

Variation of ^{15}N natural abundance in leaves and nodules of actinorhizal shrubs in Northwest Patagonia

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Abstract This study was performed to assess the N_2 -fixing capability of the native actinorhizal species *Ochetophila trinervis* (sin. *Discaria trinervis*) and *Discaria chacaye* (Rhamnaceae) in Northwest Patagonia. We measured the N concentration and ^{15}N natural abundance in leaves and nodules of *O. trinervis* and *D. chacaye*, in leaves of associated non-actinorhizal vegetation, and in the soils under each sampled plant. *O. trinervis* and *D. chacaye* had foliar N concentrations that were about twice that of non-actinorhizal shrubs growing at the same sites, even though soils varied four-fold in total N across the sites. Leaves of both actinorhizal plants had a similar $\delta^{15}\text{N}$ at any site and were close to atmospheric values. The foliar $\delta^{15}\text{N}$ of non-actinorhizal plants and soil $\delta^{15}\text{N}$ were strongly correlated across the sites. Nodules were depleted in $\delta^{15}\text{N}$ relative to the foliage of the respective actinorhizal species. In conjunction with the uniformly high foliage N concentration of these actinorhizal plants and the universal presence of vesicles observed in root nodules, these data strongly suggest that *O. trinervis* and *D. chacaye* obtain a significant amount of their N from N_2 fixation. To calculate the proportion of N derived from atmosphere, theoretical B-values were estimated. In all cases where the $\delta^{15}\text{N}$ of fixing and reference foliage were significantly different, *O. trinervis* and *D. chacaye* obtained

almost all of their N from N_2 fixation. These results are the first to demonstrate N_2 fixation by *O. trinervis* and *D. chacaye* in the field and therefore suggest an important role for these actinorhizal plants in the N economy of ecosystems in northwest Patagonia as well as their potential use for restoration of degraded lands in this region.

Keywords Actinorhizas · *Berberis microphylla* · *Discaria* spp. · *Ochetophila* · Patagonia · *Rosa rubiginosa* · Nitrogen fixation · ^{15}N natural abundance

Abbreviations

N nitrogen
pNdfa proportion of N derived from atmosphere

1 Introduction

Native actinorhizal plant species belonging to the Rhamnaceae family grow in several plant associations of the extra-Andean region of northwestern Patagonia. The actinorhizal species *Ochetophila trinervis* (Gillies ex Hook. & Arn) Poepp. ex Miers., also known as *Discaria trinervis* (Kellermann et al. 2005), and *Discaria chacaye* (G. Don) Tort. grow in the steppe, the ecotone between xeric forest and steppe, in scrublands (matorrales), along fresh water streams and lake shores, and along roadsides. Moreover, they may share same sites.

Both *O. trinervis* and *D. chacaye* have been found to be nodulated in field, with nodules belonging to the Sp(-) type (Chaia 1997). Their symbiotic nodule tissue has a seasonal behaviour, as indicated by the lower number and size of hypertrophied cells infected by *Frankia* in winter than in other seasons. In *O. trinervis* plants, the greatest development of hypertrophied cells during spring and summer coincided with

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the highest foliar N content. Nitrogen resorption before abscission of senescent leaves of nodulated *O. trinervis* (12% and 38% of the summer value in young and adult plants, respectively) was in the range of other deciduous actinorhizal plants (Chaia 1993; Chaia and Vobis 2000), but lower than other woody species in the Patagonian region, suggesting a low N-use efficiency (Mazzarino et al. 1998). *D. chacaye* and *O. trinervis* seedlings grown under experimental conditions in a growth chamber and inoculated with either nodule homogenates or pure strains of *Frankia* reduced C₂H₂, indicating the ability to fix N₂ (Chaia 1998; Chaia et al. 2006); however, it is not known if these plant species fix N₂ under natural conditions.

In the ecotone and steppe of Northwest Patagonia, several kinds of ecologic disturbances take place: fire (both natural and anthropogenic), cutting of firewood, cattle ranching, and during the last three decades, planting of exotic conifers. Similarly, the presence of fire and pine plantations affects matorral community structure (i.e., changes in the proportion of life forms and species composition, and an increase in exotic species) and regeneration strategies (Nuñez and Raffaele 2007). Such changes in these ecosystems have the potential to impact the natural communities of native actinorhizal plants. Thus, to further promote sustainable land use and management in northwestern Patagonia (Martín and Chehébar 2001), there is a need to better understand the role of rhamnaceous actinorhizal species in their native ecosystems.

