BIOLOGICAL FIXATION OF N₂ IN MONO AND POLYSPECIFIC LEGUME PASTURE IN THE HUMID MEDITERRANEAN ZONE OF CHILE

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ABSTRACT

Despite annual legume pasture are of great importance for dryland agricultural systems in Mediterranean environments, there are few studies of N₂ biological fixation (NBF) reported in Chile. In this study the NBF of four annual legume species: subterranean clover (Trifolium subterraneum L.), yellow serradella (Ornithopus compressus L.), arrow-leaf clover (T. vesiculosum L.), and crimson clover (T. incarnatum L.) (Experiment 1), as well as seven mixtures of these species (Experiment 2) were assessed. The NBF was measured by the ¹⁵N natural abundance technique. The objective was to determine NBF in the legume species and in distinct mixtures used. The study was carried out in an Andisol of the Andean Precordillera located in the humid Mediterranean zone of Chile. Pasture was evaluated for biomass; and total N and natural abundance of ¹⁵N were analyzed in plant material samples. In Experiment 1 (monospecific legume species pasture), N derived from fixation ranged between 43 and 147 kg N ha⁻¹ and where T. vesiculosum and T. subterraneum presented statistical differences (P ≤ 0.05) in connection with the other species. In the legume mixtures (Experiment 2), N derived by fixation varied between 97 and 214 kg N ha⁻¹ where the 50-50 mixtures (T. subterraneum and O. compressus, or T. subterraneum and T. vesiculosum, respectively) had the highest N fixation. Fixed N ranged between 12 and 25 kg N t⁻¹ DM, showing significant differences among mono and polyspecific legume species.

Key words: Natural abundance of ¹⁵N, Mediterranean pastures, volcanic soil.

INTRODUCTION

Legume pastures are the basis of pasture production in Mediterranean climate environments in the world as well as in Central Chile, and consequently, is the main source of nutrients for sheep and cattle production in these extensive agro-ecological areas. The two environmental factors mainly limiting pasture productivity in these environments are water availability associated to Mediterranean climatology (Loss and Siddique, 1994) and soil fertility conditions, particularly N and P supply (Lopes et al., 2004). In this context, production systems based on legume fodder crops can play a fundamental role in improving soil fertility, allowing efficient water and nutrient use, and weed control (Evans et al., 2001). Thus, for farming systems based on permanent grazing of pastures where the establishment and maintenance fertilization cost is always higher, it is possible to achieve savings in N fertilizers through the selection of species and cultivars with a high N biological fixation (NBF) potential which is key to the success of a sustainable livestock activity.

Nitrogen biological fixation can directly contribute to agricultural production providing N from the vegetative parts of leaves, pods, seeds, and tubers of plants used as livestock feed or harvested for human consumption. NBF can also be an important source of N for agricultural soils through residues rich in N subsequent to plant harvest or grazing (Unkovich et al., 2008). High value forage crops provide farmers with the capacity to diversify their production systems and are an integral part of strategies to intensify animal production (Unkovich et al., 2008).

Legume species and cultivars differ in their N fixation capacity and in the biomass N content at the stem and root level and consequently, in the capacity to contribute N to the soil (Peoples et al., 1995a; Urzúa, 2000; Fillery,
2001; Campillo et al., 2003; Ovalle et al., 2006). The transfer of N from legumes to associated species in the pasture or to other crops in a legume-crop rotation system mainly occurs through the decomposition of their residues (Danso et al., 1993; Peoples et al., 1998).

Annual legumes mixtures increase plant diversity, productivity and pasture persistence (Tilman et al., 1997; Avendaño et al., 2005). A key aspect in the design of pasture mixtures is the correct selection of species and cultivars, which must combine different reproductive strategies and be able to establish and maintain an adequate seed bank in the soil for self-seeding after the crop face in a pasture-crop rotation (Loi et al., 2000; Norman et al., 2005; Ovalle et al., 2005).

