

Lotus tenuis biological nitrogen fixation and performance contribute to defining its strategic role in the Salado River Basin lowlands (Argentina)

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Abstract

Background and Aims

: Forage production is a major challenge in the Salado River Basin, Argentina's largest livestock raising area. The successful naturalization of the European legume *Lotus tenuis* has been of productive and environmental relevance to the region. The aim of this study was to evaluate the strategic contribution of *L. tenuis*, 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* in soil plots. Non-leguminous weed species in the same plots were utilized as reference plants. Simultaneously, evaluations were carried out in the greenhouse to determine the B value of *L. tenuis* and the relative reproducibility of the field experiments.

Results

The results showed that *L. tenuis* promotion was 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 BNF with native rhizobia was about 80%, a highly relevant percentage for these constrained agroecosystems where the presence of legumes is very scarce.

Conclusions

This study supports the hypothesis that *L. tenuis* promotion plays a strategic role in ecosystem sustainability, especially in soil constrained areas. Together with previously published data, including information on ruminant management, the results contribute to supporting the idea that the naturalized legume plays a strategic role in the sustainability of marginal agricultural soils.

Introduction

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 cattle raising (León et al. 1984; 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 (Antonelli et al. 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 (Escaray et al. 2012). Due to its ability to adapt to restrictive conditions, *L. tenuis* quickly became 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 major beneficial effect on the plant community structure and ecosystem function warrants including it among the *keystone* species (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* 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 (Vignolio et al. 2010). *Lotus* promotion is thus considered to be an appealing alternative to meet the needs for cattle production during summer in soils of the Flooding Pampa (Antonelli et al. 2016; Bailleres et al. 2020). *Lotus* promotion 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 (Díaz et al. 2005). Previous studies demonstrated that this practice does not alter soil bacterial communities (Nieva et al. 2016, 2018, 2019).

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 (Fulchieri et al. 2001; Estrella et al. 2009; Sannazzaro et al. 2011). However, biological N₂ fixation by the *Mesorhizobium*-*L. tenuis* symbiosis in pastures of the Flooding Pampas has never been quantified, 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 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 provides 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 dried samples previously obtained from 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 ($\delta^{15}\text{N}$) 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 $\delta^{15}\text{N}$ in N₂ fixing plants grown with N₂ in air as the only N source ('B' value) must be determined simultaneously in 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 ^{15}N 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 ^{15}N natural abundance technique to assess the N_2 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 $\delta^{13}\text{C}$ to understand variations in carbon assimilation; and (4) to evaluate the symbiotic capacity of *L. tenuis* and native rhizobia by determining %Ndffa and the amount of N_2 fixed in soils of the Flooding Pampa. In addition to determining the %Ndffa 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 %Ndffa with commercial strains of rhizobia and co-inoculation tests with PGPR. We therefore determined %Ndffa under greenhouse conditions in parallel to the field study, to determine whether the two evaluations were replicable.

Material And Methods

Study sites description

The studies were conducted simultaneously at two sites during the experimental period September 2017– March 2018: (1) a pasture where *L. tenuis* had been promoted for 10 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 floods 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 (Lavado and Taboada 1988). Before legume promotion, plant communities consisted mainly of grasses (*Festuca arundinacea*, *Thynopirum ponticum*, *Cynodon dactylon*, *L. tenuis* and *Sporobolus indicus*) 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), twenty kilometers 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.

Pasture measurement and floristic composition

Plant biomass was harvested from five 0.25 m² quadrants located randomly 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 the Kjeldahl method, and Digestibility of Dry Matter (DDM) % by the method described by Tilley and Terry (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*.

Experiment 1: Promotion of *L. tenuis* pasture 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 six or seven annual cycles from June to August. The treatment consisted of 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. After 4 or 5 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 hours 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).

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. 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 $24/21 \pm 2^\circ\text{C}$ 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. Then, the *L. tenuis* seedlings were grown in 4 L pots containing sterile sand, and irrigated with N-free nutrient solution (Rigaud and Puppo 1975). They were inoculated following Unkovich et al. 2008, using a soil suspension with the native rhizobia present in the legume promoted field site. To do so, 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 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).

