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BIOLOGICAL FIXATION OF N_2 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 N_2 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 ^{15}N 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 ^{15}N 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 ($P \leq 0.05$) 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 ^{15}N, 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,

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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_2 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 ºC, 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 (O*rnithopus 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_2O_5 ha^{-1} (triple superphosphate), 500 kg $CaSO_4$ ha^{-1} (calcium sulfate), 48 kg K_2O ha^{-1} (potassium muriate), additionally 36 kg MgO, 44 kg K_2O and 44 S kg ha^{-1} (sulpomag), and 2.2 kg B ha^{-1} (boronatrocalcite), 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 ($\delta^{15}N$) using a Finnigan Delta Plus continuous-flow isotope-ratio mass spectrometer

Table 1. Seed ratio (%) in the different annual legume mixtures and actual contribution (%) of the species in the mixture in November (in brackets) (Experiment 2).

Treatment (mixtures)	*Trifolium subterraneum* cv. Mount Barker	*Ornithopus compressus* cv. Ávila	*T. vesiculosum* cv. Zulu	*T. incarnatum* cv. Corriente
T1	50 (60)	50 (38)	-	-
T2	50 (8)	-	50 (92)	-
T3	50 (50)	-	-	50 (41)
T4	33 (14)	33 (25)	33 (60)	-
T5	33 (32)	-	33 (57)	33 (10)
T6	33 (32)	33 (48)	-	33 (13)
T7	25 (20)	25 (23)	25 (33)	25 (15)

Saving time in april.

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 β is obtained from inoculated legume plants with effective *Rhizobium* strains in an N-free medium which is then analyzed in terms of its δ^{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):

$$\% \ Ndfa = \frac{100\ (\delta^{15}\ N_{ref} - \delta^{15}\ N_{flx})}{(\delta^{15}\ N_{ref} - ß)}$$

The ^{15}N content in reference plants provides an integral estimate of available δ^{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 β was -1‰ 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 β values described for legume species oscillate between -2 and +1‰ (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 ($P \leq 0.05$) 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 ($P \leq 0.05$) 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

Table 2. Dry matter production, N concentration, N accumulation, and natural abundance (δ^{15}N) in monospecific pastures of annual legumes (Experiment 1).

Treatments (species)	Dry matter	N concentration	N accumulation	δ^{15}N
	kg ha^{-1}	%	kg ha^{-1}	‰
Trifolium subterraneum cv. Mount Barker	6204b	3.60a	223b	-0.09b
Ornithopus compressus cv. Ávila	9772a	3.49a	341a	0.67a
T. vesiculosum cv. Zulu	8830a	4.10a	362a	0.42ab
T. incarnatum cv. Corriente	3378c	4.16a	141b	0.67a

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

(Table 2). The lowest $\delta^{15}N$ was found in *T. subterraneum*, which was significantly different ($P \leq 0.05$) from *O. compressus* and *T. incarnatum* (Table 2). This means that the natural abundance of ^{15}N 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 $\delta^{15}N$ was obtained in the mixture of *T. subterraneum* and *O. compressus* (T1), indicating lower values of ^{15}N 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 ^{15}N ($\delta^{15}N$) 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 ($P \leq 0.05$) 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 ^{15}N natural abundance ($\delta^{15}N$) in specific annual legume mixtures (Experiment 2).

Treatments (mixtures)	Dry matter	N concentration	N accumulation	$\delta^{15}N$
	kg ha^{-1}	%	kg ha^{-1}	‰
T1	8 551abc[1]	3.77a	322abc	-0.19c
T2	11 386a	3.75a	427a	0.26b
T3	5 613c	3.87a	217c	0.33ab
T4	9 453ab	3.92a	371ab	0.43a
T5	9 379ab	3.81a	357abc	0.33ab
T6	6 993bc	3.71a	259bc	0.24b
T7	7 621bc	4.00a	305abc	0.50a

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

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).

Treatments (species)	% Ndfa					Ndfa	Fixed N
	Rye grass[1]	Orchard grass	Tall fescue	Harding grass	Mean	kg N ha^{-1}	kg t^{-1} DM
Trifolium subterraneum cv. Mount Barker	60	61	62	65	62a	138a	22a
Ornithopus compressus cv. Ávila	26	28	30	35	30b	102ab	10b
T. vesiculosum cv. Zulu	37	39	41	45	41b	147a	17ab
T. incarnatum cv. Corriente	26	28	30	36	30b	43b	12b

[1] Control plants.

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

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).

Treatments (mixtures)	% Ndfa					Ndfa	Fixed N
	Rye grass[1]	Orchard grass	Tall fescue	Harding grass	Mean	kg N ha^{-1}	kg t^{-1} DM
T1	64	65	66	69	66a	214a	25a
T2	44	45	47	51	47bc	202a	18b
T3	41	43	45	49	45bc	97b	18b
T4	37	38	40	45	40cd	148ab	16b
T5	41	42	44	49	44cd	158ab	17b
T6	45	47	48	52	48b	125b	18b
T7	33	35	37	42	37d	112b	15b

[1] Control plants.

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

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 of levels of %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 $\delta^{15}N$ 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.

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RESUMEN

Fijación biológica de N_2 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 N_2 (FBN) reportada en Chile. En este estudio se evaluó la FBN en cuatro leguminosas forrajeras anuales: trébol subterráneo (*Trifolium subterraneum* L.), serradela 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 ^{15}N. 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 ^{15}N. En el Experimento 1, el N derivado de la fijación fluctuó entre 43 y 147 kg N ha^{-1}; siendo *T. vesiculosum* y *T. subterraneum* las que presentaron diferencias estadísticas ($P \leq 0,05$) con respecto a las otras especies en estudio. En las mezclas de leguminosas (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 ^{15}N, praderas mediterráneas, suelo volcánico.

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