The objective of this study was to assess the capability of naturally occurring *O. trinervis* and *D. chacaye* to fix N₂. We did this by comparing the natural abundance of ¹⁵N in the leaves and nodules of these two actinorhizal species and associated vegetation not known to form N₂-fixing associations (cf. Boddey et al. 2000).

2 Materials and methods

2.1 Study area

The study area was located in the extra-Andean region of Northwest Patagonia in Argentina. It is part of a national reserve where limited land management practices are permitted. The climate is temperate, with a maximum and minimum mean annual temperatures of 13°C and 2°C, predominant westerly winds, and a mean annual precipitation of 600 to 800 mm yr⁻¹ (Barros et al. 1983, Muñoz and Garay 1985). Soils are Mollisols, with soil N and organic matter usually concentrated beneath vegetation patches (Mazzarino et al. 1998). Four study sites with *O. trinervis* and *D. chacaye* shrubs growing in close proximity were used: the alluvial plains of two rivers, Río Ñirihuau and Río Limay, in the steppe, and a wetland and a scrubland in the ecotone between the steppe and the forest (Table 1).

2.2 Sampled plants

O. trinervis and *D. chacaye* are woody, spiny, deciduous shrubs or trees, and also take the form of prostrate bushes (Tortosa et al. 1996). *D. chacaye* has retinervate leaves and absence of petals; *O. trinervis* has leaves with three nerves (Tortosa 1983).

At each site, three non-actinorhizal shrubs were sampled as reference plant species. *Berberis microphylla* and *Rosa rubiginosa* were sampled across the sites, along with a third reference species: *Schinus marchandii* (Anacardiaceae), *Ribes cuculatum* (Saxifragaceae), *Adesmia volkmanii* (Fabaceae), or *Salix humboldtiana* (Salicaceae). *Berberis microphylla* (Berberidaceae) is a shrub up to 3 m tall, inhabiting forest-steppe ecotone in scrublands, with spine-

Table 1 Location and description of study sites that included *Ochetophila trinervis* and *Discaria chacaye* shrubs growing in close proximity

Site	Location	Elevation (m)	Soil temperature (°C)	Main vegetation and reference species
San Ramón wetland	41.08 ° S 71.12 ° W	930	16	<i>Berberis microphylla</i> , <i>Senecio</i> sp., <i>Schinus marchandii</i> , <i>Schinus patagonica</i> , <i>Juncus</i> sp., <i>Geranium</i> sp., <i>Rosa rubiginosa</i> , <i>Prunus</i> sp.
San Ramón scrubland	41.06 ° S 71.04 ° W	927	17	<i>Berberis microphylla</i> , <i>Schinus patagonica</i> , <i>Ribes cuculatum</i> , <i>Fabiana imbricata</i> , <i>Maytenus boaria</i> , <i>Crataegus</i> sp., <i>Rosa rubiginosa</i>
Limay riverbank	40.98 ° S 71.08 ° W	757	19	<i>Salix humboldtiana</i> , <i>Berberis microphylla</i> , <i>Adesmia volkmanii</i> , <i>Rosa rubiginosa</i> , <i>Acaena</i> spp.
Ñirihuau riverbank	41.17 ° S 71.13 ° W	850	13	<i>Salix humboldtiana</i> , <i>Paramela</i> sp., <i>Senecio</i> sp., <i>Fabiana imbricata</i> , <i>Discaria articulata</i> , <i>Rosa rubiginosa</i> , <i>Berberis microphylla</i>

transformed leaves; small generally solitary hermaphrodite yellow, insect pollinated flowers, fruits are blue-black berries that contain alkaloids (Dzendoletas et al. 2003; Marticorena and Rodríguez 2005). *Rosa rubiginosa* (Rosaceae) is a thorny deciduous shrub, densely branched, with composite leaves with glandulous hairs and with strong vegetative reproduction ability. It is native to Europe, and has naturalized and invaded degraded lands of the Andean Patagonia, growing in open areas in forests and in the steppe, and also in poor soils (Damascos 2008). *Berberis microphylla* and *R. rubiginosa* were chosen as reference plants because they are not N₂-fixing species and have a similar growth form as *O. trinervis* and *D. chacaye*.