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 $\delta^{15}\text{N}$.

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 Analyser 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 (‰):

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

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 $^{13}\text{C}/^{12}\text{C}$ in 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 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):

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

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 assimilable mineral 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 analyses were 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 changed in both the promoted *Lotus* and the grassland sites in all seasons (Table 1). 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).

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 difference between forage types in each seasonal period. *Conyza bonariensis* and *Lythrum* sp were included in Weeds.

	Spring		Summer		Autumn	
	Promoted Lotus (%)	Grassland (%)	Promoted Lotus (%)	Grassland (%)	Promoted Lotus (%)	Grassland (%)
Trifolium repens	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 do not differ statistically.

	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	16.70 ± 1.46 ^b	63.00 ± 2.00 ^B

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. 1, during EB and FB, there was no difference in $\delta^{13}\text{C}$ between sites 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‰) and FB (-28.75‰). The trend was the same for $\Delta^{13}\text{C}$ (Supplementary Fig. 2), 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.

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. 2). 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 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. 3).

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

Regarding N concentration (Fig. 4A), *L. tenuis* values did not differ between the greenhouse and the field in the EB and the FB stages (Fig. 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, 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. 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. 4B). 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. 4C).

$\delta^{15}\text{N}$ 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 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. 3).

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

There was no significant effect from either of the reference plant species (%Ndfa = 80%) in the EB and FB phenological stages, or from the mixture of both (Fig. 5A, 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. 5C). It is interesting to note that the %Ndfa results for *L. tenuis* in the greenhouse were similar during the EB, FB and RG stages (Fig. 5). The same was observed in the field during EB and FB. In contrast, during RG, %Ndfa was lower in the field than in the greenhouse (Fig. 5).

Discussion

Pasture improvement by *L. tenuis* promotion

Temperate pastures 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 raising cattle in a season when the productivity of native grasslands is scarce (Bresciano et al. 2019). The main ecosystem limitation is its poor ability to maintain high levels of productivity during the summer (Tejera et al. 2016). 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. 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 grasses is mainly due to edaphic conditions, and especially to the topsoil pH gradients (Perelman et al. 2001, 2005; 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 (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), 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 (Grigera et al. 2007).

Plant physiological status and photosynthetic pathways can be assessed by considering carbon isotope discrimination values (Brüggemann et al. 2011; Luo et al. 2021). 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. 1 and 2) 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, 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 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). In addition, carbon sequestration can be achieved after legume introduction (overseeding) in grazed natural grasslands, depending on grazing management practices, as 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), 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 pasture 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 (Jaurena et al. 2016; Vecchio et al. 2019).

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

Accurate estimation and quantification of BNF by legumes depends on the methodology applied (Hardarson 1993). 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 27–42 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 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. 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 reference plants. In this selection, it is important to bear in mind that soil δ¹⁵N 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 N₂ fixing species (Carlsson et al. 2006; 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 of biologically fixed nitrogen (BNF) by legumes through the ¹⁵N 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; Peoples et al. 2009; 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.

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 ¹⁵N natural abundance method to estimate %Ndfa should be circumscribed to EB and FB phenological stages in greenhouse conditions.

The δ¹⁵N value was – 1.37‰ ± 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-N₂-fixing weeds (+ 6.4‰ ± 0.6), pointing to a major contribution of N₂ 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 ¹⁵N natural abundance technique to regrowth because of the potential complication of N recycling in the N₂-fixing species. Our results confirm

the importance of forage management, 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; 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), especially 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 to C reserves (Striker et al. 2011; Aranjuelo et al. 2014). Moreover, it is accepted that a reduction in N₂-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 (Aranjuelo et al. 2014). In addition, as has been reported (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 (*Distichlis spicata* 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 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 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 cattle productivity in the most important cattle-raising region in Argentina.

