nodulation with Lotus. The mutant affected in the cyclic glucan (*cgs* mutant) induced the formation of nodules empty of bacteria (pseudonodules), and the mutant affected in the lipopolysaccharide (*lpsβ2* mutant) presented lower nodulation competitiveness than the wild type strain. To study how the nodulation process is regulated at the transcriptional level we compared, using the macroarray technology, the transcriptional profile of *Lotus japonicus* plant roots inoculated with the wild type bacterial strain with those inoculated with each of the characterized mutants. We identified hundred of genes which expression is upon the bacterial infection. Several of the differentially expressed genes are involved in the plant defense response. This is in concordance with the idea that in spite the beneficial effect of the nodulation on both symbiosis partners, it resembles in some aspects a pathogen-host interaction. Analysis results of the relative expression level for some selected genes at different post inoculation times, suggested the existence of a control of the plant defense response induced during the symbiosis. Type three secretion system (T3SS) and the effectors proteins translocated through it into the host cell, were described as one of the bacterial components involved in the modulation of the plant defense. *M. loti* has a T3SS. We found that mutation of *M. loti*

T3SS affected its competitiveness on *Lotus glaber*. Through a bioinformatics approach we identified other genes induced by the presence of NodD and the specific flavonoids such as were the T3SS components. Two of the new identified genes were able to be secreted to the external media through the T3SS. The determination of the specific role of each of the putative T3SS effectors that were identified is remaining.

In addition with our basic research, in our laboratory we are also working to create *M. loti* modified strains that could be more competitive in the process of nodulation with *Lotus* spp. As a result of biotic and abiotic stress (flooding, light, drought) plant ethylene levels increase. Ethylene has a negative effect on the nodulation. *M. loti* has the *acdS* gene that codifies for ACC deaminase, enzyme that degrades an ethylene biosynthesis precursor. Its expression only occurs in the bacteroid, inside the nodule. We have obtained a modified *M. loti* strain that expresses the *acdS* gene also in the free-living state. The modified strain presented higher competitiveness in nodulation assays carried out in stress conditions.

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Abstract, Workshop held at Chascomus, 17-19 November 2008

Arbuscular mycorrhizal colonization model in *Lotus tenuis* and dominant grasses of a natural grassland from Salado River Basin (Buenos Aires – Argentina)

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We studied the dynamic of arbuscular mycorrhizal colonization in *Lotus tenuis* and dominant grasses roots of a natural grassland from the Salado River Basin along a topographic, hydric, saline and sodic gradient (pH: 9.4 – 6.9; EC: 9.3 – 1.7 dS/m; ESP: 77 – 28%; %C: 1.1 – 2.1%; N: 0.11 – 0.19%; P Bray: 8.7 – 3.9 ppm). The main objective was to relate the seasonal changes in the mycorrhizal colonization structure with the changes in growth of plant having different affinity for mycorrhizal colonization (*Lotus tenuis*, *Paspalum vaginatum*, *Distichlis spicata* and *Stenotaphrum secundatum*). Hence, to investigate if there is a key factor controlling the pattern of mycorrhizal colonization is roots. It was reported that the climate, associated with soil and plant are the factors that control mycorrhizal colonization, but it is not possible to identify which of these factors is more important (Johnson *et al.*, 1992; Entry *et al.*, 2002). In the present work, we have studied the same plant species at the same climate growing at different soil characteristics to investigate which factor can be more important influencing the colonization pattern.

The total mycorrhizal colonization was greater and seasonally different in *L. tenuis* roots comparing with the dominant grasses. However, this pattern was similar along the soil gradient. We do not necessarily expect a similar mycorrhizal between plant species since plants differ in root system, growing period, affinity and dependence for mycorrhizal to grow in nutrient deficient soils. Then, different in length and seasonal pattern of colonized root between plants species may be ascribed to the interactions among the growth rates of both the fungi within the roots and the roots within the soil.

The arbuscular colonization (AC) pattern was similar in roots of *L. tenuis* and the grasses. Larger AC were found at the beginning of the growing season in late winter at all the sites, and this might have been related to the period of active nutrient uptake by the host plant (García and Mendoza 2007). The seasonal pattern of vesicular colonization (VC) was similar in the roots of both *L. tenuis* and the grasses. Larger VC were found at all the sites and plant species in summer, when plant growth rate decreases because of the frequency of dry periods and high temperature spells. This fact suggests the presence of a seasonal effect on vesicle formation in roots. Vesicles produced by mycorrhizal fungi are considered to function as temporary storage organs (Hirsch and Kapulnik, 1998), and are signs that the fungi speed up their life cycles in annual plants towards the end of the growing season (Gavito and Varela, 1993). From the present work, maximum AC and VC took place at

different seasons. These seasonally related effects suggest the preferential production of one kind of morphological colonization forms by the fungus during a specific season.

The number of entry points (EP) per mm of colonized root was greater in *Lotus* than in grasses, showing the differential affinity between plant species. Increases in EP suggest increases in formation of colonization units (Fig. 1). *Lotus tenuis* is a dependant plant for arbuscular mycorrhiza (Mendoza and Pagani 1997), and presents high levels of colonization at early age. In *Lotus*, the changes observed in the colonization structure are mainly because of the seasonal production of AC, VC and intraradical hyphae (HO) fungal structures, rather than the formation of new colonization units by EP. This is because with increases in EP, AM did not change and still high even with changes in EP (Fig. 1). However, in *Paspalum vaginatum*, *Distichlis spicata* and *Stenotaphrum secundatum* roots, increases in EP are positively correlated with increases in AM (Figure 1). Hence, in the grasses the changes in the colonization structure are mainly dependent of the formation of new colonization units from the EP and then the development of the intraradical mycelium within the root (HO).

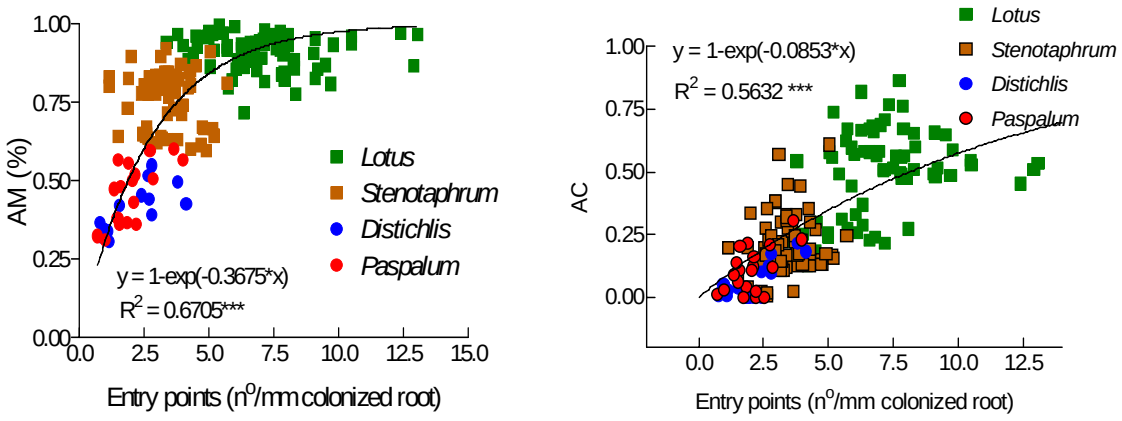

Figure 1. Changes in mycorrhizal (AM) and arbuscular colonization (AC) with the amount of entry points per millimeter of colonized root.

Different physical and chemical soil characteristics and vegetations types are present along the topographic gradient. If different plant species which grow along the gradient present a similar seasonal dynamic with respect to EP, AC, and VC at all the studied sites; it can be suggested that each mycorrhizal fungal community is adapted to the soil conditions at each site and respond seasonally associated with the stages of plant growth, but not necessarily different soil characteristics may modify the pattern of colonization in roots.

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Abstract, Workshop held at Chascomus, 17-19 November 2008

Evaluation of rhizobia immobilized in silicate matrices as an alternative inoculant formulation.

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Symbiotic nitrogen fixation results from the interaction of a legume host with rhizobia, leading to root nodulation of the host plant. One of the main problems of the inoculation industry is to keep rhizobial cells viable in large numbers in the inoculants. Furthermore, soil is a heterogeneous, unpredictable environment, where the inoculated bacteria find it often difficult to survive among the competitors, resulting in a progressive decline in the bacterial density. Immobilization of microbial cells into polymer matrices has proved to be advantageous over direct soil inoculation. A new inoculant formulation consisting of *Mesorhizobium loti* immobilized in a highly porous silicate matrix is proposed. Bacteria were immobilized using sodium silicate as the sol precursor and citric acid as the polimerization catalyst. The results obtained demonstrate the long time preservation of entrapped cells, at room temperature, for periods exceeding 10 months and the ability of rhizobia to effectively nodulate roots once they are freed from the polymer, confirming their presence in the nodules by means of PCR. Their viability at different pHs, from 3 to 7, and their survival in sterile soil were also evaluated showing better results than liquid inoculants. Further studies are being performed to use rhizobia immobilized in silicate gels as alternative inoculant formulations in real field conditions.

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Abstract, Workshop held at Chascomus, 17-19 November 2008

Lotus/Rhizobium symbiosis in contaminated soils. Importance and use for bioremediation

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The identification and characterization of contaminated areas are increasing all over the world and the pollution due to heavy metals and to arsenic had received a great attention within the last years. The main sources of heavy metals pollution are mining, industries and application of metal-containing pesticides, fertilizers and sewage sludge. Heavy metals such as Zn, Cu, Ni and Cr are essential or beneficial micronutrients for plants, animals and microorganisms, whereas others such as Cd, Hg and Pb have no known biological and/or physiological functions, being toxic even in small concentrations (Castro and Ferreira, 2006).

In polluted soils biodiversity of plants could decrease drastically and in some contaminated areas, the legume family is one of the most represented. Legumes and their associated rhizobial bacteria are important components of the biogeochemical cycles in agriculture and natural ecosystems. The fixation of atmospheric N_2 by the legume-*Rhizobium* symbiosis is a central element of the N-cycle. Also in polluted soils heavy metals have an important impact on diversity of the resident microflora, which seems to be much less variable, being some microorganisms more sensitive to heavy metals than plants growing on the same soil (Pereira *et al*., 2007).

Compared to other legumes, *Lotus* species have a higher potential for adaptability to abiotic stresses, surviving often in rather extreme conditions.

For this work we use native and apparently well adapted to heavy metals and arsenic leguminous plants of *Lotus uliginosus* Sch. (=*L. pedunculatus* Cav.) growing in soils particularly affected by the release of liquid effluents from fertilizer and chemical industries for nearly 50 years. The objective of this research was to study rhizobial population isolated from these plants in reference to genetic diversity, efficiency to fix nitrogen and host specificity nodulation and also to assess the levels of tolerance to heavy metals and As. The presence of heavy metals resistance genes was also evaluated.

The results obtained showed that *Rhizobium* isolates from polluted soil are diverse when analyzed by ERIC- PCR. It was also demonstrated the presence of an effective rhizobial population for *L. uliginosus* in these polluted soils. The experiments used to study arsenic tolerance showed the existence of few numbers of isolates which tolerate the highest

concentration tested (i.e. 20 mg ml^{-1}). The search for arsenic resistance genes enable us to admit that some *Rhizobium* isolates from contaminated soils have the genetic information that allows them to survive in these harsh conditions (Sá-Pereira *et al*., 2007).

The main impact of this study is the possible use of autochthones legume plants and their microsymbionts, such as the symbiosis *Bradyrhizobium sp./Lotus uliginosus*, for the bioremediation of contaminated soils helping his fertilization, for providing N to soils.

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Abstract, Workshop held at Chascomus, 17-19 November 2008

Preliminary results of studies of symbionts of *Lotus* and their association with solubilising of phosphate bacteria

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INTRODUCTION. Nitrogen is the nutrient that mostly determines the growth and performance of the cultivations in the soils of the Region Pampeana, followed in importance by phosphorus. An option for the use of fertilizers would be the utilization of plant growth promoting bacteria (PGPB). The action of the PGPB is manifested in different mechanisms such as; control of deleterious organisms; atmospheric nitrogen fixation; and mechanisms for moving and solubilising minerals like phosphorus and sulphur and arrange them for the plant growth. The action of the PGPB microorganisms is determined by biotic and abiotic factors, in this case it is important the salinity, sodicity, and flood conditions.

OBJECTIVE. To study the development of new alternatives of application of FBN in soils with constraints such as; salinity, and/or salinity-sodicity, and with low available phosphorus content.

MATERIALS AND METHODS. ***Isolation of native rhizobia:*** It was carried out in those rhizophere soils corresponding to the region of the depressed Pampa of the Salado (soil 3: pH=7.35, soil 5: pH =7.05, soil 8: pH =7.87, soil 10: pH =9.50), by means of the trap plant method. Isolations were preserved in tubes with specific medium for the growth of *Mesorhizobium loti*. ***Isolation of phosphorus solubilizing bacteria:*** It was carried out of the rhizophere of *Melilotus* utilized in the recovery of flooded fields in the province of La Pampa. The visual detection and the estimation of the solubilising capacity of the bacteria were determined by observing translucent areas (halos) around those colonies grown in specific media for phosphorus solubilising bacteria. ***Choice of the isolations of native strains of rizobios to be utilized in the assay of effectiveness and infectivity:*** it was done by means of the electrophoresis of isoenzymes technique (M.L.E.E) revealing for α and β-esterases**.** ***Assay in climatized chamber:*** design totally random with 10 repetitions, in tubes (20x2,5cm), with Jensen media, and light control (16-8 hours) and temperature (25-19ºC). The assay finished when senescence could be seen in the treatment test (6 weeks). ***Treatments:*** Treatment test without inoculation and plus N; Inoculated with strain LL-32 alone and coinoculated with SP21 and Spp; Inoculated with strain S83 alone and coinoculated with SP21 and Spp; Inoculated with strain S38 alone and coinoculated with SP21 and Spp. ***Evaluated parameters:*** Dry weight; Nitrogen content. ***Statistical analysis:*** Analysis of variance-ANAVA; Comparison between treatments (Tukey)

Biotechnological parameters. Determination of growth velocity and generation time of the chosen rhizobia: strains were developed in liquid media. The cell concentration was

measured every 3 hours for a period of 51 hours, by means of the turbidimetric method at 600 nm, the values obtained were expressed as units of optical density (U.D.O).