All plant species included in this study, were previously reported as arbuscular mycorrhizal symbionts (Fontenla et al. 1998, 2001).

2.3 Field sampling

Sampling was performed on 18 and 20 February 2008. Three replicates were sampled at each of the four sites. Height, stem basal diameter, and crown projection (Mueller-Dombois and Elleberg, 1974) were measured for all plants. For each actinorhizal plant and each reference plant, 12 new twigs from the current growing season, located at the four cardinal positions and at three different plant heights, were cut and placed into plastic bags. The rest of the aboveground biomass of all actinorhizal plants was cut and placed into plastic bags. Roots were excavated up to a depth of 30 cm from around the base of the stem to collect actinorhizal nodules. A rhizospheric soil sample (0-15 cm depth) was collected from underneath all sampled plants using an ethanol-rinsed shovel and placed into plastic bags. All plant material and soil was immediately stored under cold conditions in a box with ice for transportation to the laboratory.

2.4 Sample preparation and characterization

Twigs of all plants were kept at 4°C for 5 days before processing. Six leaves were separated from the twigs of all

plants to be used for ¹⁵N analysis. Another six leaves from twigs of actinorhizal plants were separated to estimate foliar area. All leaves were oven-dried at 60°C for 24 h and weighed. Leaf area of actinorhizal plants was divided by the dry mass of measured leaves, to estimate the specific leaf area (SLA). Plant material collected to estimate above-ground biomass remained at room temperature for a month, when leaves and branches were separated and oven-dried and weighed. Nodules were kept in a freezer and later thawed at room temperature and carefully washed with tap water. Three nodule lobes, belonging to one or more nodules from each plant, were assessed by light microscopy for the presence of vesicles (Wolters et al. 1997). Remaining portions of nodules were dried at 60°C for 24 h. Soil samples were air-dried at room temperature and transferred to a paper bag.

2.5 Nitrogen analyses

Leaves and nodules were ground with an agate mortar and pestle. Plant tissue and soil samples were analysed for concentrations of N and ¹⁵N abundance at the Stable Isotope Research Unit (Department of Crop and Soil Sciences, Oregon State University, Corvallis, Oregon, USA) using an isotope ratio mass spectrometer (PDZ Europa Ltd., Crewe, Cheshire, England). Peach leaves (NIST 1547) were used as standards for N concentration (2.916%) and ¹⁵N abundance (1.36‰). Results for ¹⁵N abundance are expressed in the standard notation ($\delta^{15}\text{N}$) in parts per thousand (‰) relative to the international standard (atmospheric N₂):

$$\delta^{15}\text{N} = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \right] * 1000,$$

where R denotes the ratio ¹⁵N/¹⁴N.

The proportion of N derived from atmosphere (pNd_{fa}) for both actinorhizal species were calculated from mean data of leaf samples collected at the sites. *Berberis microphylla* and *R. rubiginosa* served as reference plants. The pNd_{fa} was calculated as:

$$\text{pNd}_{\text{fa}} = (\delta^{15}\text{N reference plant} - \delta^{15}\text{N actinorhizal plant}) / (\delta^{15}\text{N Reference plant} - \text{B}).$$

2.6 Estimation of the theoretical B value

At present, there are no experimentally determined B-values for *O. trinervis* and *D. chacaye*; however, the wide range of $\delta^{15}\text{N}$ values for soils and foliage of actinorhizal and non-actinorhizal plants allowed us to estimate a likely range for B-values for these two plants. A theoretical B-

value was estimated assuming that a B-value is a constant for a given actinorhizal plant species and therefore did not vary for a given species across the four field sites or for the choice of reference plant. B-values were calculated as the intersections (i.e., solutions) of the pNd_{fa} equations for each pair of actinorhizal and reference plants for all sites. The intersection yielding the lowest pNd_{fa} value was

Table 2 Description of actinorhizal plants sampled in four different sites in Northwest Patagonia (means \pm standard error, n=3)

