

Biological Nitrogen Fixation, Carbon assimilation and plant performance of *Lotus tenuis*, contribute to define a strategic role in the lowlands in the Salado River Basin (Argentina)

MARIA PAULA CAMPESTRE

Instituto Tecnológico de Chascomús (INTECh). UNSAM-CONICET

CRISTIAN JAVIER ANTONELLI

Instituto Tecnológico de Chascomús (INTECh). UNSAM-CONICET

MATIAS ANDRES BAILLERES

Instituto Tecnológico de Chascomús (INTECh). UNSAM-CONICET

VANINA GISELLE MAGUIRE

Instituto Tecnológico de Chascomús (INTECh). UNSAM-CONICET

MIGUEL ANGEL TABOADA

Instituto Tecnológico de Chascomús (INTECh). UNSAM-CONICET

OSCAR ADOLFO RUIZ (✉ ruiz@intech.gov.ar)

Instituto Tecnológico de Chascomús (INTECh). UNSAM-CONICET

Research Article

Keywords: Legume forage, Salado River Basin, $\delta^{15}\text{N}$, $\delta^{13}\text{C}$, %Ndfa, Lotus promotion

Posted Date: August 2nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-759255/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background and Aims

Forage production constitutes a great challenge for the Salado River Basin (Argentina), the major area devoted to livestock in the country. The successful naturalization of the European legume *Lotus tenuis* has been a productive and environmental relevance for the region. This study aims to evaluate its strategic contribution, reporting for the first time the B value for this species in these marginal environments for agriculture.

Methods

The ^{15}N natural abundance method was used to evaluate the BNF of *L. tenuis* at soil plots and non-leguminous weed species in the same plots were utilized as reference plants. The assays included determinations of the ^{13}C isotope, as well. Simultaneously, evaluations were carried out in the greenhouse for the determination of the B value of *L. tenuis* and the relative reproducibility of the field experiments.

Results

The results obtained demonstrated that the *L. tenuis* promotion is accompanied by an increase in forage quality, due to the predominance of this C3 legume species, over C4 species. Moreover, its contribution to N inputs to the system, through the BNF with native rhizobia demonstrated to be about 80%, a highly relevant percentage for these constrained agroecosystems.

Conclusions

This work supports the hypothesis that *L. tenuis* promotion plays a strategic role in the sustainability of the ecosystem, especially in soil constrained areas. Joined with data previously published, the results obtained contribute to support the criteria that define the naturalized legume playing a strategic role in the sustainability of agriculture marginal soils.

Introduction

The Flooding Pampa (Argentina) constitutes a heterogeneous ecosystem covering approximately 90000 Km² area in South America (Imbellone et al. 2021). Most of the region is still covered by grasslands that are the main forage resource for cattle breeding (León et al. 1984; Cid et al. 2011) There are few native legumes in these grasslands because this area is characterized by severe phosphorus deficiency, high alkalinity and salinity levels, together with periodic exposure to waterlogging (Antonelli et al. 2019). *Lotus tenuis* Waldst et Kit (ex *Lotus glaber* Mill) is an introduced European legume species that has added sustainability improving the quality of lowlands in the Flooding Pampa (Escaray et al. 2012). Due to its ability of adaptation to restrictive conditions, *L. tenuis* became quickly naturalized and spread into the plains and humid lowland plant communities (Miñón et al. 1990; Escaray et al. 2012). *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 et al. 1999). This beneficial effect on the vegetal community observed justify its inclusion within the *keystone* species because of the large effects on community structure and ecosystem function (González-Robles et al. 2020; Campestre et al. 2020). This effect is large relative to abundance of forage supply (i.e., high fodder community importance) (Bailleres et al 2020). Moreover, *L. tenuis* have a lower phosphorus requirement than other legumes of temperate climate, reaches its maximum biomass in summer with high forage quality and maintain symbiotic associations with rhizobia, transferring the fixed N to the accompanying grasses (Vignolio et al. 2010). Due to aforementioned characteristics, *Lotus* promotion has been conceived as an appealing alternative to meet the needs for cattle production during summer season in soils of the Flooding Pampa (Antonelli et al. 2016; Bailleres et al. 2020). This agricultural practice consists of removing broad leaf weeds or aggressive grass weeds using herbicides, and therefore improving *Lotus* species establishment ability (Díaz et al. 2005). This practice does not alter soil bacterial communities and led to increased fungal diversity (Nieva et al. 2016, 2018, 2019).

The *Rhizobium*-legume symbiosis provides an alternative to N fertilizers to balance N losses in the environment. Several works were carried out on the diversity of native rhizobia and their ability to nodulate this naturalized *L. tenuis* species in the Flooding Pampa (Fulchieri et al. 2001; Estrella et al. 2009; Sannazzaro et al. 2011). However, the symbiotic capacity of the *Mesorhizobium* - *L. tenuis* association to fix biological N₂ in the field using the N isotope discrimination technique, has never been determined in this area.

The overall benefits of including a N₂-fixing *L. tenuis* promotion in marginal areas of the Flooding Pampa cannot be assessed unless a reliable and accurate field measurement is made of the levels of fixation achieved. The ¹⁵N natural abundance method, discussed in detail elsewhere (Peoples et al. 1989; Shearer and Kohl 1989; Guimarães et al. 2008; Pauferro et al. 2010), is the most appropriate technique recommended for crops and pasture legumes because it gives an overall estimate of the contribution of biological N₂ fixation (BNF) up to the time of sampling (Cadisch et al. 2000; Boddey et al. 2009). In addition, particularly in natural ecosystems, disturbance of the system is not required and measurements may be made on samples dried in the field (Shearer and Kohl 1989), determining the proportion of N in the legume plant derived from the air (%Ndfa) and comparing its ¹⁵N natural abundance value ($\delta^{15}\text{N}$) with that of a non-fixing companion species also denominated as reference plant (Pate et al. 1994). Furthermore, in naturalized species like *L. tenuis*, the $\delta^{15}\text{N}$ in N₂ fixing plants grown with N₂ in air as the only N source ('B' value), must be determined simultaneously in order to account for discrimination against ¹⁵N during N₂ fixation (Carlsson et al. 2006; Unkovich et al. 2008). As *Lotus* species are suggested to belong to the C₃ group of plants, determination of the natural abundance of ¹³C ($\delta^{13}\text{C}$) may provide useful information on carbon assimilation and plant performance during the growth period (Kurdali and Al-Shamma'a 2009). Despite the importance that *L. tenuis* implies for livestock activity and restoration of degraded environments in the Flooding Pampa, there is a lack in the bibliography of reference plants and 'B' values for this species in combination with rhizobia isolated from fields. For these reasons, our aims in this study were to: (1) identify and select a potential reference plant species for

the application of the ^{15}N natural abundance technique to assess the N_2 fixing performances of a *L. tenuis* promotion; (2) calculate the 'B' value of *L. tenuis* species in different phenological stages; (3) determine the $\delta^{13}\text{C}$ to understand variations in carbon assimilation; (4) evaluate the symbiotic capacity of *L. tenuis* and native rhizobia through the determination of %Ndfa and the amount of N_2 fixed in the field site in the Flooding Pampa. As well as determine the %Ndfa of a promoted *L. tenuis* in the field, we aimed to evaluate the possibility of making future determinations in *L. tenuis* under greenhouse assays for the specific study of the effect on %Ndfa with commercial strains of rhizobia and co-inoculation tests with growth-promoting bacteria. For this reason, the determination of %Ndfa under greenhouse was carried out in parallel to the field study to check the reproducibility between both evaluations.

Material And Methods

Study sites description

The studies were conducted simultaneously in two sites during the experimental period September 2017–March 2018: 1- In a 10- years promoted *L. tenuis* pasture at 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), which is located in the Flooding Pampa region. This region has a temperate sub–humid climate with mean temperatures averaging 8.5°C in winter and 21.5°C in summer, and annual rainfall 850–1050 mm. Short floods of 5–7 cm depth occurs at the beginning of almost every spring, sometimes followed by severe droughts may in summer. The experimental area is mostly covered by a Typic Natraquoll, (US Soil Taxonomy), whose A horizon has 35 g kg⁻¹ organic matter content and 0.22 mg kg⁻¹ of extractable Fe. Its natric and clays horizon appears at 0.17 m and contains 533 g kg⁻¹ clay (Lavado and Taboada 1988). Before promotion, plant communities were mainly composed by grasses (*Festuca arundinacea*, *Thinopyrum ponticum*, *Cynodon dactylon*, *L. tenuis* and *Sporobolus indicus*) and exotic dicots, mainly Compositae species. Native legumes were largely absent. by. 2- In a naturally-lit greenhouse located in the Instituto Tecnológico Chascomús (INTECh), twenty kilometers separated 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.