Quantification of the phosphorus solubilised by the bacteria: by means of the Fiske technique and modified by Subbarow (colorimetric). The bacterial biomass was developed in liquid media. The concentration of solubilised phosphorus was established at different times, in two media, with pH control.

RESULTS AND DISCUSSIÓN. ***Isolation of native rhizobia.*** Eight isolations were obtained, Soil 3: S38, S35, S36, Soil 5: S54, Soil 8: S83, Soil 9: S95, S96, S92.

Choice of the isolation of native strains of rhizobia to be utilized in the effectiveness and infectivity assay. we chose to work with native strains S83 and S38 and with the strain pattern LL-32, because they present a greater α and β esterase activity and because they correspond to soils with different pH, pH=7.35 and pH=8.08.

Isolation of phosphorus solubilising bacteria. it was obtained an isolation that was designed as Spp.

Assay in climatized chamber with *Lotus tenuis (=L.glaber)*

Dry weight. There are significant differences between inoculated and coinoculated treatments with the treatment test without inoculation (T). There are no significant differences among the treatments S83, S38, TN. There are significant differences among S83, S38, and LL-32, being the dry weight values obtained in the treatment with LL-32 significantly lower than the dry weight values obtained in the treatments S38 and S83. The dry weight values obtained in all coinoculated treatments with strain pattern SP21 are significantly lower than the ones obtained by inoculating with native strain Spp.

Nitrogen content. Nitrogen content is greater in inoculated and coinoculated plants than in the treatment test without inoculation (T). The coinoculation with the phosphorus solubilising bacteria Spp increases the percentages in comparison with the treatments only inoculated with the native rhizobio. It can be observed the negative effect of the coinoculation with the solubilising strain pattern (SP21).

Growth of rizobios strains. Isolations present UDO values higher than 7, which would correspond to a cell concentration of 10^9 -10^{10} viable cells/ml, adequate values if we think of a future transfer.

Quantification of the property of solubilizing phosphorus. The bacterium Spp solubilises a greater quantity of phosphorus than the pattern one, this is improved if the culture media utilized is NBRIP. Bacteria reduce the pH of the media in the first hours of their growth, the mechanism utilized for solubilising phosphorus would be the release of acids.

Conclusions. The isolations of *Mezorhizobium loti* studied possess a high infectivity and effectivity in vitro, higher than the strain pattern. Regarding biotechnological parameters in the study of the isolations of native rizobios it is concluded that they are bacteria with a high aptitude for their possible technological transfer. The native phosphorus solubilising bacterium shows and efficient solubilising power both in the quantifications done in vitro compared to the strain pattern, and in the assay in acclimatized chamber, where it was

inoculated with the isolations of *Mezorhizobium loti*, showing in this case an increase of the parameters studied in comparison with simple inoculations.

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Characterization of symbiotic bacteria from *Lotus sp*. under saline stress: preliminary results for antioxidant system activity.

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The *Lotus sp.* interaction with their symbiotic bacteria is essential in the sustainability of systems under extreme conditions such as salinity and drought. Tolerance to these stress conditions is partially associated with the antioxidative system activation in both plants and microorganism.

The aim of this work was to determinate the effect of saline and osmotic stresses in the growth of free living symbionts from *Lotus sp. Mesorhizobium loti* LL12 and *Mesorhizobium tianshanense* GGS22 (halotolerant) and to correlate these results with two enzymatic activities of the antioxidative system: Superoxide dismutase (SOD) and Glutathione reductase (GR).

Bacteria were grown in YEM medium supplemented with 0, 50, 150, and 200 mM NaCI, or 0, 8.5, 15 and 18.5% PEG respectively to mimic the same osmotic pressures of saline assays.

Biomass production of free living *M. loti* LL12 cells was affected by osmolarity at saline and osmotic conditions, in a proportional way to the osmotic pressure increase. On the other hand, biomass production and growth of *M. tianshanense* GGS22 were seriously affected by osmolarity, while these parameters were not affected by the ion content in saline treatments.

In both strains, the presence of a unique Mn-SOD isoform was observed, probably a cambialistic Fe/Mn-SOD, detected in zimogram assay.

SOD activity was notably induced in *M. loti* LL12 in presence of 200 mM NaCl, whereas in the equivalent osmotic treatments, differences respect to controls, were not observed.

The *M. tianshanense* GGS22 SOD activity did not shown differences respect to control up to 150 mM NaCl, and it was slightly reduced at 200 mM NaCl, althought the enzyme activity was strongly induced in equivalent osmotic pressures (15% and 18,5% PEG).

Growth of free living bacterial strains, *M. loti* LL12 and *M. tianshanense* GGS22, was differentially affected by the stress type which they were exposed to. The *M. loti* LL12 biomass production was affected by osmolarity, but not for the medium ionic content. While *M. tianshanense* GGS22, previously characterized as halotolerant, shown a strong growth inhibition by PEG induced osmotic pressure, this strain could also maintain growth in NaCl presence probably by Na+ inclusion and H+ exclusion, obtaining lower pH values in the culture medium after growth.

The increase of SOD and GR activity from *M. loti* LL12 was partially correlated with the saline stress in the medium. Although SOD and GR activities from *M. tianshanense* GGS22 were induced mainly by PEG generated osmotic conditions, these conditions shown a low growth rate. On the contrary, saline stress did not induce an increase of these activities, in agreement with growth data, proving that saline conditions are not a stressful situation for the microorganism.

These preliminary results contributes to *Lotus sp.* symbiotic bacteria characterization within the Lotassa project which objective is the study of differential tolerance to NaCl conferred by SOD and GR overexpression in symbiotic bacteria during both symbiosis and free living state.

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Abstract, Workshop held at Chascomus, 17-19 November 2008

Tolerance to salinity and alkalinity of rhizobia isolates from the Salado River Basin: a sustainable alternative to improve the quality of the inoculants for *Lotus tenuis* in this region

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The Salado river Basin (SRB) is the most important area for cattle ranch production in Argentina. This activity is based on the use of natural pastures because the edaphic and climatic conditions restrict the implantation and persistence of"traditional legumes"in saline-alkaline lowlands (30% of the total area). *L. tenuis* is an exotic legume well adapted to these conditions, but in spite of its successful naturalization, the survival of this species during the seedling stage is limited and is a critical step for further implantation (Miñón et al., 1990). With the aim to improve seedling survival and forage quality of this legume, the genetic diversity of native rizobia that stablish symbiosis with *L. tenuis* was evaluated, for further selection of isolates with a better symbiotic efficiency than commercial inoculants in saline soils. A high genetic diversity was found among the rizobia of the SRB (Estrella et al., 2007). The symbiotic performance of one native isolate was superior to that strains recommended for commercial formulations of inoculants tested under control and salt stress conditions. RFLP analysis of 16S rDNA genes of native isolates with better symbiotic performance revealed that the taxonomy of *Lotus tenuis* symbionts in not only restricted to *Mesorhizobium loti* species, the type specie to *L. tenuis* and *L. corniculatus* (Saeki and Kouchi. 2000), and can involve others species and genus. It can be concluded that populations of rizobia in soils of the SRB are a source of genetic and symbiotic variability that can be used to obtain high quality inoculants for *L. tenuis*.

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Abstract, Workshop held at Chascomus, 17-19 November 2008

Biochemical and molecular characterization of phosphate solubilizing bacteria and evaluation of its efficiency promoting the growth of *Lotus tenuis*

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Halophyte communities of saline-alkaline lowlands represent a variable proportion of breeding establishments, arriving sometimes to surpass 30% of its surface. It is therefore a clear need to increase the production capacity of these environments through the incorporation (adaptation and distribution) of leguminous species such as gender *Lotus*, by its condition of fixing nitrogen and its high nutritious value for ruminant (Quinos *et al*., 1998).

The improvement of soil fertility is one of the most common strategies to increase agricultural production. Maintaining high levels of available nitrogen (N) and phosphorus (P), the two most limiting nutrients in soil, remains being a challenge.

Major researches on biofertilizers have concentrated on understanding and improving N_2 fixation. However, it is known that every aspect of the process of nodule formation is limited by the availability of P. Legumes like alfalfa and clover show a high positive response to P supplementation (Gyaneshwar *et al*., 2002), but most of the supplemented P become unavailable when its reacts whith soil components.

Many soil microorganisms are able to solubilize this unavailable P through their metabolic activities exudating organic acids, which directly dissolve the rock phosphate, or chelating calcium ions that release P to the solution. Production of microbial metabolites results in a decrease in soil pH, which probably plays an important role in the solubilization (Abd-Alla, 1994).

The discovery of mutual relationship between plants and phosphate solubilizing bacteria (PSB), in which bacteria provide soluble phosphate and plants supply rootborne carbon compounds (mainly sugars), that can be metabolized for bacterial growth; encouraged the development of new technologies, such as the use of PSB for biofertilization to improve crop yield (Pérez *et al*., 2007) (Goldstein, 1995).

Our working hypothesis suggests that the use of phosphate solubilizing bacteria in saline-alkaline soils would increase the level of available phosphorus, contributing substantially to improve the implantation and development of *Lotus tenuis* in the region.

To test this hypothesis, activities undertaken were as follows:

Isolation and characterization of phosphate solubilizing bacteria. Samples of saline-alkaline soils were collected from the rizosphere of *Lotus tenuis* plants growing at fields close to IIB-INTECh and Estación Experimental de Manantiales (Latitude 35º 30' S. Longitude 58º 30' W). Isolations were made in NBRIP medium that contains $Ca_3(PO_4)_2$ as the sole P source and which allows the identification of PSB by the formation of a halo of solubilization in the culture medium (Nautiyal C. S.; 1999).

Determination of solubilized phosphate concentration. The concentration of solubilized phosphate was determined at different times (0, 24, 48 and 72 h.), allowing to observe the kinetics of solubilization of each isolate (Fiske C.H. & Subbarow Y.; 1925). Similarly the medium pH value was determined to try to establish a relationship between this parameter and the soluble phosphorus.

Based on the results, the isolates were classified into 3 groups:

	Activity		
	Low	**Intermediate**	**High**
Isolates	I26, I29, I35, I38, M22, M52	I17, M51, M56, M87	M25, M75, M76, M77, M78, M89, M91

Genetic diversity and molecular taxonomic identification of PSB. BOX-PCR fingerprinting using a BOX A1R primer (5'-CTA CGG CAA GGC GAC GAC GCT G-3'), was performed to assess the genetic diversity of the isolates, identify strains with different BOX profiles and dismiss those resulting redundant (Versalovic *et al.,* 1991). Then the taxonomic identification of strains with different BOX profiles was carried out through the amplification and subsequent sequencing of the gene coding for the ARNr 16s (Herrera-Cervera *et al*., 1999).

In vitro evaluation of Plant Growth Promoting Rhizobacteria (PGPR) activity PSB M91, one of the isolates that showed high and reproducible phosphate solubilization activity was selected and inoculated onto *L. tenuis* seedlings grown in semisolid Evans medium (Evans *et al.*, 1970). The soluble phosphate source was replaced by $Ca_3(PO_4)_2$ and several P/N ratios were used, in order to simulate different growth-limiting conditions.

Dry weight and total phosphorus content in shoots were analyzed (Murphy J. and Riley JP.; 1962). The data was subjected to two-way analysis of variance (ANOVA) (P: 0.05).

Experiment 1: Inoculation with M91 varying P/N ratio in growth medium at pH 7. Inoculation with PSB isolate M91 significantly enhanced growth of *L. tenuis* plants, as compared with non-inoculated controls. Growth of plants inoculated with PSB isolate M91 was increased by high N levels (10 ppm) in the growth medium. Shoot P content (mg P g^{-1} dry weight) was similar for all treatments.

Experiment 2: Inoculation with M91 at three levels of pH (7, 8, 9) (10 ppm N). The PGPR activity of PSB isolate M91 was not affected by a pH shift from 7 to 8. On the contrary, a

further pH increase from 8 to 9 significantly reduced the PGPR activity of this isolate.

Experiment 3: Inoculation with M91 varying P/N ratio in growth medium at pH 8. As occurred at pH 7, growth of plants inoculated with PSB isolate M91 was increased by high N levels (100% N) when the pH of the growth medium was adjusted to 8.

Conclusions

Seventeen phosphate solubilizing bacteria were isolated, identified and characterized. Most of the bacteria were isolated from soil samples with pH values close to 8. 16s ARNr sequence analysis showed a high level of identity between the isolates and bacteria from genera *Pseudomonas, Erwinia, Pantoea* and *Rhizobium*, previously reported as phosphate solubilizing bacteria. There is a close relationship between the phosphate solubilizing activity and low pH levels in the growth medium. This suggests that phosphate solubilization could be the result of organic acids released from bacterial metabolism, as reported in literature. Results from assays at pH 7 and 8 clearly demonstrate that inoculation with PSB isolate M91 enhances the growth of *Lotus tenuis*. The plant growth promoting effect was also dependent on the N content in the nutrient solution.

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Abstract, Workshop held at Chascomus, 17-19 November 2008

Phenotypic plasticity in relation to *Lotus tenuis* response to saline stress: the role of arbuscular mycorhizal and rhizobial symbionts

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We have tested the hypothesis that *Lotus glaber* displays a plastic root phenotypic response to soil salinity that may be influenced by mycorrhizal and rhizobial microorganisms. Uninoculated plants and plants colonised by *Glomus intraradices* or *Mesorhizobium loti* were exposed to either 150 or 0 mM NaCl. General plant growth and root architectural parameters (morphology and topology) were measured and phenotypic plasticity determined at the end of the salt treatment period. Two genotypes differing in their salt tolerancecapacity were used in this study. We analized root morphology, the root external and internal link lengths and the topological trend (TT). Notwithstanding the absence of a link between TTs and variations in plant growth, it is possible to predict a dissimilar adaptation of plants with different TTs. Root colonisation by either symbiotic microorganism reduced the level of root phenotypic plasticity in the sensitive plant genotype. We conclude that root plasticity could be part of the general mechanism of *L. glaber* salt tolerance only in the case of non-symbiotic plants.