Declarations

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

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Figures

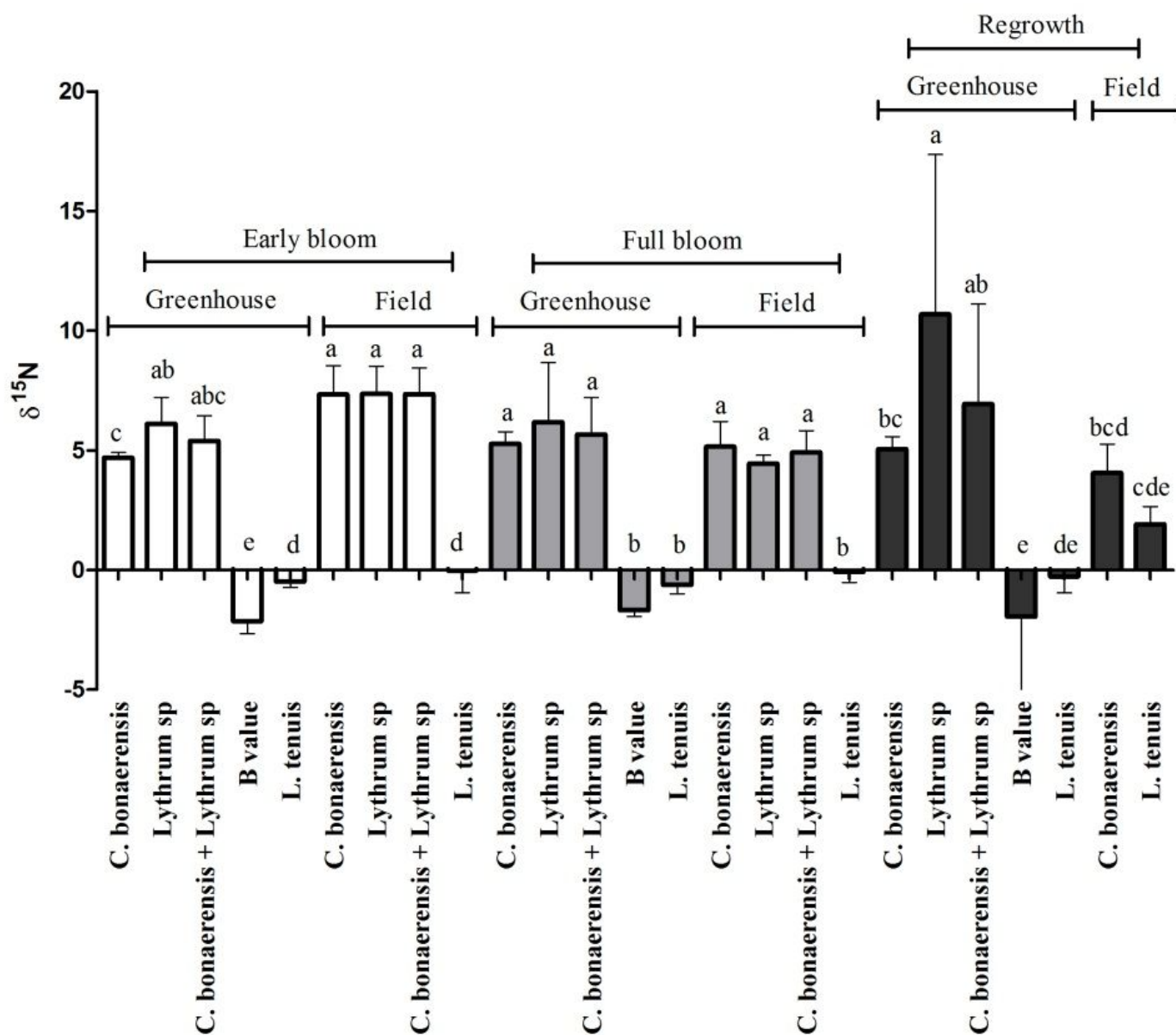


Figure 1

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; $P < 0.05$)

