

An efficiently biological nitrogen fixation of non-native *Lotus tenuis* justifies its key role in the flooding Pampas (Argentina)

Maria Paula Campestre^{a,1}, Cristian Javier Antonelli^{a,1}, Matias Andres Bailleres^b, Maximiliano Gortari^a, Vanina Giselle Maguire^a, Juan Pedro Ezquiaga^b, Miguel Angel Taboada^c, Oscar Adolfo Ruiz^{a,*}

^a Instituto Tecnológico de Chascomús (INTECh). UNSAM-CONICET, Chascomús, Buenos Aires, Argentina

^b Chacra Experimental Integrada de Chascomús (CEICh). MDA-INTA, Chascomús, Buenos Aires, Argentina

^c Instituto de Suelos. CIRN-INTA, Castelar, Buenos Aires, Argentina

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ABSTRACT

Forage production is a major challenge in the Salado River basin, Argentina (also denominated “Flooding Pampas”), the largest livestock raising area. The successful naturalization of the European legume *Lotus tenuis* (native from Mediterranean area) has been of productive and environmental relevance to the region. The main objective of this work was to quantify the efficiency of *L. tenuis* through its ability to fix nitrogen biologically and thus strengthening their strategic role in the region. The ¹⁵N natural abundance method was used to evaluate the Biological Nitrogen Fixation (BNF) of *L. tenuis* in soil plots. Non-leguminous weed species in the same plots were utilized as reference plants. Simultaneously, evaluations were carried out in the greenhouse and the relative reproducibility of the field experiments. The results showed that *L. tenuis* promotion was accompanied by an increase in forage quality improving the Crude Protein (CP) content and the Digestibility of Dry Matter (DDM). Moreover, its contribution to N inputs to the system through BNF in symbiosis with native rhizobia was about 80%, a highly relevant percentage for these constrained agroecosystems where the presence of native legumes is very scarce. In conclusion, this study supports the hypothesis that *L. tenuis* promotion plays a strategic role in ecosystem sustainability in the Salado River basin, especially in soil constrained areas. Together with previously published data, including information on ruminant management, the results obtained contribute to supporting the idea that the naturalized legume plays a strategic role in the sustainability of marginal agricultural soils.

1. Introduction

The genus *Lotus* is included in the *Fabaceae* family, characterized for a high biological diversity and became an important key role in the sustainable agricultural systems, improving marginal soil ecosystems (Bay and Cotrufo, 2022). Within the family, the tribe Loteae DC is a monophyletic group composed by four genera including to 100–130 species. Most *Lotus* species are native to Mediterranean Europe, Asia, Africa and Australia and few ones from the Atlantic and Pacific Ocean Islands (Blumenthal and McGraw, 1999). Mediterranean area is recognized for their richness in species but *Lotus* species have a worldwide distribution, except in very cold regions and certain tropical areas of Southeast Asia and Central America. All *Lotus* spp described in America were introduced (Díaz et al., 2005; Escaray et al., 2012a).

The Flooding Pampa (Argentina, South America) is a heterogeneous ecosystem of approximately 90,000 km² (Imbellone et al., 2021). Most of the region is still covered in grasslands, which are the main forage resource for cow-calf operations (Cid et al., 2011). There are few native legumes in these grasslands because the area is characterized by severe phosphorus deficiency, high alkalinity and salinity levels, and periodic exposure to waterlogging, and all of them have not relevance as cow fodder (Antonelli et al. 2016, 2019). *Lotus tenuis* Waldst et Kit (ex-*Lotus glaber* Mill) is an introduced european legume species that has added sustainability by improving the quality of lowlands in the Flooding Pampa (Blumenthal and McGraw, 1999; Díaz et al., 2005; Escaray et al., 2012a). Due to its ability to adapt to restrictive conditions, *L. tenuis* quickly became naturalized and spread into the plains and humid lowland plant communities (Escaray et al., 2012a; Paz et al., 2012, 2014,

* Corresponding author. Technological Institute of Chascomús (CONICET-UNSAM). Buenos Aires. Argentina.

E-mail address: ruiz@intech.gov.ar (O.A. Ruiz).

¹ Both authors contributed equally and should be considered as first authors.

2014a, 2014b; Nieva and Ruiz, 2021). *L. tenuis* not only contributes to forage production and influences the growth of associated plant species in this area, but also contributes to restoration and phytoremediation of degraded environments (Vignolio and Fernández, 2011; Vignolio et al., 2016). The important role played by *Lotus* spp was also evaluated in european constrained environments (Escaray et al., 2010, 2012b) and arsenic contaminated soils (Soares et al. 2023). This major beneficial effect on the plant community structure and ecosystem function warrants including it among the key species (Antonelli et al., 2016; Escaray et al. 2010; Vignolio et al., 2016, 2018; Nieva and Ruiz, 2021). This effect is large relative to abundance of forage supply (i.e., high fodder community importance) Moreover, *L. tenuis* has a lower phosphorus requirement than other legumes of temperate climate, reaches its maximum biomass in summer with high forage quality, and maintains symbiotic associations with rhizobia, transferring the fixed N to the accompanying grasses (Castagno et al., 2011, 2014; Campestre et al., 2024). *Lotus* promotion is thus considered to be an appealing alternative to meet the needs for cow-calf operations during summer in soils of the Flooding Pampa (Antonelli et al., 2016; Bailleres et al., 2020). It is an agricultural practice that consists of removing broadleaf weeds or aggressive grass weeds using herbicides, thereby improving the ability of *Lotus* species to become established. Previous studies demonstrated that this practice does not alter significantly soil bacterial communities (Nieva et al., 2016), but increases the soil fungal diversity (Nieva et al., 2018), which has given rise to studies on the differential behavior of species of the genus *Lotus* spp in their interactions with different species of the genus *Fusarium* sp (Nieva et al., 2019). Its importance in plants biodiversity in the region is under evaluation. Moreover, some studies suggest that the glyphosate used in the promotion of *L. tenuis* maybe influence their symbiosis with mycorrhizae (Druille et al., 2015) and reduce the presence of soil culturable pseudomonads (Lorch et al., 2021).

Another possible reason for the successful naturalization of *L. tenuis* in these ecosystems may be due to the lack of significant pathogenic infections. Biotic stress studies in the genus *Lotus* spp have been very scarce and molecular evaluations of plant-pathogen interactions have been limited to the model species *L. japonicus* (Bordenave et al., 2013).