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The constitutive expression of ACC deaminase in *Mesorhizobium loti* promotes nodulation on *Lotus glaber* under stress conditions

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The biosynthesis of phytohormone ethylene is accelerated in association with environmental and biological stresses. Ethylene inhibits the synthesis between rhizobia and legumes. Several rhizobia species have developed mechanisms to reduce the ethylene levels in their host legume. *Meshorhizobium loti* has the *acdS* gene which codifies for an enzyme with ACC deaminase activity. This enzyme converts the ethylene synthesis precursor, 1-aminocyclopropane-1-carboxylate (ACC), into α-ketobutyrate and ammonia. This bacterium expresses this gene only in the bacteroid state, inside the nodules. Our hypothesis is that the constitutive expression of this gene in the vegetative state will promote higher nodulation efficiency in the stress conditions to which bacteria are subjected in the soil. To test this, we integrated in the *M. loti* MAFF303099 chromosome an *acdS* copy under the activity of a constitutive promoter and we analyzed its nodulation efficiency under different stress conditions. *M. loti* expressing *acdS* constitutively resulted more competitive than the wild type strain on *Lotus glaber* that had been grown for 52 days without a nitrogen source before inoculation. On the other hand, the transconjugant strain showed a tendency to induce higher number of nodules than the wild type strain when inoculation was made on plants subjected to stress by illumination.

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Lotus corniculatus: a model species to dissect the genetic control of proanthocyanidin biosynthesis in vegetative tissues of forage legumes

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Proanthocyanidins (PAs), also known as condensed tannins, are oligomeric flavonoids generated by the sequential addition of leucoanthocyanidin, catechin or epicatechin extension units to the starter epicatechin or catechin units (Dixon *et al.*, 2005). PAs occur in a wide range of plants where play important role in defense against herbivores and pathogens and have important industrial applications. As food components, they act as antioxidants with beneficial effects for human and animal health (Marles *et al*., 2003). In forage legumes their presence prevents bloating and increases protein assimilation (Tanner, 2004). However, the major forage legumes and the model species Arabidopsis and *Medicago truncatula* accumulate these products in seed coat but not in leaves. Therefore, the advantage of inducing the PA biosynthesis in leaves of forage legumes is self evident.

The regulatory machine controlling PA biosynthesis comprises a ternary transcriptional complex consisting of WD40, MYB and bHLH proteins (Lepiniec *et al*., 2006). In order to modulate the tissue-specificity and the level of accumulation of PAs in legumes experiments of transgenesis using both regulatory and structural genes are in progress worldwide. To this purpose, the genus *Lotus* offers a model system in that it includes species that accumulate these compounds only in reproductive tissues or in both vegetative and reproductive ones. We have previously shown that as result of transformation with the maize *bHLH* transcription factor *Sn Lotus corniculatus* transgenic lines with enhanced or depleted leaf PA levels are produced (Robbins *et al.*, 2003) and that the expression/silencing of *Sn* tightly correlates with the expression of key structural genes of the pathway, such as *DFR, ANS, ANR, LAR1* but not *LAR2* (Paolocci *et al.*, 2005; Paolocci *et al.*, 2007). Conversely, the sole expression of *Sn* is ineffective in inducing PA accumulation when overexpressed in leaves of PA negative model and crop species, suggesting that the expression of MYB partners acting either as positive or negative regulators, rather than those of bHLH or their WD40 interacting proteins, de facto controls the PA synthesis in leaves of a number of species.

Here we report that the overexpression of *FaMYB1*, a strawberry gene that suppresses anthocyanin and flavonol accumulation in transgenic tobacco (Aharoni *et al.*, 2001), is sufficient to specifically suppress PA accumulation in leaves of a *L. corniculatus* genotype accumulating high amount of these compounds. *FaMYB1* reduces the transcript levels of all the structural PA-specific genes, as per real time RT-PCR analysis. The observation of sectorial patterns of PA accumulation in leaves of some transgenic lines indicates that

instable interactions among transcription factors may occur.

Present data goes along with recent findings showing that in Arabidopsis a *MYB* repressor gene of flavonoids triggers the downregulation of PAs by competing with MYB activators for binding to the bHLH/WD40 complex (Dubos *et al.*, 2008; Matsui *et al.*, 2008). All together, this evidence strongly supports the idea that in forage legumes the ability of leaf tissues to accumulate PAs depends on the competition between, and relative expression of, endogenous *MYB* activator and repressor genes.

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Seed yield components in F2 genotypes of interspecific hybrids of *Lotus uliginosus* and *Lotus japonicus*

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The compatibility among species of *Lotus* and the rhizobia that are able to nodulate them determine the efficiency of the biological fixation of nitrogen. The plants of *L. corniculatus* L. and *L. glaber* Mill. fix nitrogen with bacteria of the genus *Mesorhizobium*, while *L. subbiflorus* Lag and *L. uliginosus* (Schkuhr) form effective nodules with *Bradyrhizobium* and ineffective with *Mesorhizobium*. The genetic and metabolic mechanisms that are involved in the incompatibility of of plant-bacteria nitrogen fixation are unknown, which could be studied by means of the hybridization of two species of different symbiotic group (*L. uliginosus* x *L. japonicus* Regel (Larsen) ecotipo Gifu). *L. uliginosus* is a perennial diploid alogamous species, with 6 to 15 flowers per umbel and vegetative multiplication by rhizomes, whereas *L. japonicus* is a diploid autogamous species with 1 to 3 flowers per umbel and without vegetative reproduction. Therefore, this hybrid will also allow to analyze the possibility to combine morphological characteristic of these species, such as the natural self-pollination and the presence of rhizomes. The hybridization was carried out without emasculation, previous verification of self-incompatibility of *L. uliginosus* maternal plants. In vitro multiplication of the F1 embryos was carried out 15-21 days post-pollination because the hybrid seed aborted before reaching maturity. 170 F1 embryos were able to develop on agar and reach to adult plant, of which 3 fertile plants were identified (6-4, 6-6 and 6-81). 1800 F2 individuals were achieved from the cross of these fertile plants through a new phase of embryo rescue. The fertility of this F2 population was evaluated by its seed production as autogamous (natural self-pollination), self-pollination (forced self-pollination) and cross-pollination (polycrosses between genotypes of the same genetic origin). Umbels labeling at the beginning of blooming allowed to identify at least 30 F2 genotypes that behave as strict autogamous in the absence of pollination insects (the characteristic of the father *L. japonicus*). These genotypes are in very low frequency in different crosses (6-4 x 6-6; 6-4 x 6-81; 6-6 x 6-81; 6-6 x 6-4; 6-81 x 6-6), and their fertility was disparate. The largest number of plants identified as autogamous, including some of the most fertile ones, came from the cross of 6-81 x 6-6. Paintbrush manual pollination with pollen of the same genotype (forced self-pollination) was carried out in all F2 genotypes. Most plants recovered fertility with crossed pollination (pollen of other genotypes from the same hybrid), while 428 plants produced seed with forced self-pollination. Within the latter group of plants, 107 flowers/plant have been pollinated on average, of which 21.6 pods have been obtained, that represented a low level of fertility (25.5%). The pods harvested had 147

viable seeds and 41 immature seeds, representing 25% aborted seeds. The number of pollinated flowers, as well as the components of seed yield presented a high variability inside each cross, even though average fertility were similar for cross 6-4 x 6-6 and reciprocate cross (6-6 x 6-4), 6-4 x 6-81 and 6-6 x 6-81. Crosses of F1 genotype 6-81 with pollen of genotypes 6-4 or 6-6 produced very scarce number of embryos and no fertile plant was obtained by embryo rescue. Cross 6-4 x 6-6 presented more abundant blossom, that allowed to reach 126 pollinated flowers /plant on average, although it was recorded high variability, with a range from 10 to 298 flowers/plant. Crosses between 6-6 x 6-4 and 6-6 x 6-81 presented more morphological similarities with *L. uliginosus*, with 64% of plants with presence of rhizomes. On the other hand, cross of 6-4 x 6-6 had 56% of plants with absence of rhizomes, and the proportion increases to 85% in the cross 6-4 x 6-81 whose descendant had larger similarities with *L. japonicus*. It will be necessary to evaluate the next generation fertility to determine the stability of reproductive parameters, especially in the descendant of different F2 genotypes. Research financed by the LOTASSA project (www.lotassa.org).

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Chromosome number, seedlings and seed size in *Lotus tenuis* and *L. corniculatus*

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Lotus tenuis Waldst & Kit. and *L. corniculatus* L. are forage legumes, phenotipically similar, extensively accepted and utilized as spontaneous species in grasslands or sown in pastures *consortium*. The seeds of these species do not exhibit macromorphological characteristics that allow us to differentiate them and they have been cited as very similar in size. They cannot be identified early considering morphological characteristics of the seedlings, although differences in relation with the pubescence of them have been reported.

The purpose of our studies is to answer the need to differentiate these two species in the state of seed or during the germination utilizing methodologies that turn out to be sufficiently fast, reliable, economic and reproducible. For it, chromosomal studies in native cultivars and populations were carried out. Due to the lack of data with respect to the seed size and to the seedling pubescence and in order to know if these characters resulted to be differential among both *Lotus´*s species, they were screened on the same samples chromosomally analyzed.

Seeds from cultivars registered at National Institute of Seeds (INASE) and samples of populations sent to test at laboratories belonging to Asociación de Laboratorios Agropecuarios Privados (ALAP) were analyzed. The analysis of the mitotic chromosomes was carried out from root apexes obtained from seeds in germination. In order to estimate the seed's size 50 seeds taken at random of each origin were studied. The lengths of the longest and minor axes of the seeds were obtained with an eye micrometric incorporated into a magnifying glass triocular and an increase of 40x.. The measurements were expressed as the mean $\pm$ standard deviation. The seedling pubescence was observed when seedlings reached a length of 2 mm.

The studied cultivars turned out to be almost homogeneous in relation with the chromosomal number: those of *L. tenuis*, 2n = 2x = 12 and those of *L. corniculatus*, 2n = 4x = 24, although some few individuals with different somatic numbers were found (between 0.7 and 4%). On the other hand, the samples analysis provided by private laboratories revealed that species or mixes of seeds are distributed under different denominations. Sometimes, *L. corniculatus* (with lower commercial value) is sold as *L. tenuis*.

Data of seed's size of several cultivars of *L. corniculatus* and of *L. tenuis* showed

superimposed values, and for this reason it would not be a reliable characteristic to differentiate them, especially in samples of seed containing both species. Epicotyls and leaflets of the first leaf of *L. tenuis* seedling have been cited as glabrous and those of *L. corniculatus*, with white hairs. Nevertheless, only some cultivars as Tresur Chajá and Esmeralda of *L. tenuis*, and Gladiator of *L. corniculatus* presented between 98 and 100% with the characteristic expected.

The variability found in relation with the seedling pubescence and the dispersion of seed's size values show that these two characters are not useful to differentiate both species, although some cultivars show homogeneity in relation with seedlings pubescence. On the other hand, the chromosomal analysis allows fast, reliable and reproducible results for the specific differentiation between *L. tenuis* and *L. corniculatus*. The used cytogenetic techniques could be implemented in private laboratories with limited equipment and a relative low cost.

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Program of improvement in *Lotus corniculatus* L.: Base Germplasm characterisation

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Lotus corniculatus L. is a species that expresses many different agronomic adaptations compared with other temperate forage legumes. These adaptations include: tolerances to water-logging, drought, low levels of P, acid soils and to high levels of soil Al and Mn (Grant and Marten, 1985). In spite of this wide range of adaptations, it is very difficult to put an economic value on the species, due to the contribution given by naturalized *Lotus* material already existing in pastures. In general, the agronomic importance of Lotus has been decreasing due to the intensification of agriculture, although at present it is recovering in importance in some regions.

Even though the use of *Lotus corniculatus* is limited, the adaptative characteristics that it exhibits make it an important species to initiate an improvement programme. In 1997, AgResearch - Gentos S.A. began a joint programme aimed at developing *Lotus corniculatus* adapted to the conditions of the region and under cattle grazing.

The program was developed in two phases. Phase 1: Introduction and evaluation of material with a wide genetic base to ensure sufficient variability for the desired traits. Phase 2: Selection of the best genotypes to develop cultivars with high agronomic and forage quality potential.

In this study, we present the results of the first phase of the program. The initial evaluation consisted of 114 *Lotus corniculatus* accessions obtained from the Margot Forde Germplasm Center at Palmerston North, New Zealand. The evaluation trial was carried out at the "La Lucila " Farm, Pergamino, Argentina. The trial used a randomized complete block design with 5 replications. The trial was planted during the autumn of 1998, following establishment of accessions in trays in a green-house then transplanting to the field. The transplants were inserted in the space between furrows of tall fescue sown 3 months earlier. Every accession was represented by two rows of 10 plants, spaced 20 cm between plants in each of the replications.

During the period 1998-2000, the genetic material was characterized by visual measurements of the following traits: initial vigor (1 low - 5 high), habit of growth (1 prostrate - 5 erect), stem density (1 low - 5 high), and seasonal forage growth throughout the year (1 low – 9 high). After every measurement the plants were grazed by cattle. Intensity of

flowering was determined by a visual score (0 without flowers - 5 full flowering). The persistence of the materials was determined as percentage of surviving plants in each accession after 3 years.

The accessions used were collections of ecotypes and cultivars from different origins: South American cultivars (Group1), South American ecotypes from Argentina, Brazil, Uruguay and Chile (Group 2), North American (Group3), Mediterranean (Spain, Portugal, Italy, Israel, Greece, Ethiopia and Canary Isles) (Group 4), Central Europe (France, Yugoslavia, Hungary, Czechoslovakia) (Group 5), North European (Switzerland, Denmark, Ukraine and Russia) (Group 6) and New Zealand (Group 7).

The results showed high variation both between and among the different groups of origins for all characters. In a multivariate analysis using principal components, there was no clear separation between the groups, due to the high level of variability inside the groups. However, it is possible to observe the clustering of the materials into three main groups: the first was defined by very quick establishment with average herbage yield, and was composed by materials of Group 1. The second were slow establishers with low yield, and was composed by materials of Group 4. The third contained material of intermediate flowering and medium to high herbage yield and was composed by materials from most of the other groups.

The Group 1 material consists of the local South American cultivars and are characterized by rapid establishment vigor, good winter herbage production, erect growth habit and low numbers of stems. The Group 4 material was from the Mediterranean and had low winter vigor but average summer productivity, semi-erect growth habit and high numbers of stems. The remaining groups from North America, Central and Northern Europe, showed good production during the summer and autumn, but with slow establishment vigour, semi-erect growth habit and high stem numbers. The Group 7 material from New Zealand had good productivity throughout the whole year but particularly in summer and autumn, with a semi-prostrate habit and the highest numbers of stems compared to all groups. In general, the agronomic characterization of the different groups evaluated in Argentina was similar to the results described in New Zealand (Widdup *et al.*, 1997).