Pasture measurement and floristic composition

Plant biomass was harvested from five 0.25 m² quadrants randomly located in the paddock. 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.

For forage quality determinations, *L. tenuis* plants were clipped in spring, summer and autumn. The following parameters were evaluated: Crude Protein (CP) % by Kjeldahl method and Digestibility of Dry

Matter (DDM) % by (Tilley and Terry 1963). Samples were analyzed at the Laboratory of Animal Nutrition (INTA Balcarce).

For determination of floristic composition, a linear transect with 25 points on a 10-m-long line was used (Gaucherand and Lavorel 2007). For this, a stick was planted vertically every 40 cm and at that point each individual plant was classified as Annual grass, Perennial grass, weed, *L. tenuis* or *Trifolium repens*.

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

On a surface area of 2 ha, Glyphosate (N-(phosphonomethyl) glycine; 3.5 L/ha), followed by two applications of 2,4 DB(4-(2,4-dichlorophenoxy) butyric acid, 1 L/ha) and a single dose of Quizalofop-p-ethyl (Ethyl(R) - 2- [4- (6- chloro-2-quinoxalyloxy) phenoxy] propionate; 1.2 L/ha) were applied, in six or seven annual cycles from June to August. After 4 or 5 years of herbicide application, plant species composition in promoted paddocks shifted, and *L. tenuis* became the dominant species (Nieva et al. 2016, 2018).

L. tenuis shoots were harvested in 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). Shoots were placed into paper bags and oven-dried at 60°C for 72 hours to constant weight. Samples were reduced into a fine powder using a roller mill and homogenized for the analysis of total N, C, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$.

For the estimation of the %Ndfa is necessary to compare its $\delta^{15}\text{N}$ value with that of a non-fixing species (Pate et al. 1994) and the selection of more than one non-fixing species is recommended (Unkovich et al. 2008). In this study, *Conyza bonariensis* and *Lythrum* sp were tested as reference species because of their close similarity with the legume in terms of phenology and rooting profile with soil depth, adjacent closeness to the legume in the site and for its 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. Identical treatment was performed to process the samples used for ^{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, from the international standards (Vienna Pee Dee Belemnite, V-PDB, for ^{13}C and atmospheric N_2 for ^{15}N).

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 N_2 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, so the determination of *L. tenuis* 'B' value was performed 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 $24/21 \pm 2^\circ\text{C}$ and $55/65 \pm 5\%$ relative humidity. Light (at an intensity of $250 \mu\text{mol m}^{-2} \text{s}^{-1}$) was provided by Gro-lux F 40 W fluorescent lamps. *L. tenuis* seedlings were grown in 4 L pots with sterile sand and irrigated with N-free nutrient solution (Rigaud and Puppo 1975). Inoculation was achieved as (Unkovich et al. 2008) using a soil suspension with the native rhizobia present in the promoted field site. For this, soil was collected from the top 20 cm layer from many 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 into paper bags and oven-dried, grounded, homogenized and analyzed for $\delta^{15}\text{N}$.

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

Harvested seeds from the promoted *L. tenuis* were scarified and incubated in a growth chamber until germination. Seedlings were transferred to the greenhouse into 4 L pots containing soil obtained from the top 20 cm of the promoted *L. tenuis* land horizon. Seedlings were irrigated with water from a rain water harvesting system. In parallel, other 4 L pots were filled with promoted soil, 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, reference plants and *L. tenuis* shoot materials were harvested at the same three phenological stages, placed into paper bags, oven-dried, grounded and homogenized for total N, C, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ determination.

Estimates of Biological Nitrogen Fixation

For %Ndfa estimation, samples were reduced into a fine powder using a roller mill and homogenized for analysis. Aliquots were loaded into a Thermo Delta Advantage isotope ratio mass spectrometer couple with a Flash 2000 Elemental Analyser at the Laboratorio de Isótopos Estables en Ciencias Ambientales (LIECA, Argentine) 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 (‰):

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

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 N_2 , for N, and Pee Dee Belemnite, for C.

Although δ provides information on the $^{13}\text{C}/^{12}\text{C}$ of tissues, it is often preferable to express the values as the leaf carbon isotope discrimination (Δ) (Kurdali and Al-Shamma'a 2009):

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

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 measured value 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):

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

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 N.

Experimental Design And Statistical Analysis

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). 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 analysis was performed using the INFOSTAT statistical software package (Di Rienzo et al. 2010) (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina,).

Results

Determination of plant biodiversity in pastures and forage quality

Floristic diversity was modified in both, the promoted *Lotus* and in the grassland sites at all seasons (Table 1). In the grassland, the presence of legumes was scarce during Spring and Summer, but a slight appearance 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*, *L. tenuis* was the predominant species during the three studied seasons (61.00% - 74.00%). *T. repens* was only observed during Spring and Autumn (2.33% and 11.33%, respectively). No differences in biomass were observed between promoted *Lotus* and grassland in Spring and Summer (Table 2). Unlike, higher biomass was measured in grassland in Autumn. When summed, no difference was observed in annual biomass between promoted *Lotus* and grasslands sites ($p = 0.286$). However, better forage quality was observed in the promoted *Lotus* situation (Table 3). *L. tenuis* not only provided more CP compared to the grassland, but also a resource with higher DDM. Moreover, higher than 17% CP values were observed at promoted *Lotus* at all seasons, while only 12% CP value was observed at grassland site during spring. Differences in CP (17.53% vs 8.33% respectively) and DDM (71.83% vs 60.50%) were greater in Summer (Table 3).

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

As observed in Figure 1, during EB and FB, no $\delta^{13}\text{C}$ differences were obtained between sites for *L. tenuis* ($\delta^{13}\text{C}$ approximately -28‰). For them, differences were only observed during RG stage with more

negative $\delta^{13}\text{C}$ values in the field (-29‰). Regarding greenhouse assays, a more negative value was obtained during FB (-29.41‰) compared to its value during EB (-28.36‰) and RG (-28.76‰), while for field assays a more negative $\delta^{13}\text{C}$ was obtained during RG (-29.32‰) compared to EB (-28.70‰) and FB (-28.75‰). On the other hand, the same trend was observed in Figure 2 for the $\Delta^{13}\text{C}$, where differences were only observed during RG stage. For greenhouse assays a higher $\Delta^{13}\text{C}$ was observed during FB stage compared to EB and RG, while no differences were observed in the field during the three phenological stages.

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* (Figure 3). 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 (Figure 3). 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, observing only differences with even more negative B values 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 $7.34 \pm 1.18\text{‰}$ respectively). No differences were observed between sites during FB. For *Lythrum* sp., no differences in $\delta^{15}\text{N}$ values were obtained between sites during EB and FB. The mixture of these reference species showed no differences 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. plants were observed in the field in RG phenological stage. For this reason, 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‰) and did differ between them (Figure 3).

Proportion of N derived from the air (%Ndfa) in greenhouse and field assays according to *Lotus tenuis* phenology.

Regarding N concentration (Figure 4 A) *L. tenuis* values did not differ between the greenhouse and the field in the EB and the FB stages (Figure 4). 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, it was significantly ($P < 0.05$) lower in the RG than in the EB and FB stages. Unlike, a higher ^{15}N concentration was observed in the field in the RG stage. With regard to C accumulation, similar values were observed in EB and RG stages, while lower C accumulation was observed in the field in the RG stage (Figure 4 B). The resulting C/N ratios were similar between greenhouse and field sites at EB, slightly and not significantly ($P = 0.320$) higher in the field at FB, and significantly ($P = 0.001$) higher in the greenhouse at RG (Figure 4 C).

$\delta^{15}\text{N}$ values in the two non-leguminous reference species.

The two non-leguminous reference species selected - *C. bonaerensis* and *Lythrum* sp.- achieved mean $\delta^{15}\text{N}$ values between 4-10‰ depending on the site, phenological stage and plant species, while the mixture of both reference species achieved mean $\delta^{15}\text{N}$ values between 5-7‰, depending on site and phenological stage (Figure 3).