The *Rhizobium*-legume symbiosis provides an alternative to N fertilizers to balance N losses in the environment. Several studies have been conducted on the diversity of native rhizobia and their ability to nodulate *L. tenuis* in the Flooding Pampa (Echeverría et al., 2008, 2013; Estrella et al., 2009; Sannazzaro et al., 2011; Solans et al., 2015) and for *Glomus* mycorrhizal symbiosis (Sannazzaro et al., 2004, 2006; Echeverría et al., 2008, 2013). However, biological N₂ fixation by the *Mesorhizobium*-*L. tenuis* symbiosis in rangelands of the Flooding Pampas has never been quantified using N isotopes discrimination techniques, which limits the conclusions that may be drawn regarding the benefits of promoting a grass-legume mixture.

The overall benefits of including a N₂-fixing *L. tenuis* promotion in marginal areas of the Flooding Pampa cannot be assessed unless a reliable, accurate field measurement is made of the levels of air N fixation achieved. The ¹⁵N natural abundance method, discussed in detail elsewhere (Pauferro et al., 2010), is the most appropriate technique recommended for crops and rangeland legumes because it provides an overall estimate of the contribution of Biological N₂ Fixation (BNF) up to the time of sampling (Boddey et al., 2000). In addition, particularly in natural ecosystems, disturbance of the system is not required and measurements may be made on dried samples previously obtained from the field (Shearer and Kohl, 1989), determining the proportion of N in the legume plant derived from the air (%Nd_{fa}) and comparing its ¹⁵N natural abundance value (δ¹⁵N) to that of a non-fixing companion species (reference plant) (Pate et al., 1994). Selection of a non-fixing species as an appropriate reference plant must consider similar phenology, root architecture and distribution in the ecosystem evaluated. Furthermore, in naturalized legume species such as *L. tenuis*, the δ¹⁵N in N₂ fixing plants grown with N₂ in air as the only N source ('B' value) must be determined simultaneously in both the N₂-fixing and non-fixing species, in order to

account for discrimination against ¹⁵N during N₂ fixation (Carlsson et al., 2006). As mentioned previously, the B value can also be considered as the correction of the ¹⁵N fraction between below and aboveground tissues when only shoots are sampled for the quantification of BNF (Unkovich et al., 2008).

Despite the importance of *L. tenuis* to livestock-raising activity and restoration of degraded environments in the Flooding Pampa, there is a lack in the bibliography on reference plants and 'B' values for this species in combination with rhizobia isolated from fields. Our aims in this study were thus: (1) to identify and select a potential reference plant species for the application of the ¹⁵N natural abundance technique to assess the N₂ fixing performances of *L. tenuis* promotion; (2) to calculate the 'B' value of *L. tenuis* species in different phenological stages in order to obtain a more credible value during field evaluation and increase the samples number evaluated; (3) to determine the δ¹³C to understand variations in carbon assimilation; and (4) to evaluate the symbiotic capacity of *L. tenuis* and native rhizobia by determining %Nd_{fa} and the amount of N₂ fixed in soils of the Flooding Pampa. In addition to determining the %Nd_{fa} of promoted *L. tenuis* in the field, we wanted to evaluate the possibility of making future determinations in *L. tenuis* under greenhouse assays for the specific study of the effect on %Nd_{fa} with commercial strains of rhizobia and co-inoculation tests with PGPR (Maguire et al. 2020). We therefore determined %Nd_{fa} under greenhouse conditions in parallel to the field study, to determine whether the two evaluations were replicable.

2. Material and methods

2.1. Study sites description

The studies were conducted simultaneously at two sites: (1) a rangeland where *L. tenuis* had been promoted for five years, and (2) a natural grassland. These sites are located less than 1 km away from each other within the Chacra Experimental Integrada Chascomús (CEICh-Ministerio de Desarrollo Agrario de la Provincia de Buenos Aires – Instituto Nacional de Tecnología Agropecuaria, Argentina, 35°45'27"S, 58°3'18"W), in the Flooding Pampa region. The climate in this region is temperate subhumid with mean temperatures averaging 8.5 °C in winter and 21.5 °C in summer, and annual rainfall 850–1050 mm. Short flooding times occur at the beginning of almost every spring, sometimes followed by severe droughts during summer. The experimental area is mostly covered by Typic Natraquoll, (US Soil Taxonomy), in which the A horizon has 35 g kg⁻¹ organic matter content and 0.22 mg kg⁻¹ of extractable Fe. Natric and clays horizons were observed at 0.17 m, containing 533 g kg⁻¹ clay. Before legume promotion, plant communities consisted mainly of *Festuca arundinacea* (C3 exotic grass, perennial), *Thinopyrum ponticum* (C3 exotic grass, perennial), *Cynodon dactylon* (C4 exotic grass, perennial), *L. tenuis* (C3 exotic legume, perennial), *Sporobolus indicus* (C4 native grass, perennial) and exotic dicots, mainly *Compositae* species. Native legumes were largely absent. Simultaneously, an additional experiment under controlled conditions was conducted in a naturally-lit greenhouse located at Instituto Tecnológico Chascomús (INTECh), 20 km away from the CEICh. Temperature and irradiance were recorded throughout the experiment. Mean temperature was 26.3 ± 8.2 °C and mean maximum irradiance per day was 1150 ± 225 μmol m⁻² s⁻¹.

2.2. Experimental design

A completely randomized experimental design was used in all cases and all measurements were performed on 4 plants (=4 biological replicates for each experiment and species). A different number of pots were used depending on the type of determinations and whether non-legume species taken as references were grown simultaneously (experiment 3 with a total of 12 pots) or not (experiment 2 in greenhouse with 4 pots) together with *L. tenuis*, but keeping the number of plants evaluated unmodified (4 plants each experiment).

2.3. Rangeland measurement and floristic composition

Plant biomass was harvested from five 0.25 m² quadrats dropped at random in different places in the same paddock during the selected seasons. Plant samples were collected during three seasons (spring, summer and autumn) by clipping approximately 1 cm above the soil surface. Samples were dried at 70 °C to constant weight and dry biomass was calculated per hectare. This protocol was used in both paddocks: promoted and non-promoted.

For forage quality determinations, *L. tenuis* plants were clipped in spring, summer and autumn. The following parameters were evaluated: Crude Protein (CP) % by the Kjeldahl method, and Digestibility of Dry Matter (DDM) % by the method described by Tilley and Terry (1963). Samples were analyzed at the Laboratory of Animal Nutrition (INTA Balcarce).

Floristic composition was determined at 25 points along a 10-m linear transect (Gaucherand and Lavorel, 2007). A stake was placed vertically every 40 cm, at which points each individual plant was classified as annual grass, perennial grass, weed, *L. tenuis* or *Trifolium repens*. This protocol was used in both paddocks: promoted and non-promoted.