The South American cultivars making up the control Group 1 display the desirable traits of rapid establishment, good winter growth and high seed yield. However, overseas material from Central Europe and New Zealand show very good growth in summer and autumn, have high numbers of stems and more prostrate growth habit which was a factor in the improved persistence shown by these overseas material by the third year. The major weakness of the Europe and New Zealand material was slow establishment and late flowering resulting in lower seed yields.

Program of improvement

In Phase 2 following the characterization step, surviving plants with good agronomic features were selected and isolated into two polycrosses – the rapid establishing South American material in one polycross and the more summer-active, densely stemmed Europe and New Zealand material in the second polycross. Seed harvested from individual plants in the polycrosses were used in a progeny test to allow further selection of elite material to form

new cultivars.

In addition, the progeny material was also analyzed for the concentration of tannin in the leaves. Tannin helps reduce bloat in cattle and also improves the nitrogen metabolism in cattle. The objective has been to select material which displayed both good agronomic features and high tannin concentration in the leaves.

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Differentiation between *Lotus tenuis* and *Lotus corniculatus* as assessed by staining of leaf tannic cells

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The *Lotus* genus covers almost 200 species that are worldwide distributed as a consequence of their adaptability to diverse environments. Many *Lotus* species are important from an agronomical point of view, due to their high forage value and their use in reclamation of marginal areas. As an example, *L. tenuis* is cultivated in Salado River Basin (Buenos Aires, Argentina), where plant communities are exposed to flooding, soil salinity and alkalinity, and phosphorous deficiency, conditions that lead to the lack of native legumes and also limit the implantation of traditional ones such as lucerne and trefoil (Mazzanti *et al*., 1986; Maiola *et al*., 2003). *L. tenuis* has colonized soils in this region, thus increasing the forage value of natural grasslands and improving the edaphic conditions, as a result of biological nitrogen fixation. In addition to *L. tenuis*, *L. corniculatus* is also present in this region and shows a higher forage value, but its distribution is limited to areas less restrictive than those colonized by the former.

L. tenuis is a diploid species (2n=2x=12), morphologically similar to *L. corniculatus*, which is tetraploid (2n=4x=24). As a consequence of this morphological similarity, *L. tenuis* seeds of commercial cultivars are usually contaminated with *L. corniculatus* seeds, which diminishes the implantation success of *L. tenuis* varieties in marginal areas. Thus, techniques that allow differentiating both species at the seed or seedling stage are an important tool for the evaluation of seed contamination. Even though several methodologies have been developed for this purpose (Kade *et al*., 1997; Giorgieli *et al*., 1998; Galussi *et al*., 2006; Celotto and Sanso, 2007), they require sophisticated laboratories and specialized personnel, and are relatively expensive. Li *et al.* (1996) reported the existence of variability in the location of tannic cells in leaves of 22 legume species, including a few accessions of *L. tenuis* and *L. corniculatus*.

The aim of this work was to evaluate if the distribution of tannic cells in leaves can be used as a criterion to differentiate the two *Lotus* species previously mentioned. For this purpose, 12 *L. tenuis* populations (5 commercial varieties, 4 accessions from the germplasm collection of the *Estación Experimental Agropecuaria Pergamino*, 2 autotetraploid

populations and a population native from the Ebro River Delta in Spain) were used. In addition, 4 commercial *L. corniculatus* varieties and a population native from the surroundings of a coastal lagoon in the Mediterranean Sea (Valencia, Spain) were also used. Seeds were scarified in sulfuric acid, sown in water agar (0.8 %), and later transplanted to pots filled with peat. When plantlets reached the two-leaf state, the central leaflet of the second leaf and the root tip were withdrawn. Plantlets were kept growing in the pots for further morphological characterization. Root tips were treated with 8-hidroxiquinoline and fixed in ethanol/acetic acid (3:1). Material thus fixed was treated with cellulase (2%) and pectinase (0.5%) and hydrolyzed with 1N hydrochloric acid for 10 minutes at 60°C. Samples were then stained with propionic hematoxilin, and observed under a microscope in order to determine their chromosome numbers. Leaflets were bleached and stained according to Li *et al*. (1996), and soluble and non-soluble condensed tannin contents were determined in leaves of 4 randomly selected plants within each population. A high percentage of *L. tenuis* plants were found to be diploid (2n=12), but commercial varieties Esmeralda and Larrañaga were contaminated with tetraploid plants. Autotetraploid populations were found to contain no plants with chromosome numbers other than 2n=24. Regarding *L. corniculatus* populations, all plants were found to be tetraploid, excepting for the population native from Valencia, which contained diploid plants only. Stained leaflets could be differentiated into two groups, according to the presence or absence of tannic cells in the mesophyll. In this sense, all plants within *L. tenuis* (excepting for the contaminations found in cultivars Esmeralda and Larrañaga) were found to have no tannic cells in mesophyll cells, as opposed to leaves of all the *L. corniculatus* plants analyzed, which did contain tannic cells. Average soluble, non-soluble and total CT contents in *L. tenuis* leaves were 0.047 ± 0.038; 0.490 ± 0.223 y 0.537 ± 0.243 mg CE/g DW, respectively. Significant differences in CT content between different *L. tenuis* populations were not evident, while average soluble, non-soluble and total CT contents in *L. corniculatus* leaves were 4.102 ± 2.116; 6.028 ± 2.874 y 10.131 ± 4.584 mg CE/g DW, respectively. The *L. corniculatus* population native from Valencia, as well as cultivar San Gabriel, showed the highest leaf CT content out of all the plant materials evaluated.

Currently, a caryological technique for the determination of chromosome number is the methodolgy most frequently used for the differentiation between *L. corniculatus* and *L. tenuis*, although the occurrence of either tetraploid *L. tenuis* or diploid *L. corniculatus* plants can lead to erroneous conclusions. In addition, the caryological technique is not very practical for routine and large-scale use. As a consequence, staining of tannic cells in the first leaves is a reliable approach for differentiating both species, being a simple and low-cost method that could therefore be routinely used.

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Forage and seed production of naturalized germplasm of *Lotus corniculatus* L. multiplied by farmers

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Lotus cornicualtus L. (lotus) is a forage perennial legume of great importance because of its large distribution in Uruguay, with the consequent rising seed demand. Nevertheless its adaptation, among the restriction of the species is the slow seedling growth that hinders the establishment and the limited persistence. In spite of being a great seed producer, it is the forage legume with the largest problems at the moment of seed harvest due to pod shattering that is favoured by summer climatic conditions. Lotus lack of persistence affects seed and forage yields from the second year onward. Landvarieties are important sources of genetic variation for pasture improvement. Natural selection, as well as introgression of local populations, gave origin to the development of farmers' Creole varieties with consequent increases in adaptation, as well as intraspecific variability (Rebuffo *et al.*, 2005). The specific conditions of growth in each rural establishment can condition seed yield and its components which could generate specific genetic characteristics of the farmers' seed harvests. The present study, carried out in the frame of Project LESIS (Legumes for Sustainable Systems, FTG-787/2005), characterizes the genetic diversity of Creole varieties in Uruguay in relation to their potential of seed and forage production in the climatic and soil conditions of the Southern region of Uruguay.

In July 2006 were sowed at INIA La Estanzuela 100 accessions from farmers that produced their own seed and 4 cultivars (San Gabriel, INIA Draco, Estanzuela Ganador and LE 212), with a seed density of 12 kg/ha in micro plots (0,68 m x 2 m) with direct drilling. The experimental design utilized was incomplete random blocks (10 plots x block) with 2 replicates, analyzed by REML. Evaluations included biomass production, seed production and yield components at 4 occasions (January 23 2007; March 12 2007; January 4 2008; March 10 2008). Plot experiments (5 x 1.02 m) with 50 accessions in incomplete random blocks (10 plots x block) and 4 replicates were established to evaluate forage production. Evaluations consisted on total production of fresh and dry biomass, legume content (area evaluated 2.65 m^2) with their corresponding samples for dry matter, in 6 occasions (November 3 2006, February 8, April 10, August 28 and October 25 2007 and January 21 2008). The production of the accessions was compared with San Gabriel, the public variety mostly used in Uruguay. The experiments will be repeated 3 consecutive years, and the results of establishment in 2006 are presented in this report.

The establishment of the experiments was uniform since sowing densities were corrected by

germination. Results of the two more contrasting seed harvests, that correspond to January 23 2007 (1st crop) and March 10 2008 (4th crop), are presented in this paper, although there were 4 seed harvests. Average seed yield for the 1st harvest was 348,3 kg/ha, similar to yields obtained by the best seed farmers, even though exceptionally superior to the national average (120-150 kg/ha; García *et al*., 1991). Although the climatic conditions were very favourable for lotus seed production, the yield could be overestimated due to the size of the micro plots. In contrast, the 4th crop yielded 82,72 kg/ha. The limited persistence of most of the accessions explains the second year-old low yields. A very wide range of yields was observed in all the harvests. The accessions with the highest seed production in the harvest of January 2007 were FTG 9, FTG 22, FTG 48, FTG 53, FTG76, which yielded 90% more ($P < 0,05$) that San Gabriel (295,7 kg/ha). Production range varied from 5,07 to 192,35 kg/ha for the harvest of March 2008. Yields four times superiors to San Gabriel ($P < 0,05$) were registered for 5 accessions (FTG24, FTG 25, FTG32, FTG 46, FTG 63), that reached an average yield of 182,35 kg/ha, superior to the national average (García *et al*., 1991). Although it was recorded a great variability for seed production, the accession rank was not the same one in these harvests, since the accessions with high initial productions (January 2007) are not the same ones that present high yields in the 4th crop (March 2008). The low yield in the second summer is consequence of the water deficit that took place in this period as well as the lack of persistency of San Gabriel and some accessions.

Most of the forage evaluations registered significant differences between the accessions and San Gabriel, with the exception of February 2007 and January 2008. In the evaluations of November 2006, April and August 2007 no material rise above San Gabriel; inferior accessions were only observed in yield compared with check varieties. The establishment period was under extreme water deficit conditions. Under these conditions, only 3 accessions yielded 22,3% less forage that check varieties ($P < 0.005$; FTG 51, 132, 174). The forage production corresponding to April (2416 kg MS/ha) and August (2367 kgMS/ha) represented 16,7% less than cultivar San Gabriel. In second year Spring San Gabriel registered low persistence, therefore 28 accessions with more persistence raised above San Gabriel in 26,7% on average ($P < 0.005$). In contrast, accessions with lower persistence (FTG 14 and 23) yielded 29,4% less than the check. It was not possible to continue with the experiment evaluations after this date, consequence of the low stand of plants.

The characterization of this first experiment, allowed to identify materials with seed productions 4 times superiors to San Gabriel in each harvest, although the ranking of the accessions was not the same one along the time. The enhancement of seed production is an important characteristic because it could help to increase pasture persistence through natural reseeding, especially in the improvements of natural grasslands. The differences in production in the second year would be indicating a bigger persistence and the potential of these materials for use in plant breeding. Comparing seed production and forage yield of the accessions for both experiments, there was no direct relationship among the variables for this sowing year and climatic conditions, in spite of the identification in both harvests (January 5 2007 and March 10 2008) of accessions with low persistence (FTG 2, 3, 33, 34, 36) that would explain the low yields achieved. Results of the present research will be integrated with information from the 2007 and 2008 sowings to identify superior germplasm for their use in genetic improvement. The information obtained on seed production will be increased with damage evaluation of wasp and yield components to identify the specific

characteristics of the accessions more accurately.

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Seedling emergence and biomass production of *Lotus tenuis* sown at different densities in a grassland of the Flooding Pampa.

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Safe-sites, seed availability and physical and biological stresses occurring during seed germination and seedling establishment are critical for the species that spread by seeds. *Lotus tenuis* is a forage legume that has been naturalized in the Flooding Pampa grasslands and propagates only through seeds. This species constitutes a good alternative to improve the quality and productivity of these grasslands (Cauhépé, 2004). Seedling emergence and survival of *L. tenuis* in the grasslands depend on climatic and soil conditions of the plant community (Colabelli and Miñón, 1993; Miñón and Colabelli, 1993). The objective of the present work was to analyze under field conditions the seed density dependence for the establishment and aerial biomass production of *L. tenuis*. The study paddocks (3) were located in a private beef cattle breeding range of Ayacucho County (Buenos Aires, Argentina). Plant communities were representative of semi-natural saline-alcaline grasslands (Table 1) degraded by continuous uncontrolled cattle grazing. They were dominated by *Ambrosia tenuifolia*, *Cynodon dactylon*, *Stenostaphrum secundatum*, *Distichlis spicata*, *Carex* spp., *Juncus* spp., *Lolium multiflorum* , *Chaetotropis sp.*and *Stipa sp*., and *L. tenuis*, which was at very low plant density. Experimental treatments consisted in five seeding rates (D1: 0; D2: 0.557; D3: 2.23; D4: 8.95 and D5: 17.90 kg / ha; being 0, 57, 229, 917 and 1833 seed/m², respectively). In autumn 2004, scarified, inoculated (*Rizobium loti*, strain 733) *L. tenuis* seeds were surface-broadcast by hand on 3 replicate (plots of 1.5 x 1.5 m) by density. In each experimental paddock the plots were randomly assigned to each seeding density. Previous to sowing, the grassland was cut to 5 cm height in order to uniformize vegetation cover, and each paddock was wire fenced to exclude cattle grazing during the experimental period ending on April 2006. During the first year, seedling emergence was periodically recorded on each plot and aerial biomass was harvested on December 30, 2004; March 31, 2005; December 15, 2005 and April 25, 2006. ANOVA for repeated data in the time were used to compare ($P < 0.05$) plant density and biomass production among treatments. Maximum seedling density was recorded early in spring in all treatments and then decreased at the begging of summer (Table 2), coinciding with the low precipitations and drying soil conditions. Clorotic and dead seedlings were recorded during the experimental period. *Lotus tenuis* aerial biomass production varied significantly with seeding rate (Figure 1). Total biomass accumulated varied between 7,000 – 10,500 Kg d.m. / ha and the contribution of *L. tenuis* was between 4 to 11% of the total aerial biomass in the plot. The highest percentage of *L. tenuis* biomass was observed in the paddock 3 (Figure 2) and the lowest in paddock 2. This was consistent with the most limiting soil conditions. In paddock 1, percent *L. tenuis* biomass was also lower than in paddock 3, probably associated

to the highest proportional biomass of other dicots, competing with *L. tenuis* seedlings. *Lotus tenuis* biomass was higher for densities 4 and 5 (avg.109 ± 30 g d.m. / m²) than for densities 1, 2 and 3 (avg. 43 ± 8 g d.m./m²). The contributions of *L. tenuis* biomass also varied with seeding densities. As observed in previous studies (Colabelli and Miñón, 1993) the establishment efficiency of *L. tenuis* from seeds was very low (ca. 10 %), and most probably it decreased during summer due to that young plants are susceptibility to water stress. Management practices, such as strategic grazing exclusion, that allow increment of soil seed bank and abundant natural reseeding of *L. tenuis*, would increase the biomass production in these grasslands.