%Ndfa values in *L. tenuis* and reference plants according to phenological stages

There was no significant effect from both reference plant species (%Ndfa= 80%) in the EB and FB phenological stages, as well as from the mixture of both (Figure 5 A, B). For this reason, the use of some of the two species or the mixture of both was proved indistinct in these stages. Because *Lythrum* sp. was not found in the RG phenological stage, *C. bonaerensis* species was used as the only reference plant (Figure 5 C). Interesting to note, %Ndfa results for *L. tenuis* in the greenhouse were similar during EB, FB and RG stages (Figure 6). The same was observed in the field during EB and FB. Unlike, during RG lower %Ndfa was observed in the field than in the greenhouse (Figure 6).

Discussion

Improvement of grasslands forage contribution, by *L. tenuis* promoting

Temperate pastures of South America regions are comprised mainly by a few C3 grass and legume species, offering a quite good biomass production in winter and providing forage for raising cattle in a season when the productivity of native grasslands is scarce (Bresciano et al. 2019). The main ecosystem limitation is their poor ability to maintain high levels of productivity during the summer season (Tejera et al. 2016). Thus, the presence of C4 species in these environments plays an underlying role providing 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. As shown by Table 1, the grassland was mainly composed by annual and perennial C4 grasses such as *Paspalum dilatatum*, *Sporobolus indicus* and *Cynodon dactylon*. This agrees with previous works carried out in the region, showing that the prevalence of C4 over C3 grasses is mainly due to edaphic conditions and specially, the topsoil pH gradients (Perelman et al. 2001, 2005; Cid et al. 2011) and its relative abundances can change at a particular site and such changes may have direct consequences for ecosystem processes such as net primary production along with many other ecosystem services (Lattanzi 2010; Kim et al. 2015). Despite its contributions, this forage resource (C4 grasses) has poor quality, as shown by its low CP % and DDM % values (Table 3), which does not cover an efficient cattle production in the region, causing either a considerable increase in the need for feed supplements or a decrease in their reproductive performance (Grigera et al. 2007). Plant physiological status and their photosynthetic pathways can be assessed by considering its carbon isotope discrimination values (Brüggemann et al. 2011; Luo et al. 2021). In this sense, the *L. tenuis* $\delta^{13}\text{C}$ and $\Delta^{13}\text{C}$ values obtained from around - 28‰ and 20‰ respectively (Figs. 1 and 2, respectively) are considered typical of C3

plants following the Calvin cycle (Farquhar et al. 1989; Sun et al. 2011). The introduction of C3 forage legumes such as *L. tenuis* can modify the C3:C4 grass cover ratio, thus resulting in a richer nutritional forage resource (Table 1 and Table 3, respectively). It is also well reported that legumes can contribute improving not only the nutritive value of the pasture, but also the herbage yield of the improved pastures (Del Pino et al. 2016; Vignolio et al. 2016) and sustainability beyond current extents (Muir et al. 2014). Besides, carbon sequestration can be achieved after legume introduction (overseeding) in grazed natural grasslands, depending on grazing management practices, as was reported by Bondaruk (2020) in a case study in Argentina on commercial farms using *Lotus subbiflorus*. Successful naturalized *L. tenuis* stands (Insausti et al. 2001; García and Mendoza 2008), as well as the consociation between *L. tenuis* and *Paspalum dilatatum* (Striker et al. 2008; Vignolio and Fernández 2011) were found as keys for future forage contributions for these highly constraining environments. However, a trade-off between increased pasture production and decreased vegetation stability (long-term integrity) may be operating in legume-improved grasslands, thus requiring further studies on the effects of other mechanisms such as grazing management options (Jaurena et al. 2016; Vecchio et al. 2019).

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

The correct estimation and quantification of BNF by legumes depends on the applied methodology (Hardarson 1993). Until date, most of the work undertaken on the evaluation of N₂ fixation by *L. tenuis* species has been performed in the framework of forage mixtures and with the acetylene reduction technique or nitrogenase activity throwing a theoretical N₂ fixation (Refi and Escuder 1998). In this work we have obtained the required parameters to allow us an accurate value of N₂ fixed by this legume, and through it, obtain a 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 N₂ fixation potential is in the range of previous works using the nitrogenase activity method, such as 27–42 kg N ha⁻¹ per year in grazed tall fescue / *L. tenuis* swards growing on an alkaline hydromorphic soil, or 14–59 kg N ha⁻¹ per year tall fescue / white clover swards (Díaz et al. 2005). Furthermore, Danso et al. (1991) reported a 91 % of Ndfa by *L. corniculatus* when tall fescue was considered as the reference plant. In our study, the amount of 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. 2010) two years after planting, with the soil N being increased from 30.5 mg/kg to 66.4 mg/kg.

A very important aspect of N₂ fixation estimations using the ¹⁵N natural abundance technique is a good selection of the reference plants. In this selection is important to take in mind that the $\delta^{15}\text{N}$ of soil can vary between N pools, soil depths, and over time, the reference plant should follow the same dynamics in N uptake and take N from the same soil N pools as the N₂ fixing species (Carlsson et al. 2006; Unkovich et al. 2008). In our trials, there were no significant differences according to the 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. 3). Furthermore, no differences were observed using the species mix either

(Fig. 3). For this reason, the use of some of the two species or the mixture of both is 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. Besides, quantifying biologically fixed nitrogen (BNF) by legumes through the ^{15}N natural abundance technique requires the correct determination of the B value which can vary among species (also among accessions of the same plant species), environmental conditions and plant physiological status (Boddey et al. 2000; Peoples et al. 2009; Nebiyu et al. 2014). Most of the B values determined for legumes are usually in the range of 0 and -2‰ with few exceptions (Okito et al. 2004). In our study, the B values obtained for *L. tenuis* in two phenological stages were -2.1 and -1.7 for EB and FB, respectively, being these within the range previously mentioned. The aforementioned B values constitute the first report for *L. tenuis* in two phenological stages in these specific environments of the Salado River Basin.

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

Except for greenhouse assays during RG stage, N concentration in *L. tenuis* leaves was always higher than that in both non-fixing plants or the mixture of them (data not shown). This result demonstrated 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 tree ($+6.4\text{‰} \pm 0.6$) 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_2 -fixing species. Our results also confirm the importance of forage management, which also impacts on N fixation, especially grazing management practices that influence plant regrowth (Vecchio et al. 2019). In this sense, protocols should be designed on the basis of the legume “nutritional cycles”, avoiding the overgrazing in critical phenological periods where the relationship sink/source of C and N must be considered in a framework of grasslands conservation (Abdalla et al. 2018). As was reported in *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 imposed and specially the defoliation periods will affect the patter of reserves accumulation and subsequent regrowth (Vignolio et al. 2018; Mitchell et al. 2020). 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 (Striker et al. 2011), specially during early regrowth (Avice et al. 1996). 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 C reserves (Striker et al. 2011; Aranjuelo et al. 2014). Moreover, is accepted that a reduction in N_2 -fixation

happens in response to a lack of nodulation ability or increased N availability from regrowth stage because of a mayor input of N from the soil. Studies conducted in the past show 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 (Aranjuelo et al. 2014). In addition, as was reported previously (Skinner et al. 1999) nutrient uptake and photosynthesis can be greatly reduced following defoliation.

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 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., are growing well in these constrained soils, focused our attention on studying the BFN contribution in these harsh conditions. The results obtained 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, influencing, also, the carbon sequestration rates.

As far as we know, this is the first study where the N₂ 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 N₂ fixation of *L. tenuis* pastures 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 actual data. This, along with data previously published, brings another parameter to validate *L. tenuis* playing a significant role in the constrained areas in the Salado River Basin and, in consequence, on the cow productivity in the most important region devoted to cattle production in Argentina.

Declarations

Competing Interest statement: The authors declare that they have no competing interests.