Plant species	Site	Plant height		Plant cover		Specific leaf area		Foliage dry weight	
		(cm)		(m ²)		(cm ² g ⁻¹)		(g)	
<i>Ochetophila trinervis</i>	San Ramón wetland	98.3	± 1.7	0.14	± 0.08	94.0	± 1.9	10.5	± 3.4
	San Ramón scrubland	118.3	± 28.0	1.66	± 1.08	90.2	± 1.2	46.1	± 40.3
	Limay riverbank	94.7	± 43.2	0.59	± 0.38	126.6	± 9.0	19.7	± 16.1
	Ñirihuau riverbank	71.0	± 4.5	0.45	± 0.02	101.7	± 2.7	22.7	± 6.7
<i>Discaria chacaye</i>	San Ramón wetland	74.0	± 9.2	0.10	± 0.02	102.3	± 4.3	3.5	± 0.4
	San Ramón scrubland	107.3	± 9.4	0.87	± 0.23	97.0	± 5.1	66.4	± 31.4
	Limay riverbank	95.0	± 30.0	0.21	± 0.07	109.2	± 7.8	11.3	± 6.7
	Ñirihuau riverbank	66.3	± 12.6	0.15	± 0.02	102.4	± 7.0	24.2	± 14.4

chosen as the best, and most conservative, estimate of the B-value and was used to calculate pNdfa.

2.7 Data analysis

We analyzed variables associated with plant species (e.g., foliage mass, foliage and soil N content, etc.) by ANOVA. Variables were first analyzed with a one-way ANOVA using a randomized complete block design with sub-sampling, where sites were blocks and sub-samples were the three replicates taken at each site. The site-by-variable interaction was used to test for the effect of site and species. In some instances where these interactions were significant, we examined species effects separately by site with a one-way ANOVA using a completely random design. Species effects that were found significant ($p < 0.05$) by ANOVA were further evaluated by Tukey's HSD. Linear regression analysis was used to explore relationships between $\delta^{15}\text{N}$ of soil, foliage, and nodules.

3 Results

Plants of *O. trinervis* and *D. chacaye* were similar in height, crown diameter, specific leaf area, and foliage dry mass (Table 2). Plants were largest at San Ramón scrubland.

No significant differences in N concentration were observed in soils sampled under different plant species;

however, N concentrations were almost four times higher at the sites located in the transitional zone of the San Ramón wetland and scrubland compared to the steppe riverbank sites (Limay and Ñirihuau; Table 3). Despite these differences in soil N, foliar N concentration did not vary significantly by site for any plant species. The actinorhizal shrubs, *O. trinervis* and *D. chacaye*, had foliar N concentrations that were about twice that of non-actinorhizal shrubs growing at the same sites (Fig. 1). Actinorhizal root nodules averaged 3.1% N but did not vary significantly by species or site (data not shown).

Because site-by-species interactions were significant for soil and foliage $\delta^{15}\text{N}$ values, data are presented by site; however, this interaction was most striking for foliage N (Fig. 2). In general, soils were most enriched and actinorhizal root nodules most depleted in ^{15}N , with foliage $\delta^{15}\text{N}$ values intermediate (Fig. 2). An effect of plant species on soil $\delta^{15}\text{N}$ was only significant at San Ramón wetland, where it was significantly higher underneath *O. trinervis*. Site had the greatest effect on soil $\delta^{15}\text{N}$, being significantly higher at the San Ramón locations, intermediate at Limay riverbank, and lowest (often slightly depleted relative to atmospheric N) at Ñirihuau riverbank.

Leaves of actinorhizal plants did not differ significantly from each other in $\delta^{15}\text{N}$ at any site and were always close to atmospheric values (Fig. 2). Foliar $\delta^{15}\text{N}$ varied much more widely among non-actinorhizal plants. In general,

Table 3 Means (\pm standard error) of soil N (percentage of dry mass) at 0-15 cm depth in different sites in Northwest Patagonia under different plant species (n=3, except for soil under *O. trinervis* in San Ramón

wetland, when n=2). Other species: *Schinus molle* (in Wetland San Ramón), *Ribes cuculatum* (San Ramón scrubland), *Adesmia volkmanii* (Limay riverbank), *Salix humboldtiana* (Ñirihuau riverbank)