Isotopic methods applied to study N fixation use two techniques, the isotopic dilution which use enriched fertilizer in $^{15}$N and the natural abundance of $^{15}$N (Boddey et al., 1990). The former have been used to study the biochemistry of N$_2$ fixation and its quantification in legume crops, and factors affecting the N$_2$ fixation process, as well as to evaluate the efficiency of different microorganisms to fix N$_2$ and of the use of N fertilizer; the transfer of N in the soil or between plants and in studies of N balance. The natural abundance method has been used to evaluate NBF in natural ecosystems, because it does not involve excessive manipulation of the ecosystem, and also in legume pastures Mediterranean environments. This method has been proved to be reliable and provides accurate estimates of NBF in pastures and pulses (Ledgard and Steele, 1992; Doughton et al., 1995; Peoples et al., 1996; Unkovich et al., 1997).

Very few studies of NBF have been reported for pastures in Chile (Herrera et al., 1996; Campillo et al., 2003; Ovalle et al., 2006) and data of N fixation in Mediterranean pastures are particularly scarce. The objective of this study was to quantify NBF of four annual legume species and their respective mixtures using the $^{15}$N natural abundance technique in the humid Mediterranean in Chile.

**MATERIALS AND METHODS**

**Site description and experiment management**

The study was carried out in the Andean foothill of the Yungay county (37°10’ S, 71°58’ W, 297 m.a.s.l.), located in the humid Mediterranean zone of south-central Chile. The mean annual temperature is 14 °C, January being the warmest month and July the coldest, and with a 5-mo frost-free period (Novoa and Villaseca, 1989). Mean annual rainfall reaches 1200 mm (del Pozo and del Canto, 1999) and 4-mo (December to March) of dry season (Novoa and Villaseca, 1989). The soil at the site is an Andisol of loam texture, of the Santa Barbara soil series (Typic Haploxerands; CIREN, 1999; Stolpe, 2006). Topography of the experimental site was slightly hilly. The soil presents levels of 5.8 pH, 15% OM, 4 mg kg$^{-1}$ soil inorganic N, 9 mg kg$^{-1}$ P, and 49 mg kg$^{-1}$ K at 20 cm depth. Previous crop was wheat (Triticum aestivum L.) and residues (equivalent to 2.5 t ha$^{-1}$) were incorporated to the soil in autumn.

Nitrogen fixation of four species of annual legumes (Experiment 1) and their respective mixtures (Experiment 2) were evaluated. In Experiment 1, the species were subterranean clover (Trifolium subterraneum cv. Mount Barker), yellow serradella (Ornithopus compressus cv. Ávila), arrow-leaf clover (T. vesiculosum cv. Zulu) and crimson clover (T. incarnatum cv. Corriente). In Experiment 2, seven mixtures of annual legume forage crops were evaluated; each mixture contained a proportion of 2, 3 or 4 species, and seed ratio was calculated based on the seed number to obtain 1000 plants per m$^2$ (Table 1). Reference plants were non-N fixing: annual ryegrass (Lolium multiflorum L. cv. Tama), orchard grass (Dactylis glomerata cv. Currie), tall fescue (Festuca arundinacea cv. Exella), and harding grass (Phalaris aquatica cv. Seed Master), established in the same year and experimental site. According to Boddey et al. (2000; 2001), a minimum of three reference species should considered due to variability in the quantity of N accumulated in biomass.

Pastures were sown in rows separated by 20 cm in 5 x 4 m plots, in both experiments, in a randomized complete block design with four replicates. Seeds were inoculated with the specific Rhizobium (10 g inoculant kg$^{-1}$ seed) for each species using methyl cellulose (1%) as a glue (1 L. 25 kg$^{-1}$ seeds), and adding calcium carbonate (6-9 kg CaCO$_3$, 50 kg$^{-1}$ seeds) to cover and pellet the seeds. Fertilization in both experiments was the equivalent of 150 kg P$_2$O$_5$ ha$^{-1}$ (triple superphosphate), 500 kg CaSO$_4$ ha$^{-1}$ (calcium sulfate), 48 kg K$_2$O ha$^{-1}$ (potassium muriate), additionally 36 kg MgO, 44 kg K$_2$O and 44 S kg ha$^{-1}$ (sulphomag), and 2.2 kg B ha$^{-1}$ (boronatrocacite), were applied before sowing (broadcast). No N fertilizer was applied.