Table 1. Soil analysis of the experimental paddocks.

Paddock	pH	E.C. (mmohs)	P (Bray 1) ppm	O.M. %	Na (meq/L)
1	8.2	1.7	5.6	5.2	9.2
2	9.2	2.7	6.8	3.7	20.7
3	9.5	2.2	7.2	4.1	15.0

Table 2. Means (± SE) *Lotus tenuis* seedling density for different seed density (D) in four census dates.

Date	D1	D2	D3	D4	D5
24 May	2.46 ± 0.8	16.35 ± 4.8	29.93 ± 2.5	144.13 ± 32.5	291.97 ± 58.1
1 Jul	0.69 ± 0.6	7.63 ± 3.4	31.94 ± 6.1	212.49 ± 37.5	388.85 ± 42.2
27 Sept.	4.85 ± 3.0	20.13 ± 6.9	56.24 ± 11.0	270.83 ± 23.0	459.71 ± 25.3
14 Dec.	4.08 ± 2.6	7.77 ± 0.8	25.77 ± 6.5	89.44 ± 20.2	172.88 ±18.6

Figure 1. Mean aerial biomass of *Lotus tenuis* (n =9) for different seed density (D) in four harvest dates. References as in Table 2.

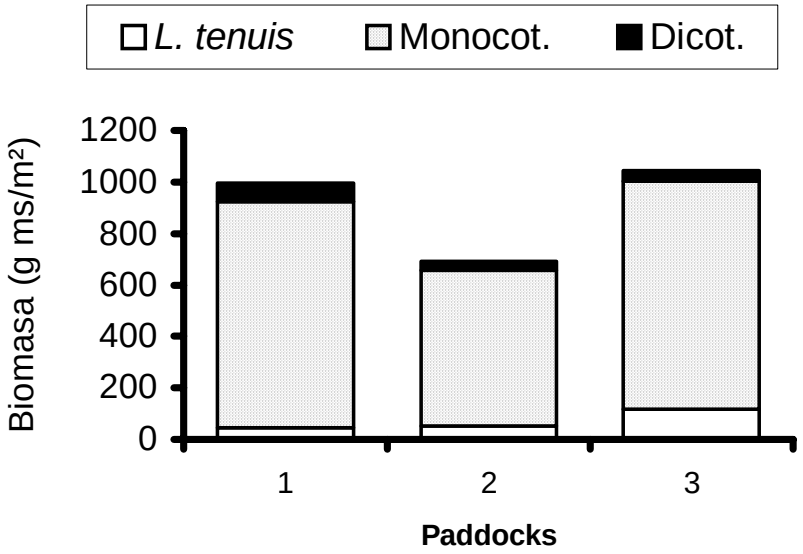

Figure 2. *Lotus tenuis*, *Monocotyledoneae* and non-legume *Dicotyledoneae* accumulated aerial biomass in 3 experimental paddocks. References as in Table 2.

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Interactive effects of trampling and flooding on the growth of *Lotus tenuis* in grassland mesocosms of the Flooding Pampa

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Flooding and trampling are two major disturbances affecting vegetation dynamics of the Flooding Pampa (Argentina). Continuous grazing and the periodic occurrence of floods determine that trampling of the flooded grassland is a common situation in the region. In these grasslands, which lack of abundant native legumes, the introduction and persistence of *Lotus tenuis* Waldst. & Kit. (syn. *Lotus glaber* Mill.) is a desirable situation because it increases the forage quality of the grassland. The objective of this work was to investigate the impact of flooding and trampling on plant cover and aerial biomass of the functional groups of graminoids and dicots, and of *L. tenuis*, growing in mesocosms of natural grassland. To this end, twenty soil monoliths supporting natural vegetation (*i.e.* mesocosms of 0.8×0.5×0.35 m depth) were extracted and transferred to the experimental garden of the Faculty of Agronomy (UBA). Mesocosms were initially similar in floristic composition, soil cover, vertical distribution of the canopy and abundance of *L. tenuis*. After 45 days of acclimatization, mesocosms were subjected to a combination of trampling and flooding in a completely randomized design (n=5). The flooding lasted 40 days while trampling was made at day 20 by covering 52% of the surface of the mesocosms. Flooding increased by two-folds the cover of graminoids ($P<0.001$) and reduced that of dicots by 20% ($P<0.05$), without affecting plant cover of *L. tenuis* ($P>0.05$). Trampling, as unique disturbance, provoked a slight lower cover (18-23%) of both functional groups and of *L. tenuis* ($P<0.05$). The combination of flooding and trampling resulted in a reduction of the cover of dicots by 76% ($P<0.001$) without affecting it in graminoids. In this case, trampling of flooded soil reduced the cover of *L. tenuis* by 80% with respect to control mesocosms ($P<0.01$). The aerial biomass was 30% higher in the flooded mesocosms due to a promotion of graminoids biomass ($P<0.01$), which compensated the lower biomass attained by dicots ($P<0.01$). Trampling and flooding, as individual disturbances, derived in a lower biomass (21-24%) of *L. tenuis* in the mesocosms in comparison to controls ($P<0.05$). Trampling of the flooded grassland did not affected total aerial biomass, but resulted in a significant decrease in the biomass of dicots species ($P<0.01$), especially of *L. tenuis* that registered only a biomass of 10-15% with respect to all other treatments ($P<0.01$). Under such conditions, (trampling plus flooding) the graminoids concentrated 90% of total aerial biomass. In conclusion, this disturbance combination did not affected biomass production of the grassland. However, trampling of the flooded grassland altered the quality of the forage by drastically reducing

the biomass of the legume *Lotus tenuis*.

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Growth responses to flooding intensity in *Lotus tenuis*

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Flooding is an essential component of the disturbance regime affecting the vegetation dynamics of the Flooding Pampa Grasslands, with variable intensity among years. *Lotus tenuis* is an exotic species introduced in these grasslands, which presence is highly desirable because their forage quality. The aim of this work was to examine the effects of different flooding levels on the growth of *Lotus tenuis* during a 30 days period, and their relation with the aerenchyma formation and functionality under intense flooding leading to plant submersion. Four treatments were applied by 30 days following a completely randomized design with six replicates: control: watered daily to field capacity, waterlogged: soil saturated with water but without water above soil surface, flooded: water maintained at 6 cm above soil surface, and submersion: plants were completely underwater without emerging leaves. Plants were established in grassland soil pots of 4L. At the end of experiment tissue porosity (shoot and root), shoot number and plant biomass were measured. Results showed that constitutive shoot porosity was higher with amounts of 26% for most treatments, and was increased only by flooding until 40% ($p<0.01$). In all treatments involving water excess, root porosity was two-fold higher than under control conditions ($p<0.05$). Shoot number was similar at control, waterlogged and flooding treatments ($p>0.2$), but was 50% lower in plants subjected to full submersion ($p<0.05$). Plant growth was negatively affected at increasing flooding intensities: waterlogged, flooded and fully submerged plants attained 75%, 54% and 22% of plant biomass when compared to control ones ($p<0.05$). In conclusion, there is a close relationship between the growth capacity of *Lotus tenuis* at flooding conditions and their ability to form functional aerenchyma. However, plant growth is severely affected, although importantly not plant survival, when the aerenchyma is not able to facilitate oxygen transport to submerged tissues due the lack of oxygen capture of the fully submerged plants.

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Persistence of *Lotus tenuis* under beef cattle grazing in a humid mesophytic grassland of the Flooding Pampa.

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Persistence of forage legumes no only implies tolerance to grazing -the ability of individual plants to recover from livestock defoliation and treading (Forde *et al*., 1989) - but also the population resilience to stand reduction through compensatory recruitment. *Lotus tenuis* regenerates only from seeds. These can accumulate in soil forming a persistent seed bank (Sevilla *et al.,* 1996). Under no limiting growth conditions seedling emerging in spring or fall can reach reproductive stage in 3 to 8 months, respectively (Cambareri *et al*., 2008). In natural grassland reproduction is most frequently delayed to the subsequent year. Commonly a summer grazing exclusion period every 3 to 4 years is recommended. This would allow for plenty seed production in order to replenish the sparse seed bank of soil, assuming that population stability is highly dependent on this source for continued recruitment and rapid turnover in lotus stands. However, when the population dynamics of *L.tenuis* in a cattle grazed mixed pasture was simulated using a Lefkovitch stage/size transition matrix (Fernández *et al.,* 1990), survival of reproductive plants showed by far higher elasticity values for the population growth rate λ than recruitment, seed production or seed bank persistence parameters. To design effective management programs aimed at promoting naturally established or sown lotus populations it is necessary to establish critical periods of cattle grazing for both plant survival and reproduction. This critical period concept as applied in this work is based on the general hypothesis that the negative livestock effects depends on the length of time of the grazing period and its timing relative to plant phenology. The objective was to determine the effects of *grazing* / *exclusion* treatments (12) which differed in the starting date (each 2 months from early spring) and duration (2 to 12 months) on survival and seed production of *L.tenuis*.

Methods

A field experiment was carried out in a paddock of about 260 has in a private range (Ayacucho County, Buenos Aires province, Argentina). This paddock was vegetated by a seminatural grassland used for extensive beef cattle breeding with a mean annual stocking rate of 0.7 AU/ha which was colonized by *L.tenuis*. The study site was located in a 100 m x 120 m short vegetation patch with a C type grassland community (*sensu* León, 1975) on a Typic Natracuol soil (pH: 7.4; MO: 6.2%; $P_{Bray\ 1}$: 4.7 ppm; CE: 1.3 mmohs; $Na_{int.}$: 3.9 meq /100 g; CIC : 19,1 meq/100g, in 0 -15 soil horizon). Mean lotus plant density at the beginning of the experiment (13/10/04) was 41.4 m^{-2} (SD 19.1 m^{-2}). Grazing and cattle

exclusion treatments were established in a complete randomized design with 5 replicates. Sixty 1 m x 1m permanent plots were marked with wood stakes. Plots were randomly assigned to 6 treatments of increasing periods of grazing and 6 of cattle exclusion (2, 4, 6, 8, 10 and 12 months from the beginning of the experiment). Either *L.tenuis* density, total vegetation cover (overall mean 86.72%, SD = 5.77 %; n=60) or *L.tenuis* cover (overall mean 9.92%, SD = 4.80%; n= 60) did not differ ($p < 0.05$) among treatments. Cattle exclusion was performed with 1 m x 1m x 0.4 m iron cages fixed to the ground with metal stakes. Lotus plants (only those with three or more crown branches) were mapped and counted to determine survivorship percentage for a year round period (Oct 2004 to Oct 2005). Seed production in each plot was assessed by counting pods and measuring pod length (10 per plot). Seed yield was calculated as number of pods x seeds per pod (S). The latter was calculated as a function of pod length (L) after fitting a linear regression (S = 1.337 L (mm) – 10.678; $R^2 = 0.824$; n = 50). In order to assess seed lost since preceding summer dispersal, the density of viable seeds recovered from 5 cm depth soil samples was determined for each plot on September 2005.

Results and Discussion

In the continuous grazing treatment mean plant mortality was about 38 % and plant survival was the highest (85.6%; SD 5.18%) in plots where grazing was permanently excluded all along the experimental period (Figure 1). The most detrimental treatments were those with spring or spring to summer cattle exclusion followed by continuous grazing where plant survival fall to less than 50%. At the end of the exclusion period in these treatments, vegetation canopy was very dense and exceeded 30 cm in height. Crown branches of lotus plants turned from a prostrate to an erect growth habit which increased their exposure to grazing. The day after the exclusion cages were removed from those plots, plant canopy was similar to (3 to 5 cm in height) but soil cover was lower than in the neighbouring vegetation. This could partially explain the highest mortality percentage observed and suggests a negative competition x grazing interaction effect on plant survival. The most critical grazing period for plant survival coincided approximately with that for seed production (Figure 2), extending from the end of spring to middle summer. However, seed production appeared to be more sensitive to continuous grazing than plant survival: while the latter was reduced in 26.7%, seed production diminished more than 90% in relation to the treatment where grazing was excluded all the experimental period. Grazing exclusion during that critical period for lotus reproduction allowed a seed yield that did not differ from the all year round exclusion treatment (1,153 m^{-2}; DE 269 m^{-2}). Density of viable seeds found in soil samples (SB) on September 01, 2005 were closely associated to the calculated seed rain (SR) for each treatment ($SBm^{-2} = 0.4806\ SRm^{-2} + 12.517$; $R^2 = 0.778$; n =60) but represented about 50% of the produced seed. Several factors could account for this apparent seed lost (embryo death, predation, deep burial), but also seed production could have been overestimated in counting all mature and immature pods for seed yield calculation. Other local studies (Sevilla *et al.,* 1996; Vignolio and Fernández, 2008) showed that about 50 viable seeds could be required to give a surviving reproductive plant the year after emergence. Under the conditions of the present study results indicate that the exclusion of cattle grazing from middle December to middle February would allow for a full compensation of plant lost through recruitment from

viable seeds remaining in the soil at the end of winter. So in designing strategic grazing schemes regular spell from defoliation of 6 to 8 weeks from the beginning of flowering stage early in summer is likely to maintain or even increase the stand of established lotus plants (Figure 3).

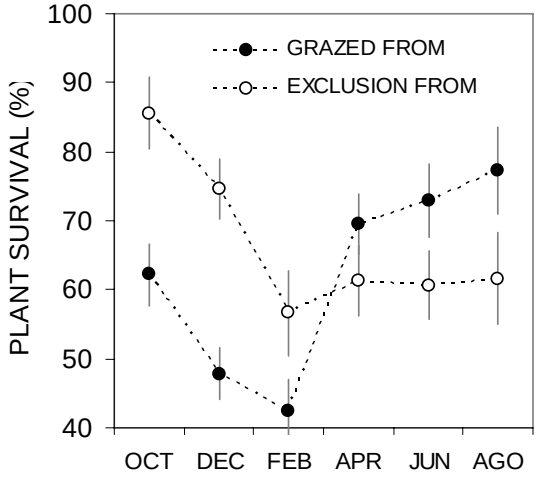

Figure 1. Plant survival as percentage of the initial plant density for grazing and cattle exclusion treatments. Bars indicate ± 1 SD (n=5).

