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Short communication

The elusive quantification of nitrogen fixation in xeric shrubs: The case of *Adesmia volckmanni*, a Patagonian leguminous shrub

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ABSTRACT

The comparison of ^{15}N natural abundance ($\delta^{15}N$) between fixing and non-fixing reference species, is the most feasible method for nitrogen fixation studies in xeric shrubs. However, it assumes that both species use the same sources of soil N, the $\delta^{15}N$ of such source is clearly different from zero, and the difference between the $\delta^{15}N$ of both species is $\geq$ 5‰. On the other hand, the analysis of the difference between the $\delta^{15}N$ of nodules and leaves ($\Delta\delta^{15}N_{n-l}$) does not require a reference species. To evaluate the effect of water availability on nitrogen fixation, we analyzed the variation of $\delta^{15}N$ and $\Delta\delta^{15}N_{n-l}$ in watered and unwatered plants of *Adesmia volckmanni* Phil. (Fabaceae) and *Mulinum spinosum* (Cav.) Pers. (Apiaceae), formerly proposed as non-fixing reference species. However, we found that *M. spinosum* was not valid as reference species to study N fixation in *Adesmia volckmann* because its $\delta^{15}N$ was closer to zero than that of the presumably fixing species, suggesting they use different soil N sources. In addition, water availability did not affect biological fixation after watering because both leaf $\delta^{15}N$ and $\Delta\delta^{15}N_{n-l}$ of *A. volckmanni* did not change either as soil dried up along the growing season nor in response to watering. However, the presence of nodules itself, the $\delta^{15}N$ closer to zero than in a former dry year, and the $\Delta\delta^{15}N_{n-l}$ (+ 1.23‰) suggest that biological fixation could have occurred before watering, probably during early spring.

1. Introduction

Quantification of nitrogen fixation of xeric shrubs is very difficult. The three main available procedures are the acetylene reduction assay, the ^{15}N dilution method, and the ^{15}N natural abundance method (Virginia et al., 1989). The acetylene reduction assay is accepted as the only method measuring potential nitrogenase activity, closely related to potential N fixation ability. However, it recquires the use of live nodules, which stay alive a very short time once detached from roots, and in the case of xeric shrubs are scarce and very deeply located in soil. The ^{15}N dilution method is the only method measuring the proportion of plant N obtained by fixation. However, it requires the use of expensive marked N in the vast and deep soil volume occupied by shrub root systems. In addition it must be applied with water, therefore confusing the measurement of N fixation itself with the effect of water addition on N fixation. Natural abundance of ^{15}N ($\delta^{15}N$) of fixing plants was proved to be more similar to that of atmosphere (atmospheric $\delta^{15}N = 0$) as more active is fixation (Dawson et al., 2002). As a consequence, the comparison between the leaf $\delta^{15}N$ of "fixing" and "non-fixing plants" may be a valid indirect evidence of N fixation (Dawson et al., 2002). However, this approach relies on two basic assumptions: both species use the same sources of soil N, and the $\delta^{15}N$ of such source is clearly different from zero (Högberg, 1997). In addition, the method relies on a $\geq$ 5‰ difference in the $\delta^{15}N$ values of both species, under active fixation (Högberg, 1997).

The objective of this work is to demonstrate the existence of N fixation in a leguminous shrub of the Patagonian steppe. In a previous work, we compared the leaf $\delta^{15}N$ of two conspicuous shrub species of the Patagonian steppes, a presumably N-fixing leguminous (*Adesmia volkmanni* Phil., Fabaceae) and a non-fixing umbelliferous (*Mulinum spinosum* (Cav.) Pers., Apiaceae) (Golluscio et al., 2006). Because of the high similarity of their root profiles and phenological patterns, we assumed that both species would access

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the same fractions of soil mineral N (Golluscio et al., 2005). However, the $\delta^{15}N$ of *Adesmia volkmanni* was similar to that of *M. spinosum* plants growing together in a xeric steppe (~ − 1.5‰ for both species), but was nearer to zero for *A. volckmanni* plants growing close to a wetland, and significantly different to the $\delta^{15}N$ of co-existing *M. spinosum* plants. As a consequence, we hypothesized that fixation in *A. volckmanni* would depend on water availability (Golluscio et al., 2006). The logical prediction from this hypothesis would be that biological fixation of *A. volckmanni* must increase with watering, and must decrease along the growing season, as water availability decreases (Sala et al., 1989). However, this may be an oversimplified hypothesis due to several logical and methodological problems. First, increasing water availability could also increase mineralization, nitrification, and soil N availability, then decreasing N fixation. Second, Patagonian shrubs usually show little response to watering, because they explore deep, humid soil layers (Golluscio et al., 1998, 2009). Third, even if watering triggers a N fixation pulse in *A. volckmanni*, it would not cause a sufficient $\delta^{15}N$ difference with respect to the reference species *M. spinosum*. Fourth, in spite of their phenology and root-structure similarites, both species may use different sources of edaphic N.