**Evaluations and determination of natural abundance of $^{15}$N**

Above-ground biomass production was carried out by random collection in two 1 x 0.5 m quadrants, in each plot, in both experiments. Samples were oven-dried with forced air ventilation at 70 °C until a constant weight was reached for dry matter (DM) determination. Subsequently, a subsample of 1-2 g of plant material was sent to the Laboratory of Agrobiology EMBRAPA, Brazil, to determine total N (by Kjeldahl digestion) and the $^{15}$N natural abundance ($^{15}$N,N using a Finnigan Delta Plus continuous-flow isotope-ratio mass spectrometer
interfaced with a Carlo Erba (Model EA 1108) automatic C-N analyzer (Finnigan-MAT, Bremen, Germany).

The $^{15}$N natural abundance technique was used to estimate plant NBF. Three values of $^{15}$N natural abundance were determined to estimate the proportion of N derived by biological fixation: a) the value $\beta$ is obtained from inoculated legume plants with effective Rhizobium strains in an N-free medium which is then analyzed in terms of its $\delta^{15}$N ratio (Peoples et al., 1995a; 1995b) the abundance of $^{15}$N-N derived from control plants, that is, non-N$_2$ fixing ($\delta^{15}$N$_{ref}$), and; c) the natural abundance of $^{15}$N from N$_2$-fixing plants ($\delta^{15}$N$_{fix}$). The percentage of N derived from air (%Ndfa), which is the proportional contribution of NBF to N in the legume, is calculated from the natural abundance of $^{15}$N of the legume and the control plant as indicated in the following equation (Shearer and Köhl, 1986):

$$\% \text{Ndfa} = \frac{100 (\delta^{15} N_{ref} - \delta^{15} N_{fix})}{(\delta^{15} N_{ref} - \beta)}$$

The $^{15}$N content in reference plants provides an integral estimate of available $\delta^{15}$N in the soil during the whole growing period. Furthermore, it is assume that the pool of available $^{15}$N in the soil is the same for both the reference plant and legumes (Boddey et al., 2000).

In this study the value of $\beta$ was -1/o/oo as have been used by different authors in other fixation studies in Chile as well as in Australia (Unkovich et al., 1994; Ovalle et al., 2006). Most of the $\beta$ values described for legume species oscillate between -2 and +1/o/oo (Köhl and Shearer, 1980; Shearer et al., 1980; Steele et al., 1983; Yoneyama et al., 1986; Ledgard, 1989; Unkovich et al., 1994; Boddey et al., 2000). This variation is due legume-rhizobium association which can affect the natural abundance of $^{15}$N in legumes (Köhl et al., 1983; Steele et al., 1983; Bergersen et al., 1986; Yoneyama et al., 1986).

All data were subjected to ANOVA (P ≤ 0.05) previous test of normality. Mean separation was done by Duncan’s multiple range test. All statistical analyses were carried out by SAS Systems for Windows V8 (SAS Institute, 1999).

**RESULTS AND DISCUSSION**

In Experiment 1, the highest production of DM was obtained in *O. compressus* cv. Ávila and *T. vesiculosum* cv. Zulu (9772 and 8830 kg ha$^{-1}$), significantly different (P ≤ 0.05) from *T. subterraneum* cv. Mount Barker (6204 kg ha$^{-1}$), the latter being higher than the production of *T. incarnatum* cv. Corriente (3378 kg ha$^{-1}$) (Table 2). The percentage of N in the biomass was similar in all species but the accumulated N showed the same tendency as DM.
The lowest δ¹⁵N was found in *T. subterraneum*, which was significantly different (P ≤ 0.05) from *O. compressus* and *T. incarnatum* (Table 2). This means that the natural abundance of ¹⁵N was lower in *T. subterraneum* compared to *O. compressus* and *T. incarnatum*.

In Experiment 2, the higher biomass production was obtained in mixtures containing high proportion of *T. vesiculosum* at sowing and in the botanical composition (T2, T4 and T5), and in the mixture of *T. subterraneum* and *O. compressus* (T1), whereas the lower production was attained in mixtures with 25-50% of *T. incarnatum* at sowing (Table 3). The same tendency was observed in accumulated N, but N concentration was significantly different in the different mixtures (Table 3). The lowest δ¹⁵N was obtained in the mixture of *T. subterraneum* and *O. compressus* (T1), indicating lower values of ¹⁵N in the dry matter (Table 3).