Funding statement: This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET-Argentina) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-Argentina)

References

Abdalla M, Hastings A, Chadwick DR, et al (2018) Critical review of the impacts of grazing intensity on soil organic carbon storage and other soil quality indicators in extensively managed grasslands. *Agric Ecosyst Environ* 253:62–81. <https://doi.org/10.1016/j.agee.2017.10.023>

Antonelli CJ, Calzadilla PC, Escaray FJ, et al (2016) LOTUS spp: BIOTECHNOLOGICAL STRATEGIES TO IMPROVE THE BIOECONOMY OF LOWLANDS IN THE SALADO RIVER BASIN (ARGENTINA). *AGROFOR Int*

J 1:43–53. <https://doi.org/10.1017/CBO9781107415324.004>

Antonelli CJ, Calzadilla PI, Vilas JM, et al (2019) Physiological and anatomical traits associated with tolerance to long-term partial submergence stress in the Lotus genus: responses of forage species, a model and an interspecific hybrid. *J Agron Crop Sci* 205:65–76.
<https://doi.org/10.1111/jac.12303>

Aranjuelo I, Arrese-Igor C, Molero G (2014) Nodule performance within a changing environmental context. *J Plant Physiol* 171:1076–1090. <https://doi.org/10.1016/j.jplph.2014.04.002>

Avice JC, Ourry A, Lemaire G, Boucaud J (1996) Nitrogen and carbon flows estimated by ¹⁵N and ¹³C pulse-chase labeling during regrowth of Alfalfa. *Plant Physiol* 112:281–290.
<https://doi.org/10.1104/pp.112.1.281>

Bailleres M, Melani G, Menéndez A (2020) Promotion of Lotus tenuis and calf early weaning as a good management practice for breeding herds in marginal soils of the Flooding Pampa (Argentina). *RIA Rev Investig Agropecu* 46:267–274

Boddey RM, Alves BJR, Soares LHDB, et al (2009) Biological Nitrogen Fixation and the Mitigation of Greenhouse Gas Emissions Substituting Nitrogen Fertilizer by. *Am Soc Agron Crop Sci Soc Am Soil Sci Soc Am* 387–413

Boddey RM, Peoples MB, Palmer B, Dart PJ (2000) Use of the ¹⁵N natural abundance technique to quantify biological nitrogen fixation by woody perennials. *Nutr Cycl Agroecosystems* 57:235–270.
<https://doi.org/10.1136/adc.47.254.671-a>

Bondaruk V, Lezama F, del Pino A, Piñeiro G (2020) Overseeding legumes in natural grasslands: Impacts on root biomass and soil organic matter of commercial farms. *Sci Total Environ* 743:140771.
<https://doi.org/10.1016/j.scitotenv.2020.140771>

Bresciano D, del Pino A, Borges A, et al (2019) Perennial C4 grasses increase root biomass and carbon in sown temperate pastures. *New Zeal J Agric Res* 62:332–342.
<https://doi.org/10.1080/00288233.2018.1504089>

Brüggemann N, Gessler A, Kayler Z, et al (2011) Carbon allocation and carbon isotope fluxes in the plant-soil-atmosphere continuum: a review. *Biogeosciences Discuss* 8:3619–3695.
<https://doi.org/10.5194/bgd-8-3619-2011>

Cadisch G, Hairiah K, Giller KE (2000) Applicability of the natural ¹⁵N abundance technique to measure N₂ fixation in *Arachis hypogaea* grown on an Ultisol. *Netherlands J Agric Sci* 48:31–45.
[https://doi.org/10.1016/S1573-5214\(00\)80003-2](https://doi.org/10.1016/S1573-5214(00)80003-2)

Campestre MP, Castagno NL, Antonelli CJ, et al (2020) Characterization of alkaline stress tolerance mechanisms in Lotus forage species modulated by *Pantoea eucalypti*. *bioRxiv* 2020.06.06.138230.

<https://doi.org/10.1101/2020.06.06.138230>

Carlsson G, Palmborg C, Huss-Danell K (2006) Discrimination against ^{15}N in three N_2 -fixing *Trifolium* species as influenced by *Rhizobium* strain and plant age. *Acta Agric Scand Sect B – Soil & Plant Sci* 56:31–38. <https://doi.org/10.1080/0906471051003131>

Cid MS, Fernández Grecco RC, Oesterheld M, et al (2011) Grass-fed beef production systems of Argentina's flooding pampas: Understanding ecosystem heterogeneity to improve livestock production. *Outlook Agric* 40:181–189. <https://doi.org/10.5367/oa.2011.0040>

Danso SKA, Curbelo S, labandera C, pastorini D (1991) Herbage yield and nitrogen-fixation in a triple-species mixed sward of white clover, lotus and fescue. *Soil Biol Biochem* 23:65–70. [https://doi.org/10.1016/0038-0717\(91\)90163-E](https://doi.org/10.1016/0038-0717(91)90163-E)

Del Pino A, Rodríguez T, Andi6n J (2016) Production improvement through phosphorus fertilization and legume introduction in grazed native pastures of Uruguay. *J Agric Sci* 154:347–358. <https://doi.org/10.1017/S002185961500101X>

Di Rienzo J, Balzarini M, Gonzalez L, et al (2010) Infostat: software para análisis estadístico

Díaz P, Borsani O, Monza J (2005) Lotus-related species and their agronomic importance. In: Márquez AJ (ed) *Lotus japonicus Handbook*. Springer Netherlands, Dordrecht, pp 25–37

Escaray FJ, Menendez AB, Gárriz A, et al (2012) Ecological and agronomic importance of the plant genus *Lotus*. Its application in grassland sustainability and the amelioration of constrained and contaminated soils. *Plant Sci* 182:121–133. <https://doi.org/10.1016/j.plantsci.2011.03.016>

Estrella MJ, Muñoz S, Soto MJ, et al (2009) Genetic Diversity and Host Range of *Rhizobia* Nodulating *Lotus tenuis* in Typical Soils of the Salado River Basin (Argentina). *Appl Environ Microbiol* 75:1088–1098. <https://doi.org/10.1128/AEM.02405-08>

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon Isotope Discrimination and Photosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* 40:503–537. <https://doi.org/https://doi.org/10.1146/annurev.pp.40.060189.002443>

Fulchieri MM, Estrella MJ, Iglesias AA (2001) Characterization of *Rhizobium loti* strains from the Salado River Basin. *Antonie Van Leeuwenhoek* 79:119–125. <https://doi.org/10.1023/A:1010221208619>

García I V., Mendoza RE (2008) Relationships among soil properties, plant nutrition and arbuscular mycorrhizal fungi-plant symbioses in a temperate grassland along hydrologic, saline and sodic gradients. *FEMS Microbiol Ecol* 63:359–371. <https://doi.org/10.1111/j.1574-6941.2008.00441.x>

Gaucherand S, Lavorel S (2007) New method for rapid assessment of the functional composition of herbaceous plant communities. *Austral Ecol* 32:927–936. <https://doi.org/https://doi.org/10.1111/j.1442->

- González-Robles A, Manzaneda AJ, Salido T, et al (2020) Spatial genetic structure of a keystone long-lived semiarid shrub: historical effects prevail but do not cancel the impact of recent severe habitat loss on genetic diversity. *Conserv Genet* 21:853–867. <https://doi.org/10.1007/s10592-020-01291-5>
- Grigera G, Oesterheld M, Pacín F (2007) Monitoring forage production for farmers' decision making. *Agric Syst* 94:637–648. <https://doi.org/https://doi.org/10.1016/j.agsy.2007.01.001>
- Guimarães AP, De Moraes RF, Urquiaga S, et al (2008) Bradyrhizobium strain and the ^{15}N natural abundance quantification of biological N_2 fixation in soybean. *Sci Agric* 65:516–524. <https://doi.org/10.1590/s0103-90162008000500011>
- Hardarson G (1993) Methods for enhancing symbiotic nitrogen fixation. In: Bliss FA, Hardarson G (eds) *Enhancement of Biological Nitrogen Fixation of Common Bean in Latin America: Results from an FAO/IAEA Co-ordinated Research Programme, 1986–1991*. Springer Netherlands, Dordrecht, pp 1–17
- Imbellone PA, Taboada MA, Damiano F, Lavado RS (2021) Genesis, Properties and Management of Salt-Affected Soils in the Flooding Pampas, Argentina. In: Taleisnik E, Lavado RS (eds) *Saline and Alkaline Soils in Latin America: Natural Resources, Management and Productive Alternatives*. Springer International Publishing, Cham, pp 191–208
- Insausti P, Grimoldi AA, Chaneton EJ, Vasellati V (2001) Flooding induces a suite of adaptive plastic responses in the grass *Paspalum dilatatum*. *New Phytol* 152:291–299. <https://doi.org/https://doi.org/10.1111/j.0028-646X.2001.257\1.x>
- Jaurena M, Lezama F, Salvo L, et al (2016) The Dilemma of Improving Native Grasslands by Overseeding Legumes: Production Intensification or Diversity Conservation. *Rangel Ecol Manag* 69:35–42. <https://doi.org/10.1016/j.rama.2015.10.006>
- Kim YC, Gao C, Zheng Y, et al (2015) Arbuscular mycorrhizal fungal community response to warming and nitrogen addition in a semiarid steppe ecosystem. *Mycorrhiza* 25:267–276. <https://doi.org/10.1007/s00572-014-0608-1>
- Kurdali F, Al-Shamma'a M (2009) Natural abundances of ^{15}N and ^{13}C in leaves of some N_2 -fixing and non- N_2 -fixing trees and shrubs in Syria. *Isotopes Environ Health Stud* 45:198–207. <https://doi.org/10.1080/10256010903084126>
- Lattanzi F a (2010) C_3/C_4 grasslands and climate change. *Grassl Sci Eur* 15:3–13
- Lavado RS, Taboada MA (1988) Water, salt and sodium dynamics in a Natraquoll in Argentina. *CATENA* 15:577–594. [https://doi.org/https://doi.org/10.1016/0341-8162\(88\)90008-2](https://doi.org/https://doi.org/10.1016/0341-8162(88)90008-2)