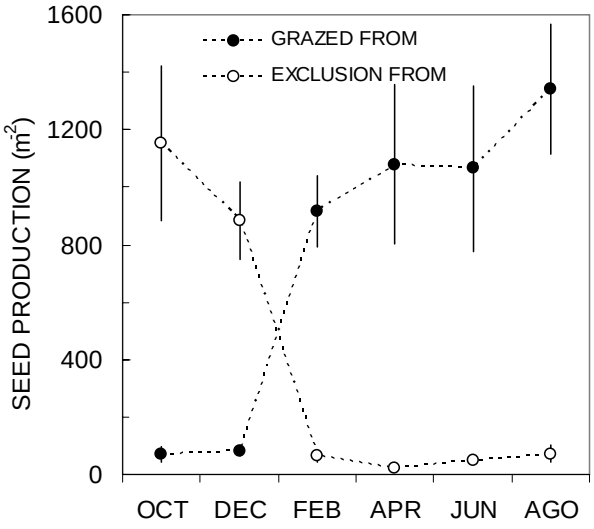

Figure 2. Seed production calculated for grazing and cattle exclusion treatments from the beginning of pod filling to Feb20, 2005. Bars indicate ± 1 SD (n=5).

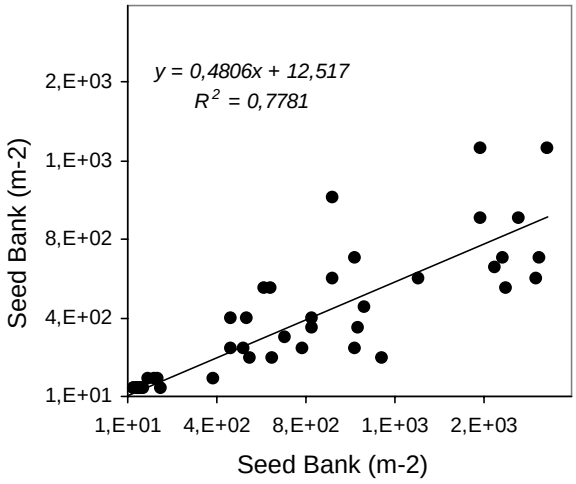

Figure 3. Relationship between seed recruitment in the soil at Sept9, 2005 and the estimated seed production (n=60).

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Changes in reproductive phenology of *Lotus tenuis* related to sowing season

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Lotus tenuis is a forage legume broadly accepted and used by the beef cattle breeders of the Flooding Pampas and its seeds are increasingly demanded in Argentina. The national production of certified seed of *Lotus tenuis* evolved from 10 t on 1999 to 84 t on 2007. Despite of this, seed yields keep low (< 200 kg ha^{-1}) because of lack of information about agronomic practices to improve them. The main goal of this work was to estimate the effects of sowing season on the reproductive phenology of *L. tenuis* under agroecological conditions of Balcarce (Buenos Aires province, Argentina). This work was performed to partially fulfil the requirements for MSc degree in Plant Production (UNMDP) of the first author. A field experiment was carried out in the Estación Experimental Agropecuaria Balcarce del INTA, (37º 45' S, 58º 18' W; 130 m.s.n.m.), from September 21th 2006 to April 1st 2008. Nine 2,1 m x 6 m plots were sown on spring 2006 (September 21 th) and autumn 2007 (April 12 th) over a Typic Argiudoll Soil using seeds of *L. tenuis* cv. Pampa INTA (González García, 2004) at an initial seeding rate of 1.62 (±SE 0.14) g / m2. After emergence, the seedling stands were hand-thinned to attain 3 plant densities (high, medium and low). Thus, a 2 seasons X 3 densities X 3 blocks (related to topography) design was established. A split – plot model was used to analyze the data with the free software R. Meteorological data were provided by Estación Agrometeorológica EEA Balcarce (INTA) and used to describe astronomical theoretic sunshine length (h) as photoperiod estimate, rainfall (mm) and daily mean air temperature (ºC). Recording phenological stages in this species at plant level is a very difficult task due to the entangled branching complex at the stand level. Because of this, we employed a reproductive bulk monitoring based on Fehr's scale for soybean (1977) and, complementary, a pods colour range was used to describe the floral initiation to maturity developmental stages (Anderson, 1955; Carámbula, 1981; Winch, 1985). At a late vegetative stage, three 1/3 m^2 frames were placed in the central area of each plot to delimit the sampling area. Within this area, 10 primary axis per frame (those emerging from the plant crown) were monitored periodically in order to determinate the bud state. Phenological stages were defined as follows: Em (seedling emergence); BF (floral bud, 50% of shoots); R1 (early flowering, 20% of flowering shoots); R2 (full flowering, 50 % of flowering shoots); R4 (full fruit setting, 50 % of shoots bearing green pods); R6 (full maturity, 50 % of shoots bearing brownish pods). Days and degree-day (growth threshold= 9ºC) accumulated from spring equinox (DFSE) were used to determinate the occurrence and length of each stage, respectively. Astronomical theoretic sunshine length was 14.06 h (± SE

0.07) and 11.73 h (±SE 0.12) for Em-R1 developmental period, and 14.16 h (± SE 0,06) and 14.23 h (± SE 0,06) for R1- R6 period, for the spring and the autumn sowing date, respectively. From the Em to the R6 stages, cumulative rainfall was 377.2 mm and 838.7 mm, and mean temperature averaged 18.6 ºC (± SE 0.36) and 13.1 ºC (± SE 0.36) for the spring and the autumn sowing date, respectively. Plant density had no significant ($p < 0.05$) effect on plant phenology. Phenological stages BF, R1, R2, R4 and R6 occurred at 92, 98, 105, 130 and 165 DFSE for the spring sowing date, whereas they occurred at 91, 93, 100, 126 and 138 for the autumn sowing date. With regards to cumulative thermal-time, the developmental periods Em-R1, R1-R2 and R2-R4 were shorter for the spring than for the autumn sowing date (Figure 1). On the contrary, the period R4 – R6 required a higher thermal sum for the spring than for the autumn sowing date.

Figure 1. Thermal sums (degree-day) during plant development periods for two sowing seasons. Em-R1: emergence to early flowering. R2-R4: full flowering to full fruit onset. On the arrows, mean sunshine length (h) is indicated.

These results suggest that the longer sunshine length recorded for the spring sown crop was associated to a shortened pre-reproductive developmental period. This resulted in a hastened reproductive development as observed for other long-day response species when a sowing delay occurred (Bodega *et al*, 2004; Miralles *et al*, 2007). However, the length of the pod ripening period (R4 – R6) for the spring sowing date was twice the same period for the autumn sowing date in terms of cumulative degree-days. Probably other environmental factors such as air humidity could have interacted with sunshine length to cause that phonological difference between sowing date. Keatige *et al.* (1998) found a conventional long-day species response in the annual legumes *Vicia faba, V. sativa and Trifolium*

resupinatum for emergence to early flowering period. However they did not observe differences in comparing the length of the complete life cycle. Summing up, our results indicate that for a wide range of stand density plant phenology is not affected. Intraspecific competition in *L. tenuis* affected the number of reproductive crown shoots but not their developmental timing for a given sowing date. By the other hand, sowing date through changes in environmental drivers such as day length, temperature and, probably air humidity have marked interactive effects on plant reproduction. These must be considered in planning and performing commercial seed crops in order to maximize the potential and the harvestable seed yield.

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Using the method of Eberhart and Russell to estimate productive adaptability and stability for three cultivars of *Lotus tenuis* on restrictive environments of the Flooding Pampas

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The Flooding Pampas is extended for over six million hectares in the Buenos Aires Province, being the main zone where extensive beef cattle breeding systems are carried out. Its topography and edaphic characteristics define strong restrictions for agriculture, being economic and bio-physic productivity highly related to grassland community growth, which has few forage legume species for building a quality forage resource. The edaphic heterogeneity determinates an herbage species community mosaic, including typical characters of highlands, medium lands, non saline and saline – alkaline lowlands (communities A, B, C and D, respectively; León, 1975). This natural design acts like a very strong environmental filter for growth and development of both exotic and native forage species, limiting pasture sowing and consequently, beef production is hardly affected. *Lotus tenuis* is a forage legume which has been successfully widespread along Flooding Pampas. This species is exotic and has high capability to tolerate environmental stresses like alkalinity and saline conditions (Montes, 1986; Stofella *et al*., 1998). The goal of this work was to estimate adaptability and productive stability for three cultivars of *L. tenuis* in actual beef cattle systems of the Flooding Pampas. This study was carried out on two ranches: one located to 20 km away from Labardén (36° 57' 0S ; 58° 6' 0W), Gral. Guido and other located to 15 km away from Chascomús (35° 34' 0S 58° 01' W), from April 2006 to May 2008. The experimental site chosen at each ranch consisted on a ca. 1 Ha plot, with high degree of environmental heterogeneity (topographic, pH, phosphorus and organic matter gradients, table 1). Cultivars Chajá (**Ch**), Esmeralda (**Es**) and Pampa INTA (**Pm**) were used. The sowing was performed by a intertill seed drill on April 2006, using 40 cm inter-row spacing and a seed rate of 6 kg ha-1, establishing a two years X two sites (macro-environments) X three cultivars X four replicates X three sub plots (micro - environments) design along the environmental gradient defined by ranges of pH, electric conductivity (EC), phosphorus, organic matter and nitrates (Table 1). The experimental unit consisted on a six rows width X 100 m long plot, from which three 1 m2 sub-samples of aerial green matter were taken (clipping with hand-scissors, leaving a 3 cm - high remnant herbage) on December and May in order to estimate cool and warm season production of

Dry Matter, respectively. After every sampling a 'cleaning' cut was applied using high cattle rates. The samples were fractioned in Lotus (L), Grasses (G) and Dicots (D), then dried at 60ºC at stove and finally, weighted in analytic scale. Data were analyzed by R statistic software, using the model, $Y_{ij} = \mu_1 + ß_1 I_j + \delta_{ij}$, which defines stability parameters that may be used to describe the performance of a cultivar over a series of environments. Y_{ij} is the cultivar mean of the i^{th} cultivar at the j_{th} environment, μ_1 is the i^{th} cultivar mean over all environments, $ß_1$ is the regression coefficient that measures the response of the i^{th} cultivar to varying environments, δ_{ij} is the deviation from regression of the i^{th} variety at the j^{th} environment, and I_j is the environmental index. (Eberhart and Russell, 1966). Indexes of both adaptability and stability are defined as follows : ß1, = 1, cultivar adapted to widest range of environments; ß1 < 1, cultivar adapted only to low aerial primary productivity environments; ß1 > 1 cultivar adapted only to high aerial primary productivity environments; d2=0, maximum stability; d2 > 0, low to high stability, where d^2 is the estimate of the variance. Average Dry Matter production of *L. tenuis* for each season, standard error, adaptability and stability estimates are showed (Table 2). Average percentage of *L. tenuis* dry matter in samples was over 25,9 % and 11,1 % on first and second year of study, respectively. Significant interactions ($p<0.05$) between years, sites and micro environments were found for dry matter production and 12 environmental index were made up taking into account this information.

Table 1. Ranges of pH, EC (electric conductivity), P (phosphorus), OM (organic matter), NO3 (nitrates) from each site.

Variable	Range
pH	9,02 - 9,61
EC (mmohs)	2,48-3,08
P (ppm)	7,23-10,98
OM (%)	3,05-4,44
NO_3 (ppm)	3,78-4,33

Table 2. Average dry matter production of *L. tenuis* ± standard error related to growing season and cultivar. Average crossing years and sites. b: regression coefficient estimate. d^2: variance of regression estimate. *: Significant regression at 5 %. 1: regression using 6 environmental indexes.

Season	Cultivar	Forage Production of *L. tenuis* (Kg DM/Ha)	ß1	d^2
Cool (Winter – Spring)	Ch	241.6 ± 20.4	0.94 *	18.9
	Es1	326.9 ± 25.4	1.45 *	20.54
	Pm	286.7 ± 15.4	0.94 *	7.38
Warm (Summer – Fall)	Ch	192.5 ± 37.1	0.81 *	37.43
	Es1	433.6 ± 57.4	1.43 *	32.14
	Pm	210 ± 35.7	1.01 *	3.12

Our results suggest that selected cultivars have distinctive attributes: *L. tenuis* cv. Chajá produces more aerial dry matter on winter – spring than does on summer - fall and is better adapted to low productivity environments but has also low stability. *L. tenuis* cv Esmeralda has got its maximum dry matter production on warm season and is better adapted to high productivity environments but with lowest stability on winter-spring. *L. tenuis* Pampa INTA has a higher production of aerial dry matter during winter-spring, being much more plastic and stable than other cultivars.

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Narrowleaf trefoil (*Lotus tenuis)* seed production with different defoliation and harvest times

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Lotus tenuis is an exotic forage legume, of indeterminate simpodial growth, cultivated in consociated pastures of the Pampa Argentina region because of its adaptation to flooded and slightly saline-alkali soils. The simultaneity of floral primordia, umbels with flowers and pods in different proportions and states of maturation is a difficulty in obtaining agronomic quantity and quality of seed harvest.

The objective was to evaluate seed yield and quality, in four dates of defoliation in the year of the implantation, at two different times of harvesting.

Two trials were sown, one by each harvest, with cultivar La Esmeralda, in plots of 25,6 m^2, 8 rows of 8 m long, spaced 0,4 m; in Latin square design. A number of 26±6 plant.m^{-2} was obtained. The defoliation date treatment were: D1: no defoliated; D2: oct-01-07; D3: nov-02-07 and D4: dec-03-07. First harvest (C1) was performed before registering pod dehiscence (shattering) and the second (C2), one week later, both with two samples of 0,5 m^2 by plot.

On average crop, in C2 was obtained a higher yield (737 kg.ha^{-1}), with no differences between treatments in weight of seed (1055 mg). In C1, the yield was higher for D2 (487 kg.ha^{-1}) with higher seed weight (1116 mg). In C2, D3 produced the best yield performance (1059 kg.ha^{-1}), with seeds of lower weight (983 mg).

The best germination (PG) was accompanied by less hard seeds (D). In C1 were higher D3 and D4 (PG 77%, D 12%); however in C2, D1, D2 and D3 (PG 86%, D 7%).