2.4. Experiment 1: promotion of *L. tenuis* rangeland and determination of total N, C, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$

In the *L. tenuis* promoted site, herbicide was applied on a surface area of 2 ha in seven annual cycles from June to August. The treatment consisted of Glyphosate (N-(phosphonomethyl) glycine) 3.5 L ha⁻¹, followed by two applications of 2,4 DB (4-(2,4-dichlorophenoxy) butyric acid) 1 L ha⁻¹ (Butyrac200®) and a single dose of Quizalofop-p-ethyl (Ethyl(R) -2-[4-(6-chloro-2-quinoxalylloxy) phenoxy] propionate) 1.2 L ha⁻¹ (Sheriff Max®). After five years of herbicide application, the plant species composition in promoted paddocks changed, with *L. tenuis* becoming the dominant species (Nieva et al. 2016, 2018).

L. tenuis shoots were harvested at three different phenological stages: 1- Early bloom (EB), 90 days after germination, 2- Full bloom (FB), 120 days after germination and 3- Regrowth (RG), 60 days post-full bloom (after simulating grazing). Harvested shoots were placed in paper bags and oven-dried at 60 °C for 72 h to constant weight. Samples were reduced to a fine powder using a roller mill, and homogenized for analysis of total N, C, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$.

For the estimation of the %Ndfa, it is necessary to compare its $\delta^{15}\text{N}$ value to that of a non-fixing species (Pate et al. 1994). To do so, it is recommended to select more than one non-fixing species (Unkovich et al. 2008). In this study, *Conyza bonariensis* and *Lythrum* sp were tested as reference species because of their similarity to the legume in terms of phenology and rooting profile with soil depth, closeness to the legume in the site, and accompaniment throughout the growth cycle. Shoot reference materials were harvested at the same time and at the same three phenological stages as the legume, and were processed in the same way for total N and $\delta^{15}\text{N}$ determination, and ^{13}C estimation. The results of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were expressed as parts per thousand (‰) deviations, in the ratio of the heavy to the light isotope of each element, based on international standards (Vienna Pee Dee Belemnite, V-PDB for ^{13}C , and atmospheric N₂ for ^{15}N).

2.5. Experiment 2: determination of *L. tenuis* 'B' value for the flooding Pampa region

The 'B' value is the $\delta^{15}\text{N}$ of shoots of legumes that are fully dependent upon N₂ fixation and sampled at the same growth stage and with the same rhizobia strains as the field plants (Unkovich and Baldock, 2008). It is best determined on plants grown in a greenhouse. The *L. tenuis* 'B' value was therefore determined in a naturally-lit greenhouse simultaneously with the field trial, and estimated for each phenological period sampled (EB, FB and RG). Harvested seeds from the promoted *L. tenuis* were scarified and disinfected with sulphuric acid for 3 min, washed 10

times with sterile distilled water, and sown in Petri dishes containing water/agar (0.8%). They were incubated for 7 d in a growth chamber with a 16/8 h day/night cycle at 24/21 ± 2 °C and 55/65 ± 5% relative humidity. Light (at an intensity of 250 μmol m⁻² s⁻¹) was provided by GroLux F 40 W fluorescent lamps. Then, the *L. tenuis* seedlings were grown in four 4 L pots containing sterile sand, and irrigated with N-free nutrient solution. They were inoculated following Unkovich et al. (2008) using a soil suspension with the native rhizobia present in the legume promoted field site. Soil was collected from the top 20 cm layer from many points (at least 20 different collected points) in the promoted area and mixed thoroughly. Then, 1 g of soil was mixed with 100 ml sterile water, and 3 ml of soil suspension per seedling were used as inoculum. At each harvest period, shoot material was placed in paper bags and oven-dried, ground, homogenized and analyzed for $\delta^{15}\text{N}$. The $\delta^{15}\text{N}$ contributed by seeds was dismissed due their small size (1000 *L. tenuis* seeds weighed less than 1 g).

2.6. Experiment 3: total N, C, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ determination of *L. tenuis* grown in pots in a greenhouse

Harvested seeds from the promoted *L. tenuis* were scarified and incubated in a growth chamber until germination. Seedlings were transferred to the greenhouse in six 4 L pots containing soil from the top 20 cm of the promoted *L. tenuis* land horizon. Seedlings were irrigated with water from a rainwater harvesting system. In parallel, another six 4 L pots were filled with legume promoted soil homogenized by sieving, irrigated and stimulated to germinate its seed bank. When the seeds germinated, all species except the ones previously selected as reference plants were removed, so that the ones kept developed accompanying the *L. tenuis* seedlings. Then, shoots of reference plants and *L. tenuis* at the same three phenological stages were harvested, placed in paper bags, oven dried, ground and homogenized for determination of total N, C, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$.

2.7. Estimates of biological nitrogen fixation and N and C isotope ratios

For %Ndfa estimation, samples were reduced to a fine powder using a roller mill and homogenized for analysis. Aliquots were loaded in a Thermo Delta Advantage isotope ratio mass spectrometer couple with a Flash 2000 Elemental Analyzer at the Laboratorio de Isótopos Estables en Ciencias Ambientales (LIECA, Argentina) to obtain the N and C isotope ratio and the total N and C content. Natural abundances of ^{15}N and ^{13}C were expressed using 'delta' notation (‰):

$$\delta = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 \quad (1)$$

where R_{sample} and R_{standard} are the $^{15}\text{N}:^{14}\text{N}$ and $^{13}\text{C}:^{12}\text{C}$ ratios of samples and of the standards, which are atmospheric N₂, for N, and Pee Dee Belemnite, for C.

Although δ provides information on the $^{13}\text{C}:^{12}\text{C}$ in tissues, it is often preferable to express the values as the leaf carbon isotope discrimination (Δ):

$$\Delta = (\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{sample}}) / (1 - \delta^{13}\text{C}_{\text{sample}}/1000) \quad (2)$$

where $\delta^{13}\text{C}_{\text{air}}$ is the $\delta^{13}\text{C}$ value of air (−8‰) and $\delta^{13}\text{C}_{\text{sample}}$ is the value measured in the plant.