Four gramineae species were used as reference plants to estimate NBF in both assays. The values of natural abundance of ¹⁵N (δ¹⁵N) for each one were: annual ryegrass (1.26‰), orchard grass (1.31‰), tall fescue (1.39‰) and harding grass (1.59‰). In Experiment 1 (monospecific legumes), the highest %Ndfa was observed in *T. subterraneum*, however Ndfa (kg N ha⁻¹) was similar (P > 0.05) to *O. compressus* and *T. vesiculosum*, due to differences in biomass production which masked differences in N fixed among species (Table 4). The N fixed, expressed as kg N t⁻¹ DM, fluctuated between 10 and 22, with statistical differences among *T. subterraneum* as compared to *O. compressus* and *T. incarnatum* (Table 4).

In Experiment 2, the %Ndfa was highest in the mixture of *T. subterraneum* and *O. compressus* (T1), which had the highest proportion of *T. subterraneum* in the biomass (Table 1 and 5). The Ndfa (kg N ha⁻¹) was higher in the mixture of *T. subterraneum* and *O. compressus* (T1), and the mixture with *T. vesiculosum* (T2, T3 and T4) (Table 5). The lower Nfda was obtained in mixtures with 10-41% of *T. incarnatum* in the biomass (Table 1 and 5). Statistical differences (P ≤ 0.05) were observed in the fixed N per unit of DM between the mixture of *T. subterraneum* and *O. compressus* (T1), and all the other mixtures (Table 5).

The results show the high potential of biomass production of legume pastures in volcanic soils of the Andean foothill which reached 11 t DM ha⁻¹ yr⁻¹ in some mixtures. These DM production are much higher than in other studies carried out in subhumid Mediterranean

### Table 3. Aerial biomass production, N concentration, N accumulation, and ¹⁵N natural abundance (δ¹⁵N) in specific annual legume mixtures (Experiment 2).

<table>
<thead>
<tr>
<th>Treatments (mixtures)</th>
<th>Dry matter kg ha⁻¹</th>
<th>N concentration %</th>
<th>N accumulation kg ha⁻¹</th>
<th>δ¹⁵N‰</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>8 551abc¹</td>
<td>3.77a</td>
<td>322abc</td>
<td>-0.19c</td>
</tr>
<tr>
<td>T2</td>
<td>11 386a</td>
<td>3.75a</td>
<td>427a</td>
<td>0.26b</td>
</tr>
<tr>
<td>T3</td>
<td>5 613c</td>
<td>3.87a</td>
<td>217c</td>
<td>0.33ab</td>
</tr>
<tr>
<td>T4</td>
<td>9 453ab</td>
<td>3.92a</td>
<td>371ab</td>
<td>0.43a</td>
</tr>
<tr>
<td>T5</td>
<td>9 379ab</td>
<td>3.81a</td>
<td>357abc</td>
<td>0.33ab</td>
</tr>
<tr>
<td>T6</td>
<td>6 993bc</td>
<td>3.71a</td>
<td>259bc</td>
<td>0.24b</td>
</tr>
<tr>
<td>T7</td>
<td>7 621bc</td>
<td>4.00a</td>
<td>305abc</td>
<td>0.50a</td>
</tr>
</tbody>
</table>

Values with distinct letters in the columns are different according to Duncan multiple range test (P ≤ 0.05).

### Table 4. Percentage of N derived from air in plants (% Ndfa) in the mixtures of four non-N fixing plants (control), N derived from air (Ndfa), and fixed N per unit of dry matter (Fixed N) (Experiment 1).

<table>
<thead>
<tr>
<th>Treatments (species)</th>
<th>% Ndfa</th>
<th>Rye grass¹</th>
<th>Orchard grass</th>
<th>Tall fescue</th>
<th>Harding grass</th>
<th>Mean</th>
<th>Ndfa kg N ha⁻¹</th>
<th>Fixed N kg t⁻¹ DM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. subterraneum</em> cv. Mount Barker</td>
<td>60</td>
<td>61</td>
<td>62</td>
<td>65</td>
<td>62a</td>
<td>138a</td>
<td>22a</td>
<td></td>
</tr>
<tr>
<td><em>O. compressus</em> cv. Ávila</td>
<td>26</td>
<td>28</td>
<td>30</td>
<td>35</td>
<td>30b</td>
<td>102ab</td>
<td>10b</td>
<td></td>
</tr>
<tr>
<td><em>T. vesiculosum</em> cv. Zulu</td>
<td>37</td>
<td>39</td>
<td>41</td>
<td>45</td>
<td>41b</td>
<td>147a</td>
<td>17ab</td>
<td></td>
</tr>
<tr>
<td><em>T. incarnatum</em> cv. Corriente</td>
<td>26</td>
<td>28</td>
<td>30</td>
<td>36</td>
<td>30b</td>
<td>43b</td>
<td>12b</td>
<td></td>
</tr>
</tbody>
</table>