León RJC, Rusch GM, Oesterheld M (1984) Pastizales pampeanos - impacto agropecuario. *Phytocoenologia* 12:201–2018

Luo W, Wang X, Auerswald K, et al (2021) Effects of plant intraspecific variation on the prediction of C3/C4 vegetation ratio from carbon isotope composition of topsoil organic matter across grasslands. *J Plant Ecol* 14:628–637. <https://doi.org/10.1093/jpe/rtab022>

Miñón DP, Sevilla GH, Montes L, Fernández ON (1990) *Lotus tenuis*: leguminosa forrajera para la Pampa Deprimida. *Boletín Técnico* 98:

Mitchell ML, Clark SG, Butler KL, et al (2020) Harvest interval affects lucerne (*Medicago sativa* L.) taproot total yield, starch, nitrogen and water-soluble carbohydrates. *J Agron Crop Sci* 206:619–629. <https://doi.org/10.1111/jac.12397>

Muir JP, Pitman WD, Dubeux JC, Foster JL (2014) The future of warm-season, tropical and subtropical forage legumes in sustainable pastures and rangelands. *African J Range Forage Sci* 31:187–198. <https://doi.org/10.2989/10220119.2014.884165>

Nebiyu A, Huygens D, Upadhyay HR, et al (2014) Importance of correct B value determination to quantify biological N2 fixation and N balances of faba beans (*Vicia faba* L.) via 15N natural abundance. *Biol Fertil Soils* 50:517–525. <https://doi.org/10.1007/s00374-013-0874-7>

Nieva AS, Bailleres MA, José M, et al (2016) Herbicide-mediated promotion of *Lotus tenuis* (Waldst . & Kit . ex Wild.) did not influence soil bacterial communities, in soils of the Flooding Pampa, Argentina. *Appl Soil Ecol* 98:83–91

Nieva AS, Vilas JM, Gárriz A, et al (2019) The fungal endophyte *Fusarium solani* provokes differential effects on the fitness of two *Lotus* species. *Plant Physiol Biochem* 144:100–109. <https://doi.org/https://doi.org/10.1016/j.plaphy.2019.09.022>

Nieva SA, Bailleres MA, Llamas ME, et al (2018) Promotion of *Lotus tenuis* in the Flooding Pampa (Argentina) increases the soil fungal diversity. *Fungal Ecol* 33:80–91. <https://doi.org/10.1016/j.funeco.2018.01.001>

Okito A, Alves BRJ, Urquiaga S, Boddey RM (2004) Isotopic fractionation during N2 fixation by four tropical legumes. *Soil Biol Biochem* 36:1179–1190. <https://doi.org/10.1016/j.soilbio.2004.03.004>

Pate JS, Unkovich MJ, Armstrong EL, Sanford P (1994) Selection of reference plants for 15N natural abundance assessment of N2 fixation by crop and pasture legumes in south-west Australia. *Aust J Agric Res* 45:133–47

Pauferro N, Guimarães AP, Jantalia CP, et al (2010) 15N natural abundance of biologically fixed N2 in soybean is controlled more by the *Bradyrhizobium* strain than by the variety of the host plant. *Soil Biol Biochem* 42:1694–1700. <https://doi.org/10.1016/j.soilbio.2010.05.032>

- Peoples MB, a.W. Faizah, Rerkasem B, Herridge DF (1989) Methods for Evaluating Nitrogen Fixation by Nodulated Legumes in the Field. *Aust Cent Int Agric Res* 11:76
- Peoples MB, Herridge DF, Rochester IJ, et al (2009) The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis* 48:1–17. <https://doi.org/10.1007/BF03179980>
- Perelman SB, Batista WB, León RJC (2005) El estudio de la heterogeneidad de la vegetación. *Fitosociología y técnicas relacionadas. La Heterog la Veg los agroecosistemas; un Homen a Rolando León Oesterheld, M al(eds) Editor Fac Agron Buenos Aires, Argentina* 321–350
- Perelman SB, León RJC, Oesterheld M (2001) Cross-scale vegetation patterns of Flooding Pampa grasslands. *J Ecol* 89:562–577. <https://doi.org/https://doi.org/10.1046/j.0022-0477.2001.00579.x>
- Refi RO, Escuder CJ (1998) Nitrogen fixation by *trifolium repens* and *lotus tenuis*-based pastures in the flooding pampa, argentina. *Agronomie* 18:285–297. <https://doi.org/10.1051/agro:19980404>
- Rigaud J, Puppo A (1975) Indole-3-acetic Acid Catabolism by Soybean Bacteroids. *J Gen Microbiol* 88:223–228. <https://doi.org/10.1099/00221287-88-2-223>
- Sannazzaro AI, Bergottini VM, Paz RC, et al (2011) Comparative symbiotic performance of native rhizobia of the Flooding Pampa and strains currently used for inoculating *Lotus tenuis* in this region. *Antonie van Leeuwenhoek, Int J Gen Mol Microbiol* 99:371–379. <https://doi.org/10.1007/s10482-010-9502-9>
- Shearer G, Kohl DH (1989) Natural ^{15}N abundance as a method of estimating the contribution of biologically fixed nitrogen to N_2 -fixing systems: Potential for non-legumes. In: Skinner FA, Boddey RM, Fendrik I (eds) *Nitrogen Fixation with Non-Legumes: The Fourth International Symposium on `Nitrogen Fixation with Non-Legumes'*, Rio de Janeiro, 23–28 August 1987. Springer Netherlands, Dordrecht, pp 289–299
- Skinner RH, Morgan JA, Hanson JD (1999) Carbon and Nitrogen Reserve Remobilization Following Defoliation: Nitrogen and Elevated CO_2 Effects. *Crop Sci* 39:1749–1756. <https://doi.org/https://doi.org/10.2135/cropsci1999.3961749x>
- Striker GG, Insausti P, Grimoldi AA (2008) Flooding Effects on Plants Recovering from Defoliation in *Paspalum dilatatum* and *Lotus tenuis*. *Ann Bot* 102:247–254. <https://doi.org/10.1093/aob/mcn083>
- Striker GG, Manzur ME, Grimoldi AA (2011) Increasing defoliation frequency constrains regrowth of the forage legume *Lotus tenuis* under flooding. The role of crown reserves. *Plant Soil* 343:261–272. <https://doi.org/10.1007/s11104-011-0716-z>
- Sun Z, Mou X, Li X, et al (2011) Application of stable isotope techniques in studies of carbon and nitrogen biogeochemical cycles of ecosystem. *Chinese Geogr Sci* 21:129–148. <https://doi.org/10.1007/s11769-011-0453-5>