Site	San Ramón wetland		San Ramón scrubland		Limay riverbank		Ñirihuau riverbank	
<i>O. trinervis</i>	0.59	± 0.08	0.45	± 0.04	0.07	± 0.01	0.15	± 0.03
<i>D. chacaye</i>	0.34	± 0.01	0.36	± 0.01	0.08	± 0.01	0.09	± 0.01
<i>Rosa rubiginosa</i>	0.43	± 0.03	0.41	± 0.09	0.17	± 0.04	0.16	± 0.01
<i>Berberis microphylla</i>	0.46	± 0.03	0.37	± 0.03	0.17	± 0.06	0.22	± 0.02
Other species	0.46	± 0.01	0.70	± 0.12	0.12	± 0.02	0.08	± 0.03

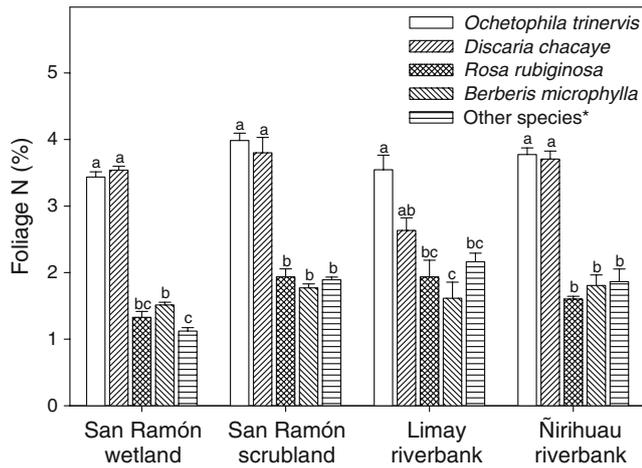


Fig. 1 Nitrogen concentrations in leaves of different plant species growing in different sites in northwest Patagonia. Means ± SE for n=3, except for *R. rubiginosa* in San Ramón wetland, when n = 2. * Other species: *Schinus marchandii* (San Ramón wetland), *Ribes cuculatum* (San Ramón scrubland), *Adesmia volkmanii* (Limay riverbank), *Salix humboldtiana* (Ñirihuau riverbank). Letters designate significant differences (p<0.05) among species within sites

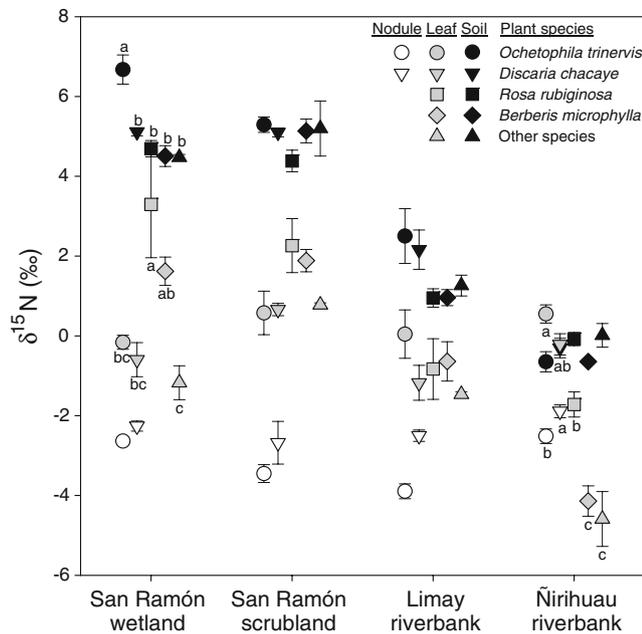


Fig. 2 Natural abundance of ¹⁵N in soils, foliage, and nodules of plant species at the four study sites of northwest Patagonia. Means ± SE for n=3, except for *R. rubiginosa* leaves in San Ramón wetland, and for soil under *O. trinervis* in San Ramón wetland when n = 2. * Other species: *Schinus marchandii* (San Ramón wetland), *Ribes cuculatum* (San Ramón scrubland), *Adesmia volkmanii* (Limay riverbank), *Salix humboldtiana* (Ñirihuau riverbank). Black symbols represent soils; gray symbols represent foliage; and white symbols represent root nodules. When present, letters above soil symbols and below leaf and nodule symbols with designate significant differences (p<0.05) among species within sites; in other cases there were no significant species differences at a site

foliar $\delta^{15}\text{N}$ of the common non-actinorhizal plants (*B. microphylla* and *R. rubiginosa*) was not different, with the exception of the Ñirihuau riverbank site. Although more variable, the unique non-actinorhizal species at each site usually had foliage $\delta^{15}\text{N}$ similar to at least one of the common species. An exception was *Schinus marchandii* at San Ramón wetland, which was similar in foliar $\delta^{15}\text{N}$ to actinorhizal plants at that site.