To deal with these problems, we not only compared the $\delta^{15}N$ of both watered and unwatered plants of both species but also analyzed the difference between the $\delta^{15}N$ of *A. volckmanni* nodules and leaves. This isotopic index may be useful to qualitatively evaluate biological N fixation, and has the advantage of being independent of the existence of a valid reference species. In N-fixing legume species, nodules are enriched in ^{15}N (i.e. its $\delta^{15}N$ is higher than that of their soil source) while leaf $\delta^{15}N$ is lower than that of nodules (Shearer et al., 1982), and the ^{15}N enrichment of nodules increases with fixation activity (Khadka and Tatsumi, 2006a). This pattern would respond to the discrimination against ^{15}N during the process of transference of fixed N from nodules to other sites, and also during the subsequent allocation and circulation of fixed N from shoot to root (Khadka and Tatsumi, 2006a). As a consequence, the difference between the $\delta^{15}N$ of nodules and leaves ($\Delta\delta^{15}N_{n-1}$) increases proportionally to biological fixation (Wanek and Arndt, 2002). This hypothesis was successfully tested for annual legume crops as soybean (Khadka and Tatsumi, 2006a; Schweiger et al., 2012; Wanek and Arndt, 2002), bean and cowpea (Khadka and Tatsumi, 2006a), but also for perennial legumes, as *Lespedeza cuneata* (Khadka and Tatsumi, 2006b). Although we do not know the exact relationship between $\Delta\delta^{15}N_{n-1}$ and N fixation for *A. volckmanni*, we will adopt $\Delta\delta^{15}N_{n-1}$ to qualitatively estimate N fixation. We expect this approach may be useful for other researchers aiming to assess N fixation in shrubs growing in arid environments.

2. Materials and methods

We performed a manipulative experiment from November 2006 to March 2007 within a 1 ha plot closed to the access of big herbivores since 1983, located at the experimental farm of INTA (Instituto Nacional de Tecnología Agropecuaria) at Río Mayo, SW of Chubut province, Argentina (45°41′ S, 70° 16′ W). The sampling area is representative of the Patagonian semiarid shrub-grass steppes. Soils have a 45–60 cm deep upper layer, sandy with high gravel content, over a calcareous layer with even higher gravel content (Paruelo et al., 1988). Mean annual temperature is 8.4 °C, with 14 °C in the hottest month (January) and 2 °C in the coldest one (July) (Golluscio et al., 1998). Mean annual precipitation is 150 mm, 70% falling in autumn-winter, the water recharge season (Jobbágy and Sala, 2000; Sala et al., 1989). Most water used by plants during the experiment fell during 2006, a humid year 24% over the historical mean (186 mm, concentrated between May and September). As usual, precipitations during the first three months of 2007 were scarce (6.1 mm between January and March 2007).

We irrigated plants with a unique water pulse at the beginning of growing season (november 7), until a 1 m deep soil cilinder of $R + 0.5$ m of radium (R = mean radium of the individual shrub) attained field capacity (see water retention curves in Paruelo et al., 1988). We compared irrigated *vs.* non-irrigated plants growing in the field, focusing on (a) the variation of the leaf natural abundance of ^{15}N ($\delta^{15}N$) along the growing season between *Adesmia volckmanni*, presumably "fixing" species, and *M. spinosum*, utilized as reference "non-fixing" species (Golluscio et al., 2006), and (b) the difference in $\delta^{15}N$ between nodules and leaves of the fixing species. We also measured leaf water potential and nitrogen content of both species, nitrogen content in *A. volckmanni* nodules, and $\delta^{15}N$, N content, and gravimetric water content at five soil depths (5, 15, 30, 45 and 60 cm) under plants of both species. We sampled all variables at four sampling dates during the growing season (Southern Hemisphere: December 6, January 7, February 7, and March 7).

The sampling units were 24 plant pairs, each one composed of one individual of each species, separated ≤1.5 m. At the time of watering, we randomly defined 6 pairs to be sampled at each of four sampling dates, and half of these pairs to be watered. At each sampling date, on the six randomly chosen pairs, we measured leaf water potential with a BioControl model 6 (Argentina) pressure bombe (Scholander et al., 1965). We also sampled leaves of both species to measure their $\delta^{15}N$ and N content, and extracted one 1 m deep soil parelellepiped (0.8 × 0.8 m section), with one vertical face below the canopy of each individual. At five depths of both faces we gathered the soil samples, on which we measured all the above mentioned soil variables. In addition, on the face below *A. volckmanni*, we harvested nodules to measure their $\delta^{15}N$ and N content.

We performed the analyses of soil and plant N content and $\delta^{15}N$ (standard: atmospheric N_2) at CATNAS (Centro de Aplicaciones de Tecnología Nuclear en Agricultura Sostenible) of University of the Republic, Uruguay, using a Finnigan MAT Delta Plus XL mass spectrometer. The precision of the device, calculated as the standard deviations of the repeated measurements made on laboratory standards were 0.1% and 0.1‰ for plant samples, and 0.2% and 0.2‰ for soil samples (for N content and $\delta^{15}N$ respectively). Since both species produce green leaves at the same time of the year and did not retain green leaves of previous years (Golluscio et al., 2005), the leaves of both species had essentially the same age at each sampling date. Soil and leaf samples were oven dried at 60 °C for 48 h, then ground with mortar and pestle until samples could pass through a 40 μm mesh.

To measure gravimetric soil water content, immediately following gathering, we placed <2 mm sieved soil samples within double polyethilene bags to avoid water losses. We kept samples at air temperature during field work and afterwards in refrigerator at 5 °C until processing. Then, we weighed mass of samples, before and after dry them in oven at 105 °C until constant weight, and calculated gravimetric water content as the mass ratio beween water and dry soil.

We analyzed the results by Analysis of Variance (ANOVA) for linear mixed models. For plant variables (leaf water potential, $\delta^{15}N$ and N content of leaves and nodules), we used a two way array (4 dates × 2 watering levels), with plots splitted by species (2 levels: *M. spinosum* or *A. volckmanni*, except in the case of nodules, which only existed below *A. volckmanni*). To analyze soil variables ($\delta^{15}N$, N content and gravimetric water content), we used the same statistical layout (4 dates × 2 watering levels), with plots splitted by species (2), but assessing the depth effect (5 depths) below the respective plant species. To do this, we incorporated into the linear model a simple correlation structure associated to each plot and species. All studied effects were fixed, except for plots. In all cases