- Teixeira EI, Moot DJ, Brown HE, Fletcher AL (2007) The dynamics of lucerne (*Medicago sativa* L.) yield components in response to defoliation frequency. *Eur J Agron* 26:394–400. <https://doi.org/10.1016/j.eja.2006.12.005>
- Tejera M, Speranza P, Astigarraga L, Picasso V (2016) Forage biomass, soil cover, stability and competition in perennial grass–legume pastures with different *Paspalum* species. *Grass Forage Sci* 71:575–583. <https://doi.org/https://doi.org/10.1111/gfs.12208>
- Tilley JMA, Terry RA (1963) A Two-Stage Technique for the in Vitro Digestion of Forage Crops. *Grass Forage Sci* 18:104–111. <https://doi.org/10.1111/j.1365-2494.1963.tb00335.x>
- Unkovich M, Baldock J (2008) Measurement of asymbiotic N₂ fixation in Australian agriculture. *Soil Biol Biochem* 40:2915–2921. <https://doi.org/10.1016/j.soilbio.2008.08.021>
- Unkovich M, Herridge D, Peoples M, et al (2008) Measuring plant-associated nitrogen fixation in agricultural systems
- Vecchio MC, Bolaños VA, Golluscio RA, Rodríguez AM (2019) Rotational grazing and exclosure improves grassland condition of the halophytic steppe in Flooding Pampa (Argentina) compared with continuous grazing. *Rangel J* 41:1–12. <https://doi.org/10.1071/RJ18016>
- Vignolio O, Cambareri G, Maceira N (2010) *Lotus tenuis* (Fabaceae). Productividad y manejo agronómico. *Rev Argentina Prod Anim* 30:97–116
- Vignolio O, Cambareri G, Petigrosso L, et al (2016) Reproductive Development of *Lotus tenuis* (Fabaceae) Crop Defoliated at Different Times and Intensities. *Am J Plant Sci* 7:1180–1191. <https://doi.org/doi:10.4236/ajps.2016.78113>
- Vignolio OR, Fernández ON, Maceira NO (1999) Flooding tolerance in five populations of *Lotus glaber* Mill. (Syn. *Lotus tenuis* Waldst. et. Kit.). *Aust J Agric Res* 50:555–560
- Vignolio OR, Fernández ON (2011) *Lotus tenuis* Seedling Establishment and Biomass Production in Flooding Pampa Grasslands (Buenos Aires , Argentina). *Chil J Agric Res* 71:96–103. <https://doi.org/10.4067/s0718-58392011000100012>
- Vignolio OR, Petigrosso LR, Rodríguez IM, Murillo NL (2018) Effects of temperature, photoperiod and defoliation on flowering time of *Lotus tenuis* (Fabaceae) in Buenos Aires, Argentina. *Exp Agric* 54:417–427. <https://doi.org/10.1017/S0014479717000126>

Tables

Table 1. Relative seasonal percentage distribution of C3 / C4 species in promoted *Lotus tenuis* and natural grasslands paddocks. Results with the same letter mean no statistical differences between both forage in each seasonal period.

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

Table 2. Seasonal productivity (Kg DM/ha) of promoted *Lotus tenuis* and natural grassland paddocks.

	Promoted <i>Lotus</i>		Grassland		
Period	Kg DM/ha	SD	Kg DM/ha	SD	p-value
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 are not statistically different.

	Promoted Lotus		Grassland	
Period	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	1.67 ± 1.46 ^b	63.00 ± 2.00 ^B

Figures

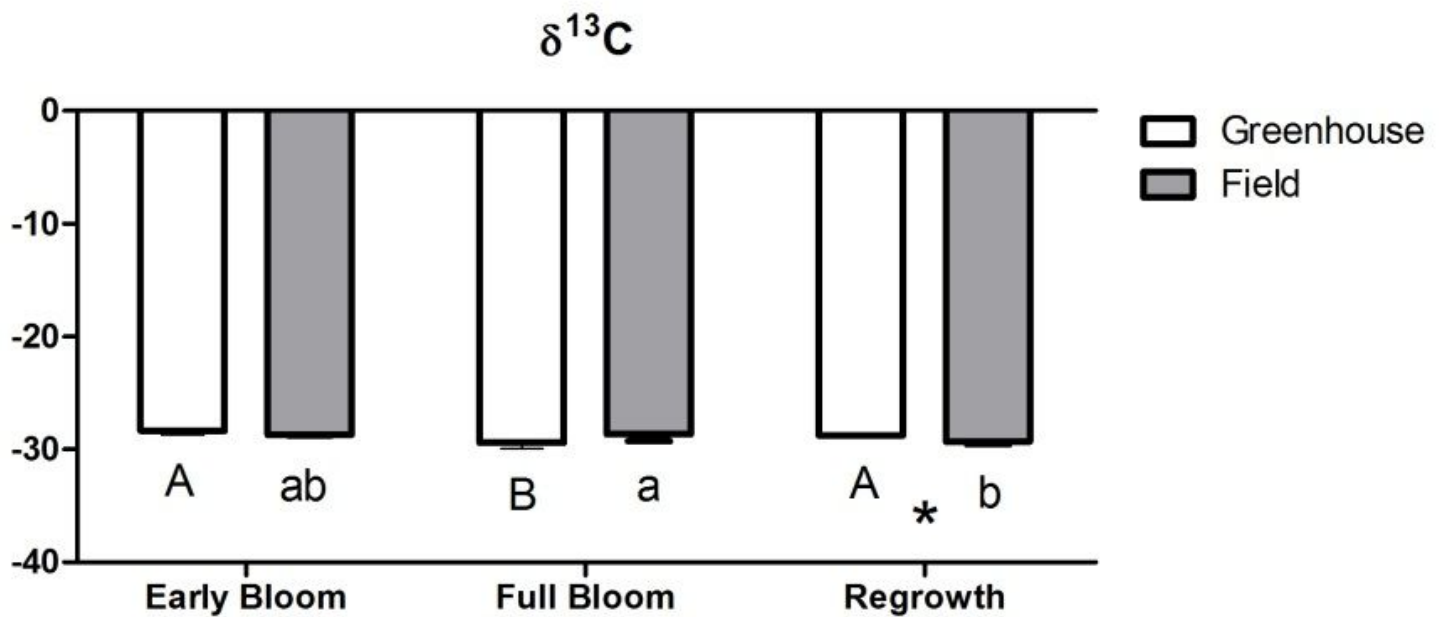


Figure 1

Modification of $\delta^{13}\text{C}$ in Greenhouse (white bars) and Field (grey bars) assays according to *Lotus tenuis* phenology (Early Bloom, Full Bloom and Regrowth). Bars with the same uppercase (Greenhouse analysis) and lowercase (Field analysis) letter are not statistically different (Duncan test; $P < 0.05$). Bars with asterisks represent significant differences between study sites in a specific phenological stage (T test; $P < 0.05$).