Estimates of the %Ndfa were made using the ^{15}N natural abundance technique. The proportion of fixed N in the plant was calculated using the following formula (Shearer and Kohl, 1989):

$$\% \text{Ndfa} = [(\delta^{15}\text{N}(\text{reference}) - \delta^{15}\text{N}(\text{fixing})) / \delta^{15}\text{N}(\text{reference}) - \text{B}] \times 100 \quad (3)$$

where; $\delta^{15}\text{N}(\text{reference})$ is the average value of $\delta^{15}\text{N}$ signals of reference plants (*C. bonariensis* and *Lythrum* sp), $\delta^{15}\text{N}(\text{fixing})$ is the average value of $\delta^{15}\text{N}$ signals of *L. tenuis* and 'B' is the $\delta^{15}\text{N}$ value for fixing plants grown in the absence of assimilable mineral N.

2.8. Statistical analysis

Data were subjected to t-tests for differences between experimental sites and one-way ANOVA analysis for differences between harvest times for each experimental site. Duncan's test was used for multiple comparisons ($P < 0.05$). All statistical analyses were performed using the INFOSTAT statistical software package (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina) and are showed in [Supplementary Fig. 1](#).

3. Results

3.1. Determination of plant biodiversity in rangelands and forage quality

Relative floristic diversities between C4 and C3 species changed in both the promoted Lotus and the grassland sites in all seasons ([Table 1 A and B](#)). In the grassland, the presence of legumes was scarce during spring and summer, but slight growth of *Trifolium repens* (8.00%) and *L. tenuis* (8.66%) was observed in autumn. Perennial grasses clearly prevailed (67.00%–75.00%) over annual grasses (8.66%–19.33%). As expected, in the promoted Lotus site, *L. tenuis* was the predominant species during the three study seasons (61.00%–74.00%). *T. repens* was only observed during spring and autumn (2.33% and 11.33%, respectively). No difference in biomass was observed between promoted Lotus and grassland in spring and summer ([Table 2](#)). In contrast, higher biomass was measured in grassland in autumn. When summed, no difference in annual biomass was observed between promoted Lotus and grasslands sites ($p = 0.286$). However, forage quality was better in the promoted Lotus site ([Table 3](#)). *L. tenuis* provided not only more CP compared to the grassland, but also a resource with higher DDM. Moreover, CP values higher than 17% were observed in promoted Lotus in all seasons, while only 12% CP was observed in the grassland site during spring. Differences in CP (17.53% and 8.33%, respectively) and DDM (71.83% and 60.50%, respectively) were greater in summer ([Table 3](#)).

3.2. Comparison of $\delta^{13}\text{C}$ and $\Delta^{13}\text{C}$ in greenhouse and field assays according to Lotus tenuis phenology

As observed in [Supplementary Fig. 2](#), during EB and FB, there was no difference in $\delta^{13}\text{C}$ between greenhouse and field assays for *L. tenuis*. ($\delta^{13}\text{C}$ approximately -28‰). Differences were only observed during the RG stage, with more negative $\delta^{13}\text{C}$ values in the field (-29‰). Regarding greenhouse assays, $\delta^{13}\text{C}$ was more negative during FB (-29.41‰) than during EB (-28.36‰) and RG (-28.76‰), while for field assays, $\delta^{13}\text{C}$ was more negative during RG (-29.32‰) than during EB (-28.70‰)

Table 1.A

Relative seasonal percentage distribution of C3/C4 species between promoted Lotus tenuis pastures and natural grasslands using Student's t-test. Results with the same letter mean that there is no statistical difference between forage types in each seasonal period. Conyza bonariensis and Lythrum sp were included in Weeds.

	Season	Promoted Lotus (%)	Grassland (%)
<i>Trifolium repens</i>	Spring	2.33 ± 0.58a	0.33 ± 0.58b
	Summer	0.00 ± 0.00a	0.33 ± 0.60a
	Autumn	11.33 ± 6.03a	8.00 ± 5.30a
<i>L. tenuis</i>	Spring	74.00 ± 12.49a	5.00 ± 2.00b
	Summer	71.33 ± 5.51a	7.66 ± 3.10b
	Autumn	61.33 ± 3.22a	8.66 ± 2.52b
Annual grass	Spring	3.00 ± 2.00b	19.33 ± 2.52a
	Summer	13.33 ± 2.31a	8.66 ± 3.51a
	Autumn	4.00 ± 2.00b	12.33 ± 3.22a
Perennial grass	Spring	14.33 ± 9.87b	70.00 ± 2.00a
	Summer	5.33 ± 3.10b	75.00 ± 2.65a
	Autumn	13.66 ± 5.51b	67.00 ± 5.00a
Weeds	Spring	6.33 ± 1.53a	5.33 ± 0.58a
	Summer	10.00 ± 5.00a	8.33 ± 2.52a
	Autumn	9.66 ± 2.52a	4.00 ± 1.00b

Table 1.B

Comparison of relative seasonal percentage distribution of C3/C4 species within promoted Lotus tenuis paddocks and natural grasslands using one-way ANOVA to compare seasonal moments within each experimental site.

	Promoted Lotus			
	Spring (%)	Summer (%)	Autumn (%)	p-value
<i>Trifolium repens</i>	2.33 ± 0.58	0.00 ± 0.00	11.33 ± 6.03	0.046
<i>L. tenuis</i>	74.00 ± 12.49	71.33 ± 5.51	61.33 ± 3.22	0.0001
Annual grass	3.00 ± 2.00	13.33 ± 2.31	4.00 ± 2.00	0.160
Perennial grass	14.33 ± 9.87	5.33 ± 3.10	13.66 ± 5.51	0.097
Weeds	6.33 ± 1.53	10.00 ± 5.00	9.66 ± 2.52	0.585

	Grassland			
	Spring (%)	Summer (%)	Autumn (%)	p-value
<i>Trifolium repens</i>	0.33 ± 0.58	0.33 ± 0.60	8.00 ± 5.30	0.002
<i>L. tenuis</i>	5.00 ± 2.00	7.66 ± 3.10	8.66 ± 2.52	0.126
Annual grass	19.33 ± 2.52	8.66 ± 3.51	12.33 ± 3.22	0.003
Perennial grass	70.00 ± 2.00	75.00 ± 2.65	67.00 ± 5.00	0.065
Weeds	5.33 ± 0.58	8.33 ± 2.52	4.00 ± 1.00	0.168

Table 2

Seasonal productivity (kg DMha⁻¹) of promoted Lotus tenuis and natural grassland paddocks.