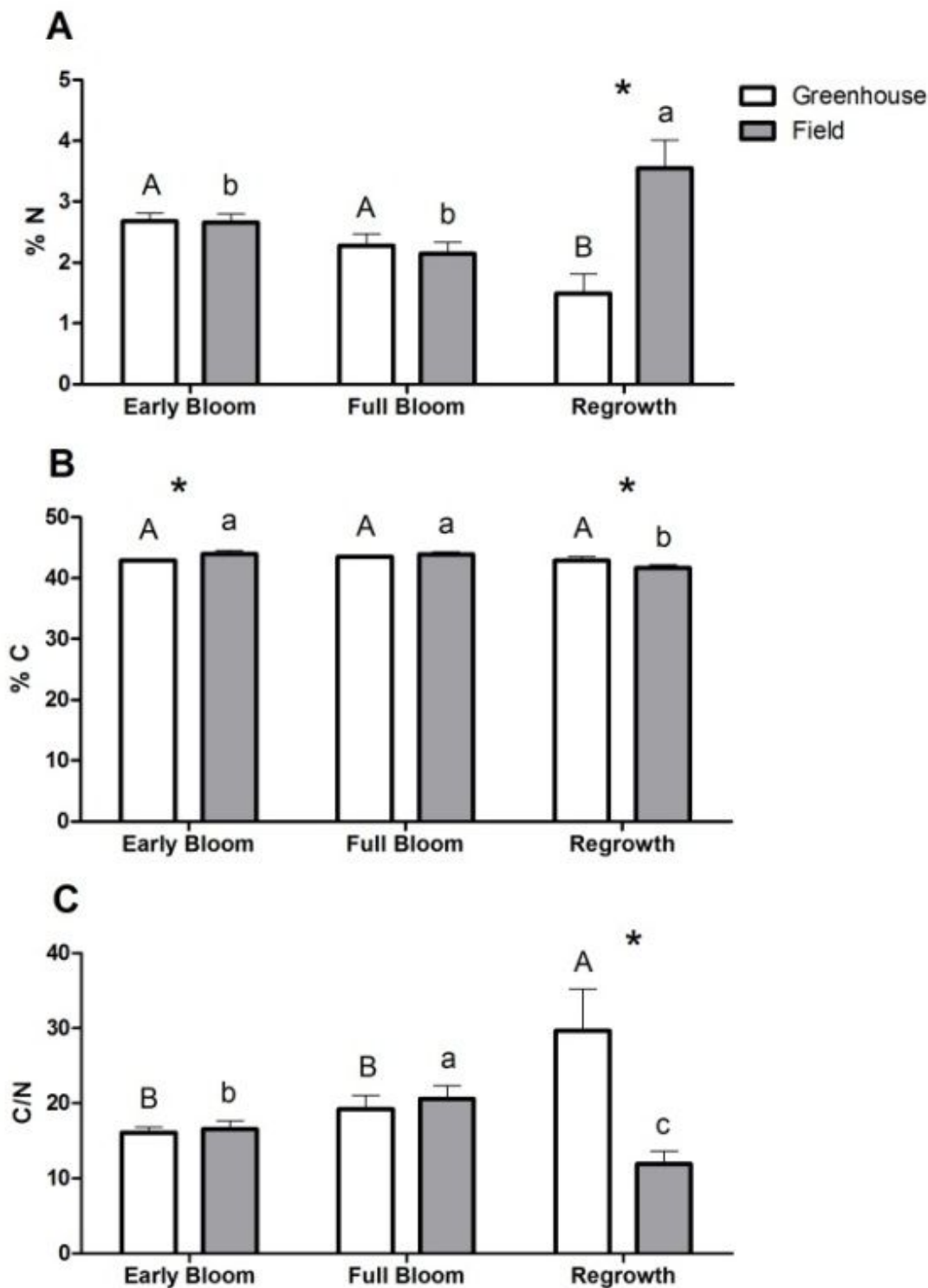


Figure 2

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; $P < 0.05$).