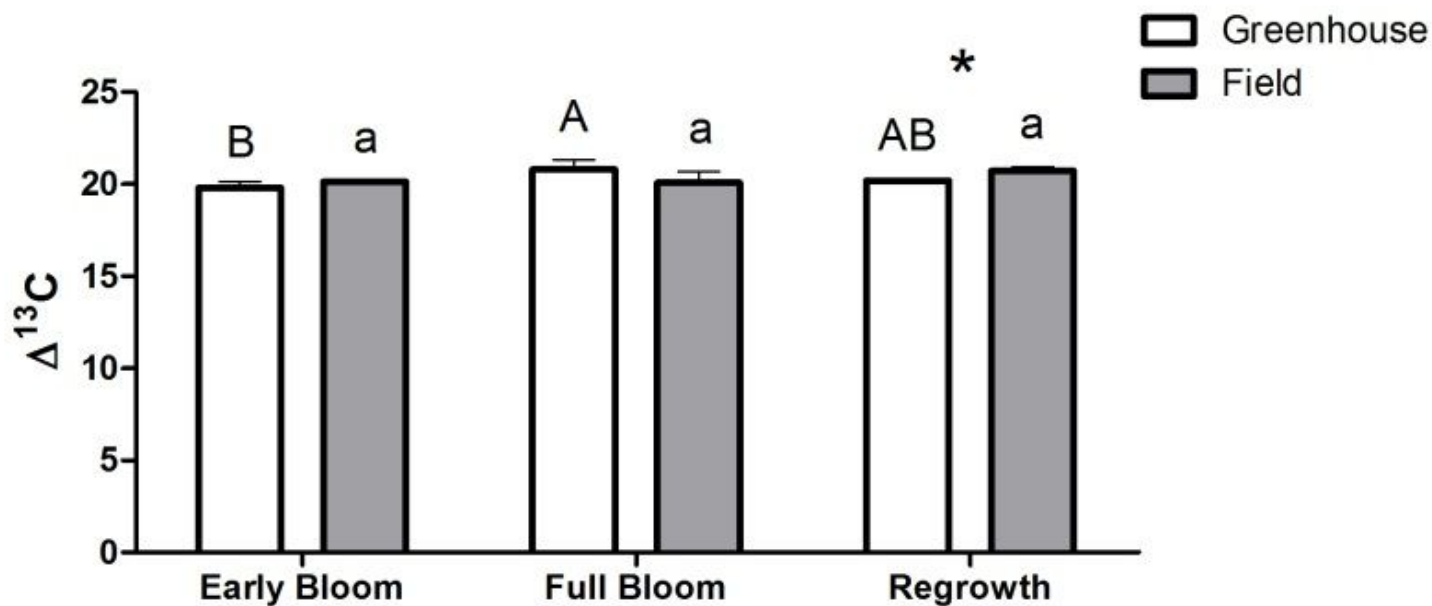


Figure 2

Modification of $\Delta^{13}C$ in greenhouse (white bars) and field (grey bars) assays according to *Lotus tenuis* phenology (Early Bloom, Full Bloom and Regrowth). Bars with the same uppercase (Greenhouse analysis) and lowercase (Field analysis) letter are not statistically different (Duncan test; $P < 0.05$). Bars with asterisks represent significant differences between study sites in a specific phenological stage (T test; $P < 0.05$).

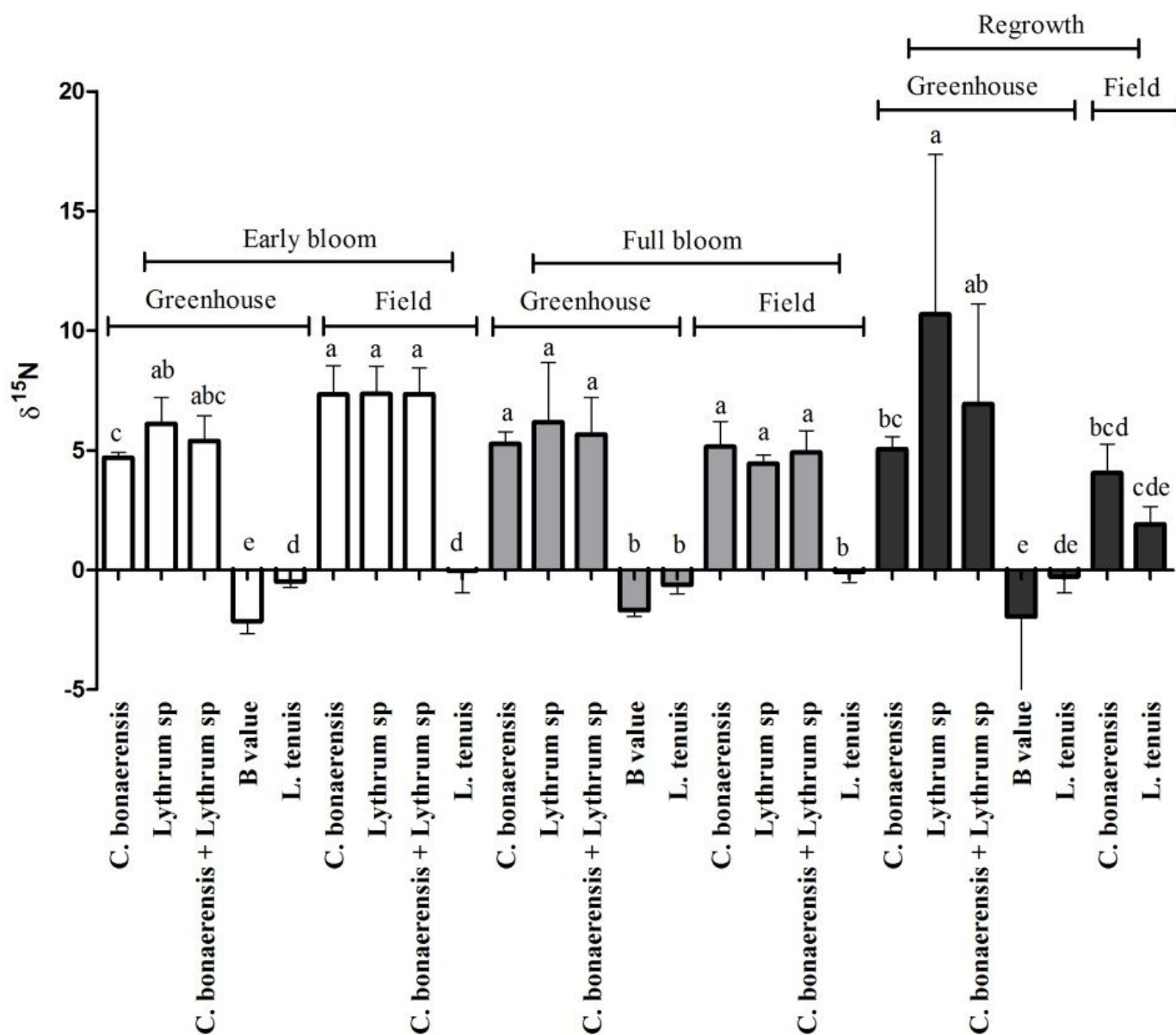


Figure 3

Natural abundance of ^{15}N ($\delta^{15}\text{N}$) in *L. tenuis* and non-legume plant species grown at Greenhouse and Field sites from 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 are not statistically different (Duncan test; $P < 0.05$)

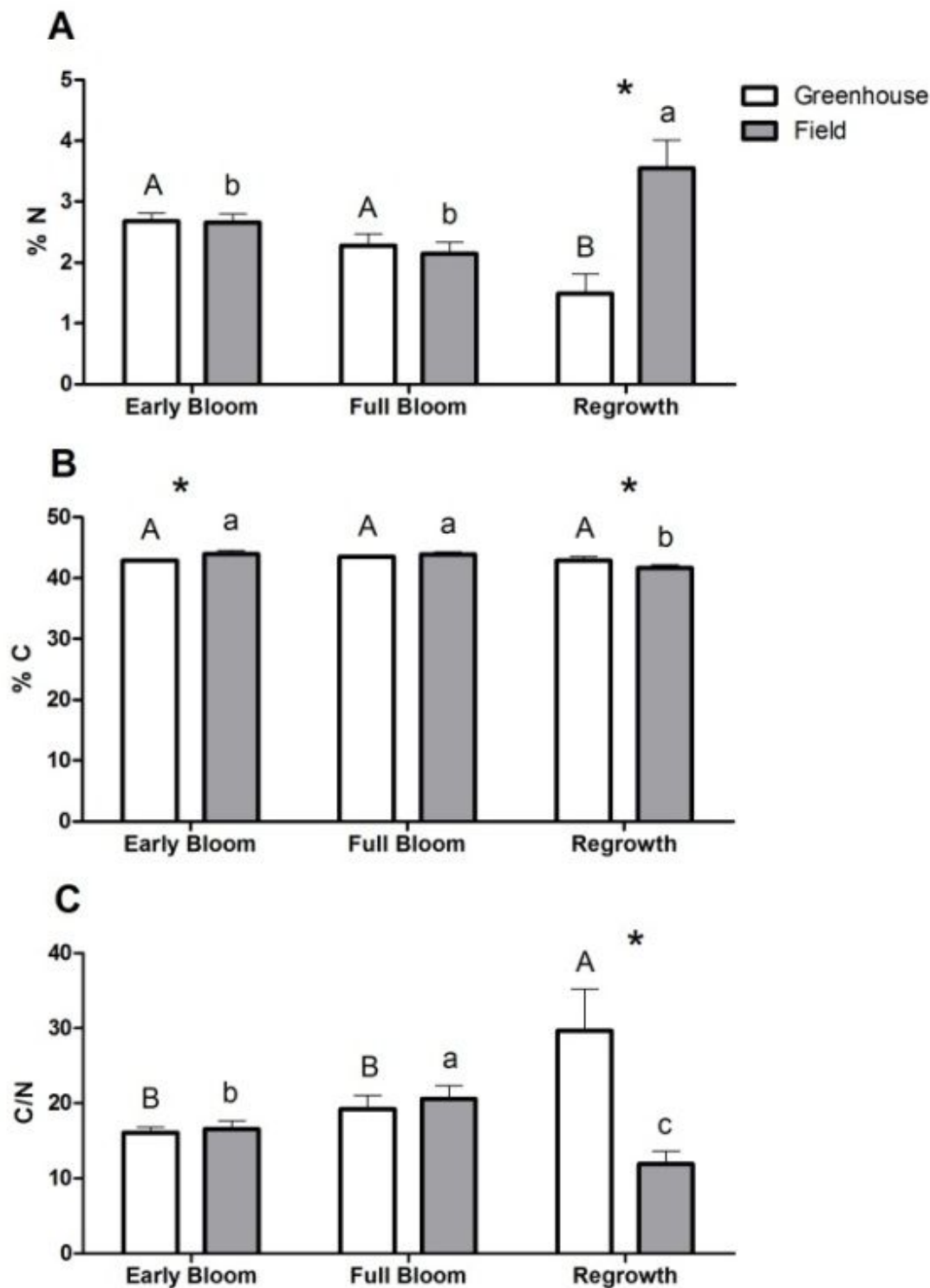


Figure 4

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) letter are not statistically different (Duncan test; $P <$