Period	Promoted Lotus		Grassland		p-value
	kg DM ha ⁻¹	SD	kg DM ha ⁻¹	SD	
Spring	1769.33	668.09	2167.00	396.30	0.4253
Summer	2897.73	205.00	2542.00	151.21	0.0729
Autumn	1641.42	84.85	2323.50	154.00	0.026
Annual Biomass	6308.49	930.41	7032.50	701.51	0.2867

Table 3

Forage quality determinations by Crude Protein (CP) % and Digestibility of Dry Matter (DDM) % of promoted Lotus tenuis and natural grassland paddocks. Results with same lowercase (CP analysis) and uppercase (DDM analysis) letter do not differ statistically. The results are presented with standard deviations.

Period	Promoted Lotus		Grassland	
	CP (%)	DDM (%)	CP (%)	DDM (%)
Spring	20.33 ± 2.51 ^a	75.17 ± 5.35 ^A	11.63 ± 3.80 ^b	62.33 ± 1.15 ^B
Summer	17.53 ± 1.08 ^a	71.83 ± 2.02 ^A	8.33 ± 2.00 ^b	60.50 ± 1.00 ^B
Autumn	18.20 ± 0.17 ^a	73.67 ± 2.56 ^A	16.70 ± 1.46 ^b	63.00 ± 2.00 ^B

and FB (-28.75‰). The trend was the same for $\Delta^{13}\text{C}$ ([Supplementary Fig. 3](#)), for which differences were only observed during RG stage. For greenhouse assays, $\Delta^{13}\text{C}$ was higher during FB than during EB and RG, while no difference was observed in the field during any of the three phenological stages.

3.3. Modification of $\delta^{15}\text{N}$ in greenhouse and field assays according to Lotus tenuis phenology

The $\delta^{15}\text{N}$ values from the reference plants, which should be derived entirely from the soil, were always positive and different from the $\delta^{15}\text{N}$ values of *L. tenuis* ([Fig. 1](#)). As expected, the $\delta^{15}\text{N}$ value determined in *L. tenuis* shoots was lower than that of the reference value ($\delta^{15}\text{N}$) for non- N_2 -fixing plants grown at the same site ([Fig. 1](#)). Because of the very high proportion of N derived from BNF, $\delta^{15}\text{N}$ values of *L. tenuis* nodulated by native rhizobia were negative both in the greenhouse (EB -0.5‰ and FB -0.60‰) and in the field (EB -0.02‰ and FB -0.08‰). In addition, these values were close to the B values, with differences with even more negative B values only in the EB stage (EB -2.1‰ and FB -1.7‰). For *C. bonaerensis*, the $\delta^{15}\text{N}$ value was significantly different in greenhouse and field during the EB stage ($4.70 \pm 0.21\text{‰}$ vs $7.34 \pm 1.18\text{‰}$). No difference was observed between sites during FB. For *Lythrum* sp., there was

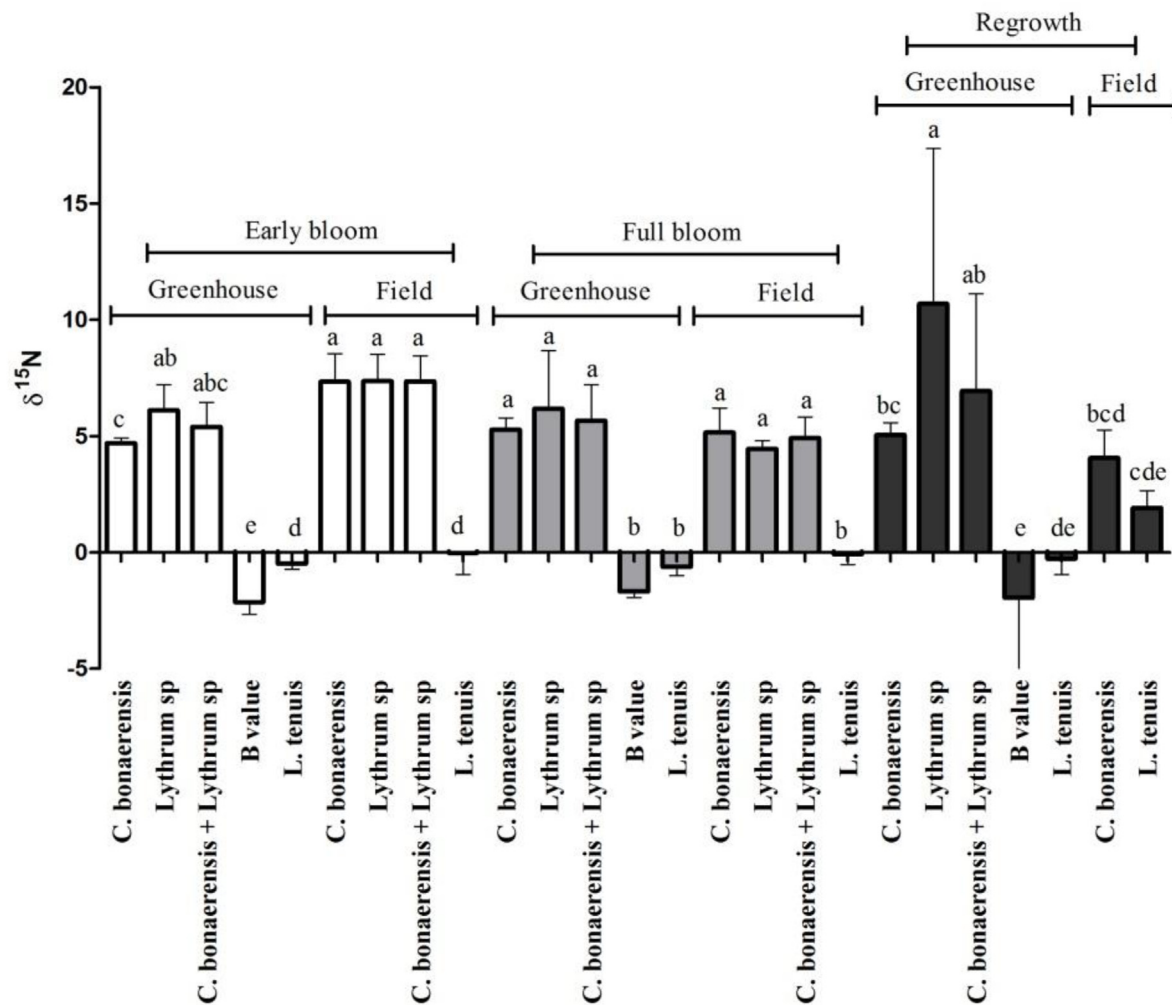


Fig. 1. Natural abundance of ^{15}N ($\delta^{15}\text{N}$) in *L. tenuis* and non-legume plant species grown in Greenhouse and Field sites in the Flooding Pampa during three different phenological stages (Early Bloom = white bars, Full Bloom = grey bars and Regrowth = black bars). B value is also included for each greenhouse analysis. Bars with the same letter do not differ statistically (Duncan test; $P < 0.05$).