The opportune defoliation of the culture can increase the yield and the quality of seed harvested in *Lotus tenuis*, but the moment for realizing the harvest is determining.

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Birdsfoot trefoil (*Lotus corniculatus)* seed production with different defoliation and harvest times

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Lotus corniculatus is a perennial forage legume species introduced in Argentine because its adaptation to grow and survive in low fertility and poorly drained soils. In addition birdsfoot trefoil presents good nutritional value, efficient forage accumulation and it is a non bloating legume. Since growth of the plant is indeterminate, flowers and fruits of all stages of development may occur simultaneously on the same plant. The main reason for low harvest seed yields, is the indeterminate flowering and unpredictability of pod shatter. Since lotus has an extended flowering period, deciding when to harvest lotus seed crops is very difficult.

This feature, along with the sudden rupturing of the seed at maturity, makes the harvesting of a large proportion of the total seed crop difficult under many conditions. The objective is to evaluate seed yield and quality, with four dates of defoliation in the year of implantation, at two different time of harvest.

Two trials were sown, one by each harvest, with cultivar Gladiador, in plots of 25,6 m^2, 8 rows of 8 m long, spaced 0,4 m; in Latin square design. A number of 18,1±3,6 plant.m^{-2} were obtained. The defoliation date treatment were: D1: no defoliated; D2: 10/01/07; D3: 11/02/07 and D4: 12/03/07. First harvest (C1) was performed before registering pod dehiscence (shattering) and the second (C2) one week later, both with two samples of 1 m^2 by plot.

In C1, D3 and D4 (255,5 kg.ha^{-1}) in average they rendered more than D1 and D2 (81,5 kg.ha^{-1}). In C2 was obtained higher yield of seed (461 kg.ha-1) and heaviest (1306 mg); D3 treatment produced the maximum, 1033 kg.ha^{-1}, with seeds of lower weight 1227 mg.

In C1, D3 and D4 in average had more hard seeds (D, 12%) without germination differences (PG). In C2, the PG average (74%) was superior, with the greater number of D (16%) and minor of died (4%).

High yield of quality seed is obtained when the defoliation occurs early in November and the harvest is done at the start of pod dehiscence.

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Abstract, Workshop held at Chascomus, 17-19 November 2008

Genetic differences between *Lotus glaber* populations tolerant and susceptible to salinity, growing in a non restrictive soil condition

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Lotus glaber Mill (*Lotus tenuis* Waldst. et Kit) is a perennial forage legume native from the Mediterranean Basin. It grows during spring-summer and autumn. In Argentina it has been naturalized in the lowlands of the Salado River (Buenos Aires Province). In this area, *L. glaber* has shown adaptation to ecological conditions due to its ability to overcome frequent drought and flooding, the adaptation to soils poor in phosphorus, poor drainage and its ability to natural reseeding.

At Pergamino Gene Bank are conserved populations of *L. glaber* collected in the lowlands of Salado River, and landraces that seed growers maintain by their own. Several studies were done by INTECH and Pergamino Experimental Station (EEA Pergamino) INTA on 23 accessions in order to evaluate tolerance to salinity, and several morphological, physiological and biochemical attributes were evaluated (Pesqueira *et al.*, 2007). Other studies performed under spaced plant conditions indicated appreciable genetic variation between and within *L. glaber* populations on forage and seed production attributes (Andrés and Rosso, 2007). In accordance with the results obtained 2 pools of 3 populations each: tolerant to salinity (TOL) and susceptible to salinity (SUS), were transplanted on 28 November 2006, at the experimental grounds of EEA Pergamino under spaced plant conditions, in a randomized block design with 2 replicates. Each pool was compound by 3 populations (20 genotypes/population), and all genotypes were measured or scored for a range of morphological attributes related to forage and seed production during 2006 and 2007. Analysis of variance was performed on the attributes considering individual populations and the 2 pools. Estimates of genetic and environmental variances were calculated from one-way ANOVA performed on each individual population.

The results showed highly significant differences between pools (SUS vs TOL) for all attributes analyzed. SUS populations had plants with larger diameter (plant diameter: 150 cm vs 120,17 cm), higher branching number (274 vs 168), higher dry matter weight (253,83 g vs 130,83 g), early flowering (92 vs 115 days), higher seed yield (7,42 g vs 1,68 g) than TOL populations. The better performance of SUS populations suggested that they were grown or selected under environment with no salinity stress, and they should be tested under both restricted and non restricted soil conditions. There were significant differences between populations for all attributes and the broad sense heritability reached values higher than 0.41,

suggesting that this variability may account for future selection response.

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Lotus corniculatus and toxic Aluminum: an update of the progress obtained by the LOTASSA Project

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Introduction

Environmental stresses are among the most important production limiting factors in the Southern Cone Region, and soil acidity and the presence of toxic aluminum (Al) are very important in this scenario, especially in Brazil. There are several Al tolerance mechanisms described in literature, root exudation of organic biomolecules being the most studied and explored by researchers (Kang and Ishii, 2006). These molecules form stable complexes with Al, reducing its activity (MA, 2000). Plant populations and genotypes, in a same species, may differ deeply in tolerance to several soil fertility problems and in nutrient utilization efficiency. A correct understanding of toxicity caused by Al and related tolerance levels is important for developing appropriate tests for the selection of tolerant germoplasms and for developing genotypes with superior performance in soils that present this characteristic. Compared to other grazing leguminous, several *Lotus* species present a high adaptation potential to soil acidity, making this genus a strong candidate to be used for this condition. This paper aims characterize the *L. corniculatus* genotypes' response to toxic Al.

Material and methods

Three experiments were realized for the characterization of Al tolerance. The first was realized in soil, where six levels of liming were applied: 0, 10, 20, 30, 40 and 80% of the recommended dose by the SMP method (Comissão de Fertilidade, 2004). Five *L. corniculatus* genotypes (*São Gabriel, San Gabriel, Estanzuela, Draco e UFRGS 2004/2*) were tested. Additionally, alfalfa was used as an Al sensitive species. The dry matter production of the aerial part (DMPAP), foliar area (FA), height (H), number of ramifications and number of live trifoliate leaves were evaluated. In a second experiment, two genotypes of *Lotus corniculatus* (Draco and UFRGS) which presented contrasting responses to the Al in the previous experiment, were evaluated in nutrition solution. The solutions used were composed of 200 µmol L^{-1} of Ca ($CaCl_2$) and three levels of Al^{+3} (50, 100 or 200 µmol L^{-1} of Al, $AlCl_3$), using a control solution (0 µmol L^{-1} of Al). The initial and final root lengths and

root growth were evaluated after 20 days of growth. Finally, a third experiment designed to evaluate the exudation of organic acids by three *Lotus corniculatus* genotypes (Draco, São Gabriel e UFRGS) and alfalfa was realized. Plants with 15 days were transplanted to potd containing a complete nutrition solution and maintained in these conditions for 67 days. Between the 30^{th} and 37^{th} and after the 60^{th} day, 200µM of Al was added to half the pots of each genotype, being the other half maintained without aluminum. The nutrition solution samplings for the evaluations of the organic acids exudation were carried out 24, 72 and 120 hours after the last Al addition. Two ml of each pot were collected and analyzed through chromatography (HPLC). The exudation of oxalic, malic and citric acids was evaluated. After the third sampling (120 h), the plants were collected, evaluating the dry matter of the aerial and root parts, root length and plant height.

Results and discussion

Evaluating the six genotypes in each liming level, a severe limitation in the 57,9% treatment of saturation by Al treatment was observed. In the 34,4% treatment, the UFRGS and São Gabriel genotypes were superior to the others, with average DMPAP values of 0,06g, while the alfalfa presented the lowest values (0,0227g). In the 22,2% level, the UFRGS genotype was better, with an DMPAP of 0,1165g, with the alfalfa being the less productive genotype once again. The 14,1% level promoted, in general, the highest productions in all genotypes, including alfalfa, with an average of 0,0832g. Overall, the UFRGS and São Gabriel genotypes were the most efficient in situations of higher soil acidity (34,4% SMP treatment), presenting good DMPAP productions, giving them potential for breeding.

Regarding the data in nutrition solution, in the absence of Al, the three genotypes presented similar root length, while in the presence of Al (50, 100 and 200 µmol L^{-1}) an average of 60% reduction of root length of alfalfa seedlings were observed at the 27^{th} day of growth . However, for the Draco and UFRGS genotypes, no significant reduction was observed in any of the three doses of Al tested at the 27^{th} day. At this time, the Draco and UFRGS genotypes presented similar root length in the 0 and 50 µmol L^{-1} doses. In the 100 µmol L^{-1} dose, the UFRGS genotype presented the longest root length, while the Draco genotype was intermediate and the alfalfa presented the shortest length. For the root growth, evaluated by the difference between the length at the 20^{th} and the 7^{th} day after germination, it was observed that between the 100 and 200 µmol L^{-1} level there was no significant reduction, indicating that the 200 µmol L^{-1} level may have been too high. Amongst the tested genotypes, the UFRGS had a good response, presenting a higher growth compared to the alfalfa, while the Draco genotype presented a growth 32% smaller than the UFRGS genotype and 40% superior to the alfalfa.

The root exudation results demonstrated that of the three organic acids tested, the only one quantified by the chromatography was the oxalic. An average exudation of 14,0 mMolar, 24 and 72 hours after the Al exposure was observed, reducing significantly to 6,6 mMolar (53%) 120 hours after the addition of Al. In all three evaluations, the three Lotus genotypes treated with Al presented exudation three times superior to the alfalfa, indicating that this is a mechanism of tolerance activated by the presence of Al. Of all the Lotus genotypes evaluated, the UFRGS genotype exhibited a greater oxalic acid exudation compared to the

others, 24 and 72 hours in the presence of Al. In the third evaluation (120 hours) the three Lotus genotypes tested did not differ among each other, but were superior to the alfalfa. Regarding the parameters evaluated after the last sampling (120 hours), in the absence of Al an accumulation of DM (aerial and roots) about 200% superior to the alfalfa was observed, which presented an accentuated reduction in production after exposure to Al, even for short periods. This production reduction was not observed in the Lotus genotypes. The protective role played out by the oxalic acid has already been demonstrated in experiments realized with other species and could be related to the tolerance observed in *Lotus corniculatus*.

Conclusions

The 34,4% level of Al saturation was the most efficient for the selection in soil due to the great differentiation presented among the genotypes in this level.

The UFRGS and São Gabriel genotypes presented moderate tolerance to soil acidity, while the DRACO genotype was the most effected by the Al.

The results obtained in nutrition solution confirmed those obtained by using acid soils, indicating that this methodology can be used to characterize Al tolerance in a faster and cheaper manner.

The alfalfa confirmed its low tolerance to Al, reducing greatly its growth in doses greater than 50 µmol L^{-1}.

There was a significant increase in the oxalic acid exudation in *Lotus corniculatus* genotypes cultivated in nutrition solution in the first 72 hours after exposure to toxic Al, specially in the UFRGS genotype. This is the first report of production of organic acid in response to Al in *L. corniculatus*.

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Lotus RILs analyses under salt stress: establishing discriminative growth conditions

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The aim of this work was to study some functional markers associated to saline stress tolerance in Lotus recombinant inbreed lines (RILs) as an activity inside the LOTASSA project (EU). Tolerance responses to NaCl based on growth parameters were characterized in RILs obtained by *Lotus filicaulis* x *Lotus japonicus* ecotype Gifu (NS- RILs) and *Lotus japonicus* ecotype Gifu x *Lotus burttii* (GxLb-RILs) crossings.

Differential tolerance to 0, 75, 100, 125, 150 mM NaCl in each diploid parents was previously assayed using 7 days old plantlets. Salt treatments were applied in a stepped-increase fashion. There were defined length of salts stress treatment, salt levels, and growth parameters in which genotypes differences in growth were statistically significant.

On control conditions, shoot length and growth velocity were higher in *L. filicaulis.* Salt stress diminished shoot length in all genotypes. Lj Gifu was the less affected genotype. Salt effects were notably evident at 13 d after the beginning of 125 mM NaCl treatment (9 days under final salt concentration). Both total and shoot length, allowed discriminating Lj Gifu from the other genotypes; being a useful data given that Lj Gifu is a common parent in both kinds of RILs.

RILs seed were scarified and cultured during 5 days on agar (0.8%). Before salt treatments, percentage of germination and development of NS-RILs (Lotus filicaulis x Lotus japonicus ecotype Gifu) and GxLb-RILs (*Lotus japonicus* ecotipo Gifu x *Lotus burttii*) untreated plantlets were determined. Seventy five percent of NS-RILs showed a range of germination between 50-100%, however 4% of these RILs showed germination lower than 9%. In GxLb-RILs, 61% showed a range of germination between 50-100% but in 37% of this RILs germination percentage was between 10-49%. Normal plantlets development was also analyzed by ranges. In NS-RILs 34% developed more than 50% of normal plantlets whereas in 54% of these RILs viable plantlets were between 10-49% of germinated seeds. Sixty two percent of GxLb-RILs showed between 50-100% of normal plantlets.

NS-RILs and GxLb-RILs were acclimated at 125 mM NaCl in hydroponic trays and growth parameters were evaluated 9 d after reaching final salt level.

Total length showed two different groups in NS-RILs, one of them included 46 RILs with smaller total length and without differences respect to parental Lj Gifu. The second group,

with higher total length, included 10 NS-RILs and did not show statistically significant differences respect to *L. filicaulis*. GxLb-RILs also showed two statistically different groups: one of them comprised 13 lines with smaller total length, and the other one included 8 RILs and both parents Lj Gifu and *L burttii* without differences among them.

The protocol designed to analyzed salt tolerance in Lotus parents and NS and GxLb-RILs allowed differentiating responses against salt stress. These results will contribute to correlate physiological responses with the molecular markers developed for *Lotus*. Under the defined conditions to discriminate salt stress responses in both groups of RILs, none of them showed any improved responses in growth on respect to their parents, for this reason analyzing the responses in RILs with a higher susceptibility and correlating functional markers associated with NaCl susceptibility, could be of interest.