¹Control plants.
Values with distinct letters in the columns are different according to Duncan multiple range test (P ≤ 0.05).
environments, in both Australia and Chile (Peoples et al., 1995b; Dear et al., 2004; del Pozo and Ovalle, 2009; Ovalle et al., 2008; 2010). The lower water and nutrient availability in subhumid areas explained the lower productivity of pastures (Peoples et al., 1995b; Unkovich et al., 1997; Dear et al., 2004), factors which are closely related to the fixed N quantities of the pastures.

Results of %Ndfa show important differences in the efficiency of the NBF among legumes species in mono and polyspecific pastures, in Andisols of the humid Mediterranean zone of Chile. In general, pastures with high proportion of *T. subterraneum* presented higher %Ndfa. In soils with more limited fertility such as granites and Vertisols of the interior dryland of the subhumid zone, the Ndfa (%) values of *T. subterraneum* and *O. compressus* were 82-95% (Ovalle et al., 2006).

The high organic matter (16%) content and subsequent high mineralization capacity and N mineral availability in Andisols would explain the lower efficiency in the fixation process (Zagal et al., 2003).

Despite the lower %Ndfa detected in some species, the amounts of fixed N per unit of area were high, especially in *T. vesiculosum* and in mixtures containing this species, as well as *T. subterraneum* and *O. compressus* (112 to 214 kg N ha⁻¹) (Table 5). This can be explained by the high biomass production of these pastures except the one containing 50% *T. incarnatum* (Table 3). These results agree with studies carried out in a perhumid environment in southern Chile, with similar N fixation values (190 kg N ha⁻¹) in *T. subterraneum* (Campillo et al., 2003). In granitic soils and Vertisols of the subhumid Mediterranean zone (650 mm) the N fixation values were 41 and 56 kg N ha⁻¹ mainly as a consequence of the lower biomass productions (Ovalle et al., 2006). In *Lotus corniculatus* L. in Vertisols under irrigation and different cutting regimes, the amount of N fixed ranged from 112 to 173 kg N ha⁻¹ yr⁻¹ (Ruz et al., 1999).

In other Mediterranean environments in Australia, Peoples et al. (1995b) reported a wide range of N fixation from 2 to 206 kg N ha⁻¹ yr⁻¹ in pasture of *T. subterraneum*. Other studies carried out by Peoples et al. (1998) in northern Victoria and southern New South Wales indicate that efficiency was 20 to 25 kg N t⁻¹ DM produced by *T. subterraneum* pastures. Our results showed higher levels of N fixed per ton of DM in both monospecific pastures (10-22 kg N t⁻¹ DM, Experiment 1) and mixtures (15-25 kg N t⁻¹ DM, Experiment 2).

In summary, the amount of NBF by a legume pasture is depending of the species composition; however, other factors like the effectiveness of the Rhizobium strains, edaphoclimatic conditions, pasture management, and eventually, livestock management are also important. All these factors have an influence on δ¹⁵N values but also in the DM production, which are the basis for estimating N fixation (Eriksen and Hogh-Jensen, 1998). In addition, soil N availability as well as small topographical variations provoking differences in water content or flooding can also influence NBF (Stevenson et al., 1995).

**CONCLUSIONS**

From the studied annual forage species, *T. subterraneum*, *O. compressus* and *T. vesiculosum* fixed more N (average 129 kg N ha⁻¹) than *T. incarnatum* (43 kg N ha⁻¹). The higher NBF was reached in 50:50 legume mixtures of *T. subterraneum* and *O. compressus*, or *T. subterraneum* with *T. vesiculosum*, reaching an average of 208 kg N ha⁻¹, demonstrating synergy between these species in Chilean Mediterranean humid zone.