no difference in $\delta^{15}\text{N}$ between sites during EB and FB. The combination of these reference species showed no difference between sites for the EB and FB stages ($5.4 \pm 0.6\text{‰}$ and $5.7 \pm 1.4\text{‰}$, respectively). In the RG stage, *Lythrum* sp. $\delta^{15}\text{N}$ values varied greatly in the greenhouse. No *Lythrum* sp. plant was observed in the field in RG phenological stage, so only $\delta^{15}\text{N}$ values were obtained for *C. bonaerensis* in this site. $\delta^{15}\text{N}$ of the B value did not differ between EB and FB (-1.9‰ ; $p = 0.910$). In *L. tenuis*, $\delta^{15}\text{N}$ values were negative in the greenhouse (-0.26‰) and positive in the field (1.92‰), differing from each other (Fig. 1).

3.4. Proportion of N derived from the air (%Nd_{fa}) and C/N ratios in greenhouse and field assays according to *Lotus tenuis* phenology

Regarding N concentration, *L. tenuis* values did not differ between the greenhouse and the field in the EB and the FB stages (Fig. 2A). In contrast, in the RG stage, N concentration was higher in the field than in greenhouse. N was accumulated differently according to the phenological stage. In the greenhouse, N concentration was significantly lower ($P < 0.05$) in the RG than in the EB and FB stages. In contrast, ^{15}N concentration was higher in the field in the RG stage (Fig. 3C). With regard to C accumulation, similar values were observed in the EB and RG stages, while C accumulation was lower in the field in the RG stage (Fig. 2 B). The resulting C/N ratios were similar between greenhouse and field sites

during EB, slightly though not significantly higher ($p = 0.320$) in the field during FB, and significantly higher ($p = 0.001$) in the greenhouse during RG (Fig. 2C).

3.5. %Nd_{fa} values in *L. tenuis* and reference plants according to phenological stages

The two non-leguminous reference species selected – *C. bonaerensis* and *Lythrum* sp. – achieved mean $\delta^{15}\text{N}$ values of 4–10‰, depending on the site, phenological stage and plant species, while the mixture of the two reference species achieved mean $\delta^{15}\text{N}$ values of 5–7‰, depending on site and phenological stage (Fig. 1).

There was no significant effect from either of the reference plant species (%Nd_{fa} = 80%) in the EB and FB phenological stages, or from the mixture of both (Fig. 3A and B). Thus, it was found that there was no difference in using either of the two species or the mixture of both in these stages. Because *Lythrum* sp. was not found in the RG phenological stage, *C. bonaerensis* was used as the only reference plant (Fig. 3C). It is interesting to note that the %Nd_{fa} results for *L. tenuis* in the greenhouse were similar during the EB, FB and RG stages (Fig. 3). The same was observed in the field during EB and FB. In contrast, during RG, %Nd_{fa} was lower in the field than in the greenhouse (Fig. 3).

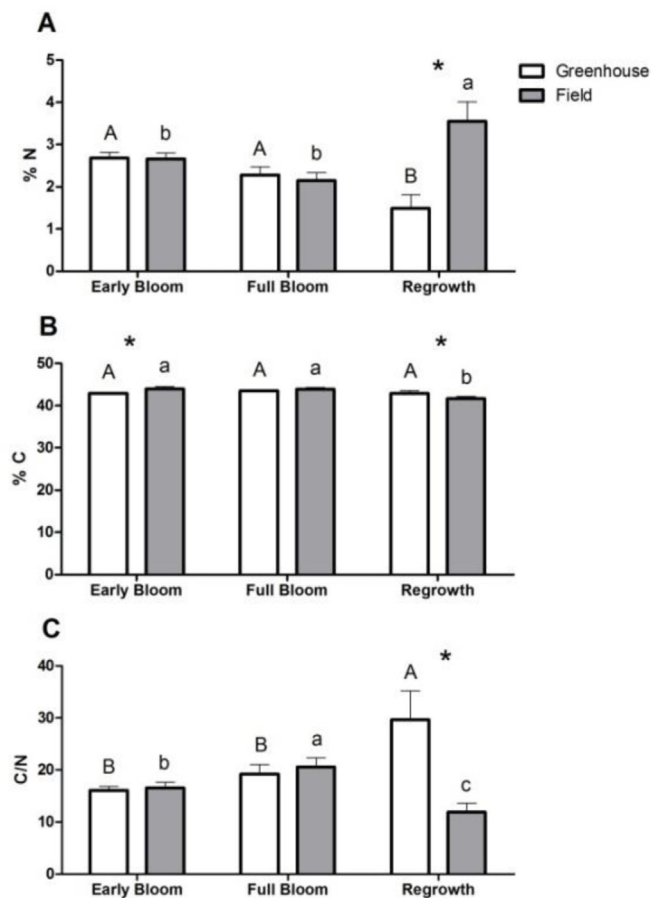


Fig. 2. Percentages of Nitrogen-%N- (A) and Carbon-%C- (B) accumulation in *L. tenuis* and changes in C/N ratio between evaluation sites (C) during three different phenological stages (Greenhouse determinations = white bars and Field determinations = grey bars). Bars with the same uppercase (Greenhouse analysis) and lowercase (Field analysis) letters do not differ statistically (Duncan test; $P < 0.05$). Bars with asterisks represent significant differences between study sites in a specific phenological stage (T test; $P < 0.05$).