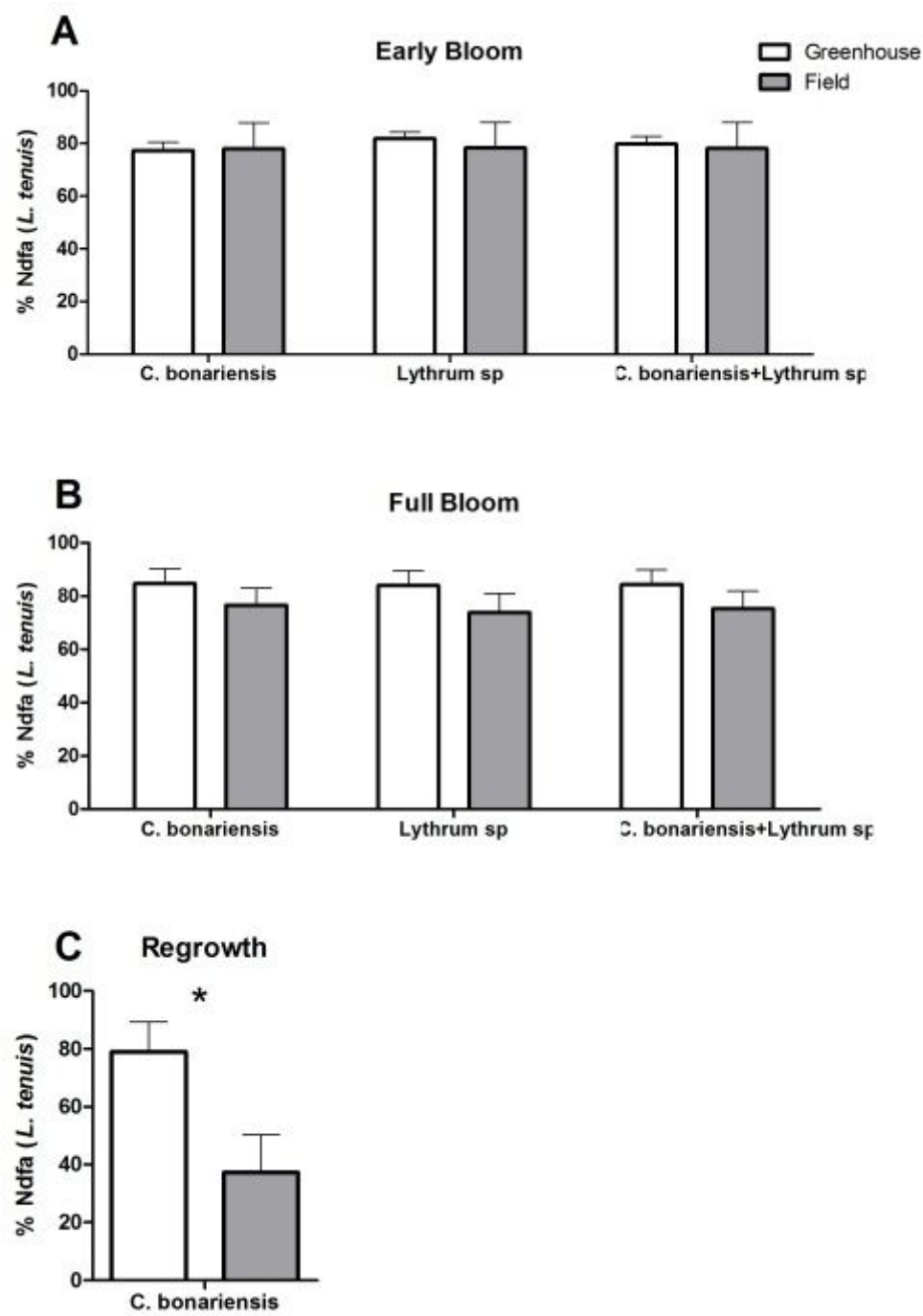


Figure 3

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; $P < 0.05$).

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Figure 4

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Image not available with this version

Figure 5

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