**Table 5. Percentage of N derived from air in plants (% Ndfa) in mixtures of four non-N fixing plants (control), N derived from air (Ndfa), and fixed N per unit of dry matter (Fixed N) (Experiment 2).**

<table>
<thead>
<tr>
<th>Treatments (mixtures)</th>
<th>Rye grass¹</th>
<th>Orchard grass</th>
<th>Tall fescue</th>
<th>Harding grass</th>
<th>% Ndfa</th>
<th>Ndfa kg N ha⁻¹</th>
<th>Fixed N kg t⁻¹ DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>64</td>
<td>65</td>
<td>66</td>
<td>69</td>
<td>66a</td>
<td>214a</td>
<td>25a</td>
</tr>
<tr>
<td>T2</td>
<td>44</td>
<td>45</td>
<td>47</td>
<td>51</td>
<td>47bc</td>
<td>202a</td>
<td>18b</td>
</tr>
<tr>
<td>T3</td>
<td>41</td>
<td>43</td>
<td>45</td>
<td>49</td>
<td>45bc</td>
<td>97b</td>
<td>18b</td>
</tr>
<tr>
<td>T4</td>
<td>37</td>
<td>38</td>
<td>40</td>
<td>45</td>
<td>40cd</td>
<td>148ab</td>
<td>16b</td>
</tr>
<tr>
<td>T5</td>
<td>41</td>
<td>42</td>
<td>44</td>
<td>49</td>
<td>44cd</td>
<td>158ab</td>
<td>17b</td>
</tr>
<tr>
<td>T6</td>
<td>45</td>
<td>47</td>
<td>48</td>
<td>52</td>
<td>48b</td>
<td>125b</td>
<td>18b</td>
</tr>
<tr>
<td>T7</td>
<td>33</td>
<td>35</td>
<td>37</td>
<td>42</td>
<td>37d</td>
<td>112b</td>
<td>15b</td>
</tr>
</tbody>
</table>

¹Control plants. Values with distinct letters in the columns are different according to Duncan multiple range test (P ≤ 0.05).
ACKNOWLEDGEMENTS

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RESUMEN

Fijación biológica de N₂ en praderas mono y poliespecíficas de leguminosas en la zona mediterránea húmeda de Chile. A pesar de la gran importancia que las praderas de leguminosas tienen en los sistemas agrícolas de secano en ambientes mediterráneos, existe muy poca información sobre la fijación biológica de N₂ (FBN) reportada en Chile. En este estudio se evaluó la FBN en cuatro leguminosas forrajeras anuales: trébol subterráneo (Trifolium subterraneum L.), serradella amarilla (Ornithopus compressus L.), trébol vesiculoso (T. vesiculosum L.) y trébol encarnado (T. incarnatum L.) (Experimento 1), además de siete mezclas de estas especies (Experimento 2). La FBN se midió mediante la técnica de la abundancia natural de ¹⁵N. El objetivo fue determinar la FBN en las especies de leguminosas y en las diferentes mezclas. El estudio se llevó a cabo en un suelo Andisol, de la Precordillera Andina, localizada en la zona mediterránea húmeda de Chile. En la pradera se evaluó producción de biomasa y en submuestras se analizó N total y abundancia natural de ¹⁵N. En el Experimento 1, el N derivado de la fijación fluctuó entre 43 y 147 kg N ha⁻¹; siendo las mezclas 50-50 (T. subterraneum - T. vesiculosum) las que presentaron diferencias estadísticas (P ≤ 0,05) con respecto a las otras especies en estudio. En la pradera se evaluó producción de biomasa y en submuestras se analizó N total y abundancia natural de ¹⁵N. En el Experimento 2 el N derivado de la fijación fluctuó entre 97 y 214 kg N ha⁻¹; siendo las mezclas 50-50 (T. subterraneum - O. compressus y T. subterraneum - T. vesiculosum, respectivamente) las que presentaron la mayor fijación de N por hectárea. El N fijado fluctuó entre 12 y 25 kg N t⁻¹ MS, presentando diferencias significativas entre especies de leguminosas mono y poliespecíficas.

Palabras clave: abundancia natural de ¹⁵N, praderas mediterráneas, suelo volcánico.

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