0.05). Bars with asterisks represent significant differences between study sites in a specific phenological stage (T test; $P < 0.05$).

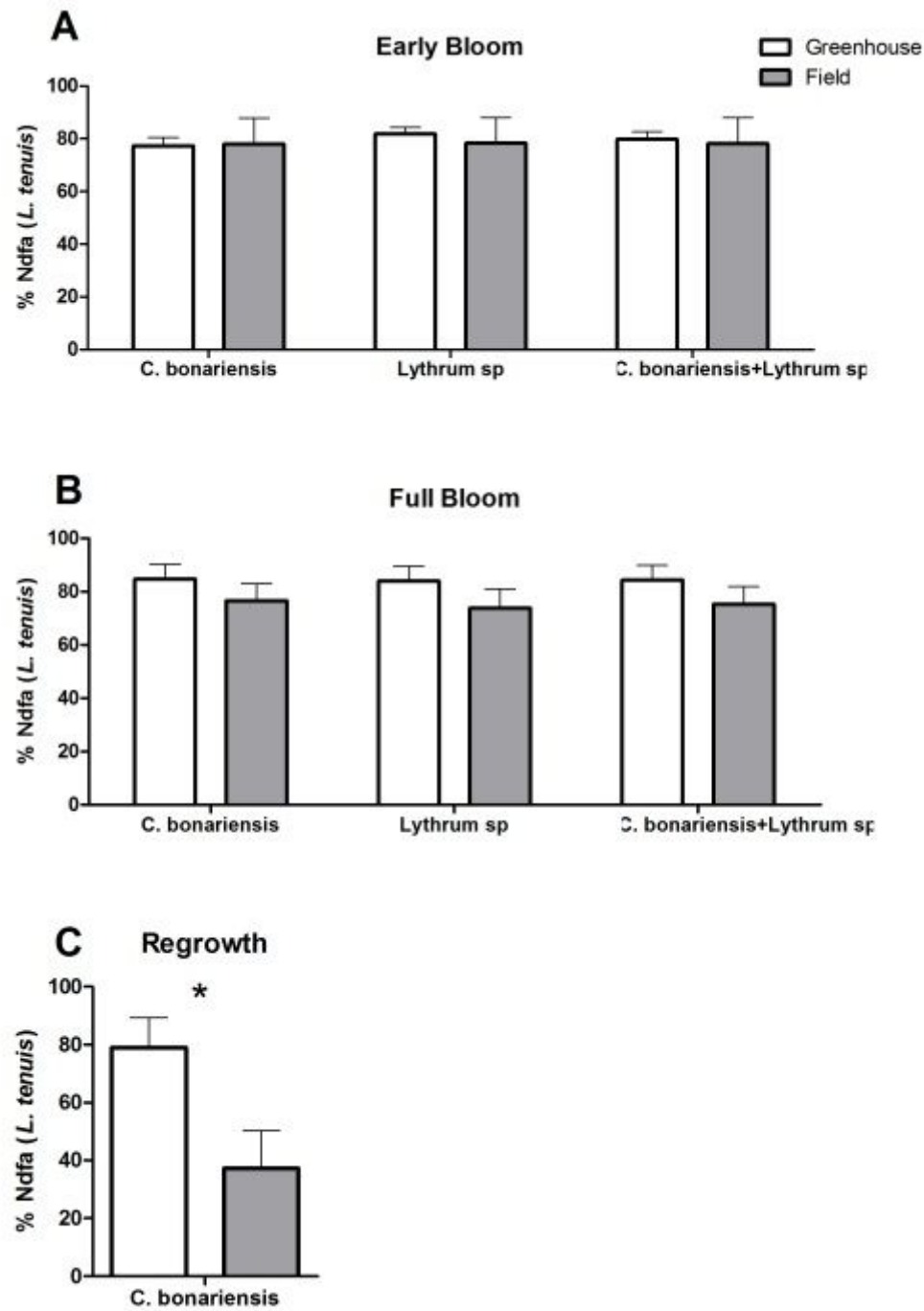


Figure 5

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; $P < 0.05$).

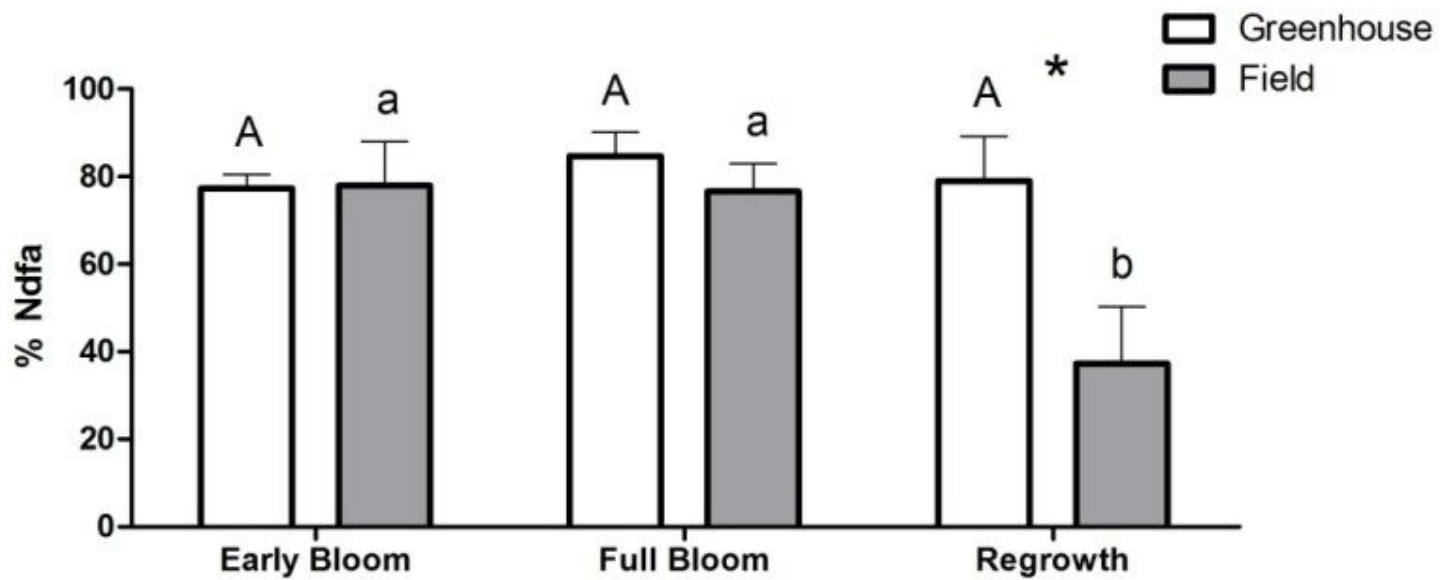


Figure 6

L. tenuis proportion of N derived from the air (%Ndfa) with *C. bonaerensis* as reference plant species in the different evaluation sites (Greenhouse determinations=white bars and Field determinations=grey bars) during three different phenological stages. Bars with the same uppercase (Greenhouse analysis) and lowercase (Field analysis) letter are not statistically different (Duncan test; $P < 0.05$). Bars with asterisks represent significant differences between study sites (T test; $P < 0.05$).