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Potential biotechnological application of the inter-specific hybrid *Lotus tenuis* x *Lotus corniculatus*

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The so-called Salado River Basin (Buenos Aires, Argentina) is a region of high economical importance, due to the agronomical activities developed therein (Mendoza, 1981; Montes, 1988). Approximately 80% of this region is marginal for agriculture and the main productive activity is cattle breeding, which is based on the management of natural grasslands that lack native legumes (Noailles, 1998). Plant species that grow in this region are exposed to soil salinity and alkalinity, phosphorous deficiency and alternating cycles of drought and flooding, conditions that seriously limit the implantation of traditional legumes such as lucerne and trefoil (Mazzanti *et al.*, 1986; Soriano, 1991; Maiola *et al.*, 2003). *Lotus tenuis* has successfully colonized soils in this region, due to its ability to tolerate salinity (Vignolio *et al*., 1999) and flooding (Teakle *et al*., 2007) and to grow in a wide range of soil pHs (Miñón *et al.*, 1990) and nutrient levels (Montes, 1988). *L. corniculatus* is also present in this region and shows a high forage value and condensed tannin (CT) levels adequate for cattle nutrition. However, commercial *L. corniculatus* varieties available at the present are able to grow only in small zones of good edaphic conditions, being less tolerant than *L. tenuis* to salinity and flooding (Lagler, 2003). Anyway, many *L. corniculatus* populations are adapted to diverse environments, this being the case of a population found in the *Parque Natural de la Albufera de Valencia* (Spain). Part of the landscape in this area consists of basins located close to the sea, which harbour plant species adapted to flooding, nutrient deficiency and salinity (Rubio-Delgado *et al*., 1998; Benavent-Olmos, 2004). A *L. corniculatus* subsp *corniculatus* population native from this area exhibited a higher level of salt tolerance than other *Lotus* species, and was also found to contain medium to high CT levels (Escaray *et al*., 2008). On the contrary, CT levels in *L. tenuis* are very low, being well below those considered to be optimal for cattle nutrition (Escaray *et al*., 2007). Moreover, both intra an inter-population variability is extremely low in *L. tenuis*, thus preventing traditional breeding approaches for the selection of germplasm with higher CT content. In order to obtain a *Lotus* population well adapted to restrictive conditions typical of the Salado River Basin and containing adequate CT levels, it was attempted to obtain an inter-specific hybrid between *L. tenuis* and the above-mentioned *L. corniculatus* population native from *Albufera de Valencia*, using *L. tenuis* Pampa INTA as the female parental. Plants were cultivated in 600 cm^3 pots filled with sand, placed in a closed chamber free of insects under controlled conditions (16 hours light, 10,000 to 12,000 lux; 24/20°C, day/night temperature) and were

irrigated with half-strength Hoagland solution. Once plants reached the reproductive stage, *L. tenuis* flowers were emasculated and pollen collected from *L. corniculatus* was manually disposed on them. This procedure led to the development of seeds whose viability was low, but a few plants were obtained, whose hybrid nature was confirmed by PCR amplification and sequencing of internal transcribed sequences 1-4 (ITS 1-4).

CT levels were evaluated in leaves of both parental materials and the hybrid progeny, by the method of Li *et al*. (1996). Soluble, non-soluble and total CT content in leaves of *L. tenuis* Pampa INTA were 0.120 ± 0.087, 0,540 ± 0,040 y 0.660 ± 0.047 mg CE/g DW, respectively. In leaves of the *L. corniculatus* population native from *Albufera de Valencia*, soluble, non-soluble and total CT contents were 6.410 ± 2.730, 10.090 ± 2.850 and 16.500 ± 4.470 mg CE/g DW, respectively, while in leaves of the hybrid plants, soluble, non-soluble and total CT contents were 1.890 ± 0.850, 3.420 ± 2.740 and 5.320 ± 3.060 mg CE/g DW, respectively. The CT levels found in the hybrid progeny are thus intermediate to those of both parental materials, and fall within the concentration range considered to be optimal for cattle nutrition (Li *et al.*, 1996; Aerts *et al.*, 1999).

Preliminary assays demonstrated that the hybrid plants obtained in the present work have a good level of salt tolerance, as compared with both parental populations. In addition, an F2 hybrid progeny has recently been obtained and seed viability was found to be high, thus suggesting that the approach employed in the present work could contribute to the development of a stable population with adequate CT and salt tolerance levels, which could be used for forage production in the Salado River Basin.

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Resúmen, Taller realizado en Chascomus, 17-19 Noviembre 2008

Experimental design optimization for the study of saline, alkaline and mixed stress in *Lotus* sp.

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Mixed saline-alkaline stress severely restricts primary production of forages in the Flooding Pampa, the most important area for cattle breeding in Argentina. About 68 % of the soils in this region contain alkaline salts like $NaHCO_3$ and Na_2CO_3 (Mendoza, 1981; Vazquez *et al*, 2001; Costa and García, 1998). In soil solution, alkalinity produced by CO_3^{-2} and HCO_3^- anions of the above-mentioned salts causes a reduction of nutrient availability to the plants, and also produces root damage (Lucena *et al.*, 2007; Cartmill *et al.*, 2008) and leads to chlorosis, growth inhibition and adaptation to nutrients uptake (Alhendawi *et al.*, 1997).

Lotus tenuis is a perennial legume naturalized in saline-alkaline lowlands of the Salado River Basin (Vignolio *et al.*, 1999), highly valuable because of its contribution to forage offer in the region and its influence on growth of associated species (Papadopulous and Kelman, 1999). For this reason, *L. tenuis* is considered as a Keystone specie (Quinos *et al.*, 1998; Paz *et al.*, 2007). These characteristics are common to other species of *Lotus* genus, and for this reason, many efforts were destined to evaluate the mechanisms of salt stress responses in this genus. However, adaptive characteristics of *L. tenuis* and other *Lotus* species indicate that these species are also able to tolerate soil alkalinity, thus being important to discriminate between responsive mechanisms to saline, alkaline and mixed saline-alkaline stresses (Mokhtar *et al.*, 2006).

Preliminary results indicate that mixed saline-alkaline stress is more severe than either the saline or the alkaline stresses, and that there are different degrees of tolerance among species of the genus. In addition, anatomical and morphological were found to be induced by these stresses, with an increase in leaf and stem succulence and plastic differential responses in roots.

In this way, tolerance mechanisms might be acting at different levels (genetic, biochemical, physiological and/or anatomical) and these responses might be different among diverse *Lotus* species. On the other hand, the symbiotic interaction with highly efficient and competitive rhizobacteria able to fix atmospheric nitrogen and well adapted to restrictive

soils might contribute to adaptive responses of *L. tenuis* (Parniske and Downie, 2003; Paz *et al.*, 2008; Bergottini *et al.*, 2008; Estrella *et al.*, 2007).

Finally, it is worth to point out that, in spite of the lack of information about molecular genetics of *L. tenuis*, genomic sequencing of the model species *L. japonicus* is well advanced (>67%) (Udvardi *et al.*, 2005; Sato *et al.*, 2008). As a consequence, the existence of a high level of macro and micro-sinteny between different *Lotus* species was found, which could be relevant for the identification of *L. tenuis* genes induced by stress conditions.

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Characterization of genes induced by drought stress in nodules of *Lotus tenuis*

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Water status of plants is the most important factor determining their productivity. One of the most affected processes by drought stress is nitrogen fixation, which involve nodule function and develop. This is accompanied by changes in expression of genes related principally with cellular homeostasis regulation, reactive oxygen species (ROS) detoxification and signal transduction.

In this work we present evidences of genes induced during drastic change of water content in the plant, which constitute emergency first response. Mediating use of GeneFishing DEG-Kit were isolated twelve fragments, which were cloned and sequenced. Results obtained mediating DEG Kit were validated using northern reverse and northern blot analysis. Genes identified have not previously reported in nodules, however, have been described in other organs for several species and related with mechanisms of drought tolerance. DEG Lt7 coding for class III endochitinase was studied in more detail. This show a high homology with gene *Srchi13* from *Sesbania rostrata*, which is described by modulate signal generated by Nod factors during nodule formation. Endochitinase gene is induced in nodules and roots of *L. tenuis* during drought stress. Detailed studies will allow elucidate role of endochitinases in mechanisms of nodule tolerance to drought stress in legumes.

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Ion distribution at the whole plant level in *Lotus* species exposed to salinity

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More than 800 millions Ha of soils are affected by salinity worldwide (FAO, 2008). Soil salinity restricts plants' ability to obtain water through the roots, an effect known as the osmotic component of salt stress. In addition, high salt levels within plant cells exert toxic effects on them. Different levels of salt tolerance exist within plants, a higher degree of variation being evident within dicots (Munns and Tester, 2008).

The Salado River Basin (Buenos Aires, Argentina) is a big area of prime importance for cattle breeding. *Lotus tenuis* has been naturalized in this region, successfully becoming adapted to its restrictive conditions, such as the low phosphorus content and high salt levels in soils, as well as alternating cycles of drought and flooding, which limit the implantation of other forage legumes such as trefoil and lucerne. The knowledge of the physiological mechanisms underlying the adaptive success of *L. tenuis* to salt stress could contribute to the development of cultivated varieties with improved tolerance to soil salinity. Moreover, the study of salt stress responses in *Lotus* species other than *L. tenuis*, could contribute to draw a picture of salt tolerance within this genus, and thus identify species that could be used as sources of tolerance genes for breeding programs.

Ion homeostasis plays a key role during plant growth under salt stress, given that sodium and chloride accumulation results toxic to plant tissues and also provokes a nutritional imbalance. In the present work, the consequences of salt stress on ion accumulation and distribution at the whole plant level were evaluated in several *Lotus* species that differ in salt tolerance levels.

The following species were studied: *L creticus*, *L. burtii*, *L. filicaulis*, *L. japonicus* (ecotypes Gifu and MG-20), *L. corniculatus* (cv San Gabriel and a natural population native from the Ebro River Delta in Spain) and *L. tenuis* (cv Esmeralda). Plants were cultivated in pots containing vermiculite and irrigated with half-strength Hoagland solution. Once plants had two leaves, a progressive salinization protocol was initiated, by adding sodium chloride to the nutrient solution, till reaching three different final NaCl concentrations (50, 100 and 150 mM). Plant material was harvested 20 days after reaching the final sodium chloride concentration, and shoots were separated from roots. Sodium and potassium levels were determined by flame photometry, and chloride was determined by a colorimetric method. Under control conditions, *Lotus* species differed in their growth rates, *L. corniculatus* San Gabriel showing the highest level of dry matter production. Growth of all species was reduced when plants were irrigated with either 100 or 150 mM NaCl, *L. tenuis* showing the

highest level of dry matter production when cultivated in the presence of 150 mM NaCl. Salinity induced the accumulation of sodium and chloride ions in leaves of all *Lotus* species tested. When plants were irrigated with 150 mM NaCl, leaf sodium levels were similar for all species, but *L. japonicus* MG-20 showed the lowest levels of this ion. Leaf chloride levels were higher in *L. corniculatus* San Gabriel than in any other species. *L. creticus* and *L. corniculatus* San Gabriel exhibited contrasting results regarding the sodium/chloride ratio in leaves of plants cultivated in the presence of NaCl. In this way, *L. creticus* leaves accumulated sodium in a higher degree than chloride, while the opposite was valid for leaves of *L. corniculatus* San Gabriel.

Salinity had no effect on root sodium levels of any species tested, while on the contrary, root chloride was significantly enhanced in all species. At the highest NaCl concentration tested, *L. japonicus* MG-20 was the plant material that accumulated the highest chloride concentration in roots, as opposed to *L. japonicus* Gifu and *L. tenuis*, which showed the lowest chloride levels in the above-mentioned organ.

.The high amounts of chloride accumulated in leaves of *L. corniculatus* San Gabriel could be one of the causes of the strong growth inhibition of this cultivar under salt stress, given the toxicity of this anion. This observation is in good agreement with results obtained by Teakle *et al* (2007), who found *L. corniculatus* to accumulate higher chloride concentrations than *L. tenuis* under combined conditions of salinity and flooding. Interestingly, in the present work it was found that under salt stress, a natural *L. corniculatus* population native from a saline soil in the Ebro River Delta accumulated chloride levels much lower than those found in *L. corniculatus* San Gabriel. In this way, this population could be used as a germoplasm source for the development of new *L. corniculatus* varieties able to restrict chloride accumulation in leaves under salt stress and thus tolerate higher levels of soil salinity. Moreover, results of the present work emphasize the importance of non-domesticated *Lotus* populations, native from marginal environments, for the search of agronomical traits of interest as well as for the elucidation of physiological responses to stress conditions.

In addition, the differential selectivity for sodium and chloride accumulation in leaves of some *Lotus* species, suggest the existence of different strategies for keeping ion homeostasis at the whole plant level within this genus. As a whole, results suggest that the mechanisms involved in ion transport and the regulation of ion levels in *Lotus* species deserve further attention, which could contribute to the understanding of salt tolerance mechanisms and the development of cultivars well adapted to soil salinity.

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Effect of photoperiod and phosphorus nutrition on *Lotus tenuis* flowering

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The genus *Lotus* is widely distributed, being found within 26 and 58 ^{o} of latitude in both hemispheres. Occurrence of *Lotus* in Argentinean grasslands is strongly influenced by temporal and spatial variation. Phosphorus availability is a major constrain for *Lotus* productivity and flowering particularly depends on latitude. The interactive effects of P nutrition and photoperiod have received scarce attention. Here we studied the combined effect of photoperiod and P availability on flowering time and flower production in *Lotus tenuis*. For this goal two *Lotus tenuis* cultivars, "Tresur Chajá" and "La Esmeralda", were grown in a natracuol soil with or without the addition of 100 ppm P. Plants were grown at 8 (natural), 14 or 16 h photoperiod.

Results obtained unveil a strong influence of the genotype since flowering took place early for "La Esmeralda" than for "Tresur Chajá" regardless of the P level and the photoperiod. In turn, P-fertilization led to a reduction of the flowering time for both cultivars. Extended photoperiods led to early flowering and to increased number of flowers. Interestingly, anthesis in plants exposed to a 16 h photoperiod occurred in September (South hemisphere) while in those receiving a natural photoperiod it took place in December. Plants exposed to a 16h photoperiod displayed large internodes and small leaves with chlorosis symptoms.

It is concluded that *Lotus tenuis* is a long day plant. Optimal flower production requires a 16 h photoperiod but a 14 h photoperiod could be sufficient to ensure an adequate production of flowers. P nutrition positively affects flowering. Because *Lotus tenuis* is grown in the Flooding Pampa (Buenos Aires, Argentina) at the limits of the above mentioned photoperiods and that most soils in this zone are affected of low P availability, both factors are likely to constrain flower production under natural conditions thus limiting *Lotus tenuis* distribution. The existence of genotypic differences in the response to photoperiod suggests the suitability of *Lotus tenuis* breeding for improved photoperiodic response.