4. Discussion

4.1. Rangeland improvement by *L. tenuis* promotion

Temperate rangelands in South America consist mainly of a few C3 grass and legume species, offering a fairly good biomass production in winter and providing forage for cow-calf operations in a season when the forage quality of native grasslands is scarce (Bresciano et al., 2019) due to the lignification process of certain species, which contrasts with what was observed for *L. tenuis*. Thus, the presence of C4 species in these environments provides a chance to improve biomass stability, ensuring year-round productivity, particularly in the Flooding Pampa, where specific edaphic limitations such as high levels of halo-hydromorphism are very common. This statement is based on the fact that the spring-summer-autumn growth cycle of the C4 species in the ecosystem (*Paspalum dilatatum*, *Sporobolus indicus*, *Cynodon dactylon*) can improve biomass stability. Therefore, we understand that they complement the forage gap left by the C3 species (*Festuca arundinacea*, *Thinopyrum ponticum*) with an autumn-spring-summer growth cycle. In this sense, *L. tenuis*, a species with a spring-summer-autumn growth cycle, would be replacing the aforementioned C4 species, improving the productivity and nutritional quality of the forage. As shown in Table 1, the grassland was mainly composed of annual and perennial C4 grasses such as *Paspalum dilatatum*, *Sporobolus indicus* and *Cynodon dactylon*. This agrees with previous studies in the region showing that the prevalence of C4 over C3

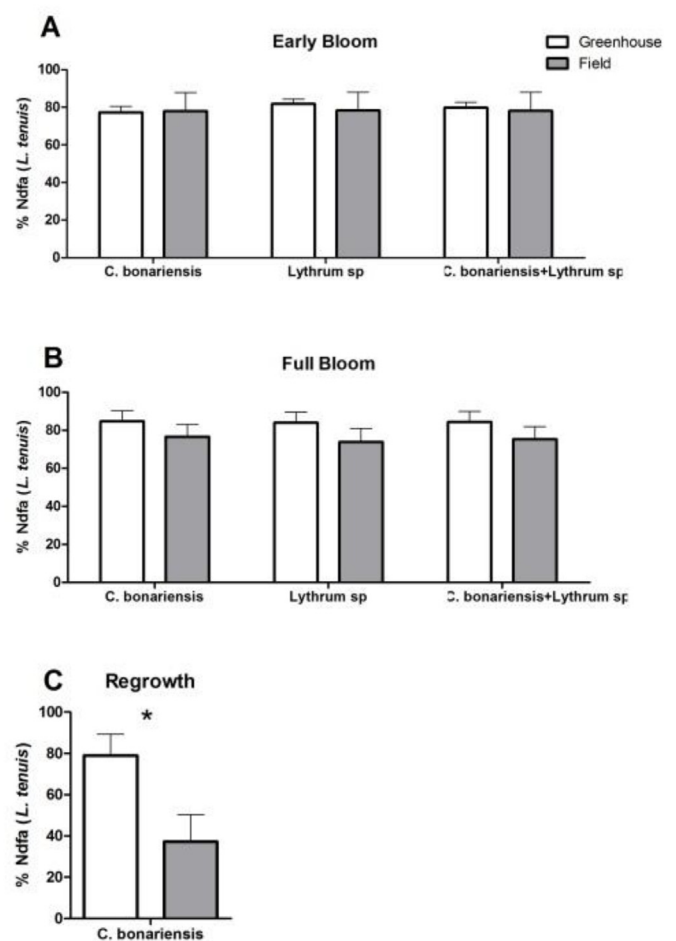


Fig. 3. *L. tenuis* proportion of N derived from the air (%Ndfa) in the different evaluation sites (Greenhouse determinations = white bars and Field determinations = grey bars) during three different phenological stages: Early Bloom (A), Full Bloom (B), Regrowth (C). Bars with asterisks represent significant differences between study sites (T test; $P < 0.05$).

grasses is mainly due to edaphic conditions, and especially to the topsoil pH gradients (Perelman et al., 2001; Cid et al., 2011). Their relative abundances can change at a particular site, and such changes may have direct consequences on ecosystem processes such as net primary production and many other ecosystem services. Despite its contributions, this forage resource (C4 grasses) has poor quality, as shown by its low CP % and DDM % values (Table 3), and does not enable efficient cattle production in the region, causing either a considerable increase in the need for feed supplements or a decrease in livestock reproductive performance.

It is probably controversial to favor the promotion of an exotic species to the detriment of the native flora and to associate this change with the sustainability of the ecosystem. However, it must be taken into account that the Salado River basin environment is subject to cattle grazing despite its ecological fragility. Furthermore, it should be taken into account that *L. tenuis* has a tolerance to abiotic stresses that is superior to other forage legumes such as alfalfa, clover, etc. That is a key factor that justifies its successful naturalization. The periods of flooding and dryness alternating between winters and summers respectively, in soils with high salinity and, in some sectors, extreme alkalinity, explain their natural and increasing presence in soils that do not have presence significantly of native forage legumes. The naturalization of *L. tenuis* in these environments maybe allowed an increase in the N/C ratio. The promotion using herbicides, constitute a experimental protocol that accelerates the implantation and persistence of *L. tenuis*.

4.2. Plant physiological status and photosynthetic pathways can be assessed by considering carbon isotope discrimination values

In this regard, the *L. tenuis* $\delta^{13}\text{C}$ and $\Delta^{13}\text{C}$ values found in this study, of about -28‰ and 20‰ , respectively (Supplementary Figs. 2 and 3) are considered typical of C3 plants following the Calvin cycle (Luo et al. 2021; Sun et al. 2011). The introduction of C3 forage legumes such as *L. tenuis* can modify the C3:C4 grass cover ratio, resulting in a richer nutritional forage resource (Tables 1 and 3). It has also been widely reported that legumes can contribute to improving not only the nutritional value of the rangeland, but also the herbage yield of the improved rangelands (Vignolio et al. 2016) and sustainability beyond current extents. In addition, carbon sequestration can be achieved after legume introduction (overseeding) in grazed natural grasslands, depending on grazing management practices, as reported by Bondaruk et al. (2020) in a case study in Argentina on commercial farms using *L. subbiflorus*. Successful naturalized *L. tenuis* stands, and the consociation between *L. tenuis* and *Paspalum dilatatum* (Striker et al. 2008; Vignolio and Fernández, 2011) were found to be key to future forage contributions for these highly constraining environments. However, a trade-off between increased rangeland production and decreased vegetation stability (long-term integrity) may occur in legume-improved grasslands, requiring further studies on the effects of other mechanisms such as grazing management options (Vecchio et al. 2019).

4.3. Parameters for the determination of the BNF by *Lotus tenuis*

Accurate estimation and quantification of BNF by legumes depends on the methodology applied. To date, most of the work undertaken on the evaluation of N_2 fixation by *L. tenuis* has been performed in the framework of forage mixtures, using the acetylene reduction technique and estimated by weighing the nitrogenase activity of samples by legume cover (Refi and Escuder, 1998). In this study, we have obtained the parameters required to enable determination of an accurate value of the N_2 fixed by this legume, and thereby, gain better understanding of its economic and environmental importance for the region. The %N accumulated by field plants was approximately 2.7% during spring (47 kg N ha^{-1}) and 2.15% during summer (62 kg N ha^{-1}). This N_2 fixation potential is within the range found in previous studies using the nitrogenase activity method, such as $27\text{--}42\text{ kg N ha}^{-1}$ per year in grazed tall fescue/*L. tenuis* swards growing on an alkaline hydromorphic soil, or $14\text{--}59\text{ kg N ha}^{-1}$ per year in tall fescue/white clover swards (Díaz et al., 2005). Furthermore, Danso et al. (1991) reported 91 % Ndfa by *L. corniculatus* when tall fescue was considered as the reference plant. In our study, Ndfa was determined to be 80 %, without significant differences between the reference plants considered in the calculation. The amount of nitrogen in the soil can also be increased with the presence of *L. tenuis*, as found by (Vignolio et al., 2018) two years after planting, with the soil N being increased from 30.5 mg kg^{-1} to 66.4 mg kg^{-1} .

A very important aspect of N_2 fixation estimations using the ^{15}N natural abundance technique is a good selection of reference plants. In this selection, it is important to bear in mind that soil $\delta^{15}\text{N}$ can vary between N pools, soil depths, and over time, and that the reference plant should follow the same dynamics in N uptake and take N from the same soil N pools as the N_2 fixing species (Unkovich et al. 2008). In our trials, there was no significant difference according to reference plant species used in the contribution of BNF of *L. tenuis* inoculated with native rhizobia (% Ndfa = 80%) for the EB and FB phenological stages (Fig. 1), nor was any difference observed using the species mix (Fig. 1). The use of either species or the mixture of both is thus indistinct in these stages. However, although the bibliography recommends using a mixture of reference species, *Lythrum* sp. was not found in the RG phenological stage and therefore the species *C. bonaerensis* was used as the reference plant. Moreover, quantification BNF by legumes through the ^{15}N natural abundance technique requires accurate determination of the B value, which can vary among species (and among accessions of the same plant

species), environmental conditions and plant physiological status (Boddey et al., 2000; Nebiyu et al., 2014). Most of the B values determined for legumes are usually in the range of 0 to -2‰ , with few exceptions (Okito et al. 2004). In our study, the B values for *L. tenuis* in two phenological stages were -2.1 and -1.7 for EB and FB, respectively, which are within said range. These B values constitute the first report for *L. tenuis* in two phenological stages in these specific environments of the Salado River basin.

4.4. BNF from *L. tenuis*, in different phenological states in greenhouse and field sites

Except for the greenhouse assays during the RG stage, N concentration in *L. tenuis* leaves was always higher than that in either of the non-fixing plants or the mixture of them (data not shown). These results also evidenced that the ^{15}N natural abundance method to estimate % Ndfa should be circumscribed to EB and FB phenological stages in greenhouse conditions.

The $\delta^{15}\text{N}$ value was $-1.37\text{‰} \pm 0.6$, which was close to the atmospheric value but different from that of the available soil N determined in a large number of non- N_2 -fixing weeds ($+6.4\text{‰} \pm 0.6$), pointing to a major contribution of N_2 fixing in this plant species. As has been discussed in a previous publication (Unkovich et al., 2008), there can be some issues with applying the ^{15}N natural abundance technique to regrowth because of the potential complication of N recycling in the N-fixing species. Our results confirm the importance of forage management avoiding overgrazing, which also impacts N fixation, especially grazing management practices that influence plant regrowth (Vecchio et al., 2019). In this regard, protocols should be designed on the basis of the legume “nutritional cycles”, avoiding overgrazing during critical phenological periods when the sink/source relationship of C and N must be considered in a framework of grasslands conservation (Abdalla et al., 2018). As was reported for *Medicago sativa*, total or partial removal of the photosynthetic area results in the mobilization of C and N reserves from roots to shoots, generating an inversion of source and sink organs (Teixeira et al., 2007). The management system used, and especially the defoliation periods, will affect the pattern of reserve accumulation and subsequent regrowth (Vignolio et al. 2018). After shoot removal, regrowth of the new shoots in *L. tenuis* plants must be supported by non-structural carbohydrates along with N compounds (such as proteins and amino acids) stored in the taproots especially during early regrowth (Striker et al., 2011). These non-structural carbohydrates are used to support respiration of the crown and taproots until photosynthesis is re-established. Although a larger reduction in non-structural carbohydrates in comparison with N root reserves has been observed, regrowth is linked to N reserves in roots rather than to C reserves (Striker et al. 2011). Moreover, it is accepted that a reduction in N_2 -fixation occurs in response to a lack of nodulation ability or increased N availability from regrowth stage because of a major input of N from the soil. Studies have shown that after shoots are removed, nodule functioning is reduced during the first days of regrowth, and is concomitant with an 88 % decrease in N fixation 24 h after cutting. In addition, as has been reported nutrient uptake and photosynthesis can be greatly reduced following defoliation (Skinner et al. 1999).

5. Conclusions

We consider that *L. tenuis* could aid rehabilitation of disturbed or marginal edaphic areas by adding fixed atmospheric nitrogen that can be used by other plants (non-legumes), including typical C4 species (*Distichlis* spp and others) growing in association. However, many leguminous species do not grow well under adverse soil conditions such as flooding soils. The knowledge that some leguminous plants, mainly *Lotus* spp., grow well in these constrained soils focused our attention on studying the BFN contribution in these harsh conditions. The results of the study confirm that in the Salado River basin, the naturalization of *L. tenuis* to

more constrained areas where there is no significant presence of native legumes could substantially modify the volume of N cycling, also influencing carbon sequestration rates. As far as we know, this is the first study where the N_2 contribution of *L. tenuis* in symbiotic association with native rhizobia is effectively determined in the Flooding Pampa. Also, no information on seasonal variation or levels of N_2 fixation of *L. tenuis* rangelands was available. The observation that *L. tenuis* promotion is an appealing forage alternative in marginal areas to meet the nutritional requirements of livestock in the summer season has been confirmed by the current data. This, along with data previously published, provides another parameter to validate *L. tenuis* playing a significant role in the constrained areas in the Salado River basin and, in consequence, on cow-calf operations productivity in the most important livestock breeding region of Argentina.

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CRedit authorship contribution statement

Maria Paula Campestre: Writing – original draft, Investigation, Conceptualization. **Cristian Javier Antonelli:** Methodology, Investigation, Conceptualization. **Matias Andres Bailleres:** Visualization, Methodology. **Maximiliano Gortari:** Visualization, Methodology, Investigation, Data curation. **Vanina Giselle Maguire:** Visualization, Methodology, Data curation. **Juan Pedro Ezquiaga:** Methodology, Investigation. **Miguel Angel Taboada:** Visualization, Validation, Supervision, Methodology, Conceptualization. **Oscar Adolfo Ruiz:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.farsys.2024.100122>.

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