At all sites nodules were depleted in $\delta^{15}\text{N}$ relative to the foliage of the respective actinorhizal species. Overall, *O. trinervis* nodules were more depleted in $\delta^{15}\text{N}$ than those of *D. chacaye*, although this was only significant on a site basis at Limay riverbank (Fig. 2). All actinorhizal nodules of *O. trinervis* and *D. chacaye* had vesicles.

4 Discussion

Soils of the study sites varied four-fold in total N. The steppe soils at San Ramón, which had the most N, were also more enriched with ¹⁵N. This enrichment of ¹⁵N probably reflects a longer history of N accumulation and more active N cycling (Högberg 1997). In contrast, the riverbank sites are subject to periodic disturbances, which remove older N that is more enriched in ¹⁵N, and reflects only recent N inputs that are closer to atmospheric N₂ in their $\delta^{15}\text{N}$ signature.

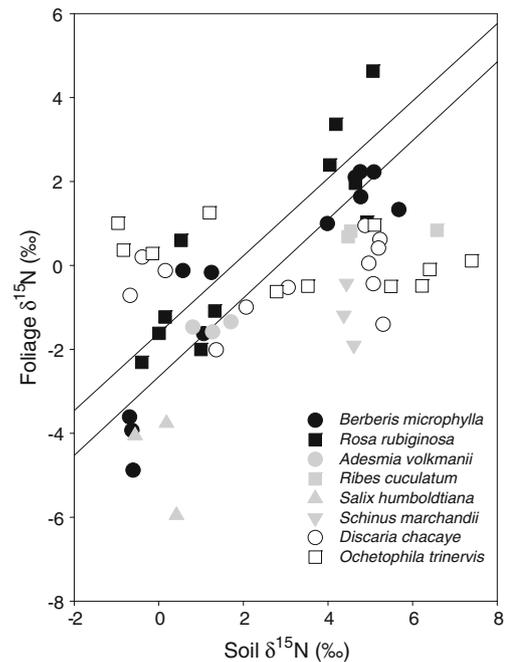


Fig. 3 Relationship between the $\delta^{15}\text{N}$ of foliage and soil found at all four study sites. Black symbols represent non-N₂-fixing species found at all sites; white symbols represent N₂-fixing species found at all sites; and gray symbols represent unique non-actinorhizal species found at each site (see Table 1 for locations)

Table 4 Regression statistics for the relationship between leaf $\delta^{15}\text{N}$ and soil $\delta^{15}\text{N}$. Means \pm standard error

Plant species	Slope	Y-intercept	r ² -value	p-value
<i>Berberis microphylla</i>	0.94 \pm 0.13	-2.65 \pm 0.44	0.848	<0.0001
<i>Rosa rubiginosa</i>	0.92 \pm 0.17	-1.61 \pm 0.54	0.761	0.0005
<i>Discaria chacaye</i>	0.10 \pm 0.11	-0.65 \pm 0.41	0.084	0.3602
<i>Ochetophila trinervis</i>	-0.10 \pm 0.06	0.50 \pm 0.29	0.221	0.1443

Unlike soil N concentrations, we found no differences in the foliar N concentration of non-actinorhizal plants across the four sites, suggesting little difference in soil N availability despite the four-fold difference in total soil N. There was, however, a strong correlation between the foliar $\delta^{15}\text{N}$ of non-actinorhizal plants and soil $\delta^{15}\text{N}$ across the sites (Fig. 3). In fact, for *B. microphylla* and *R. rubiginosa*, which occurred at each of the sites, this positive correlation was highly significant with a slope that was not significantly different from 1.0 (Table 4), suggesting a constant fractionation in plant N uptake of about -2‰. The non-actinorhizal plants that were unique to each site followed a similar trend, although the scatter was much greater as would be expected due to plant-to-plant differences in N uptake processes. In both riverbank sites, $\delta^{15}\text{N}$ in leaves of reference plants had negative values. Some studies found

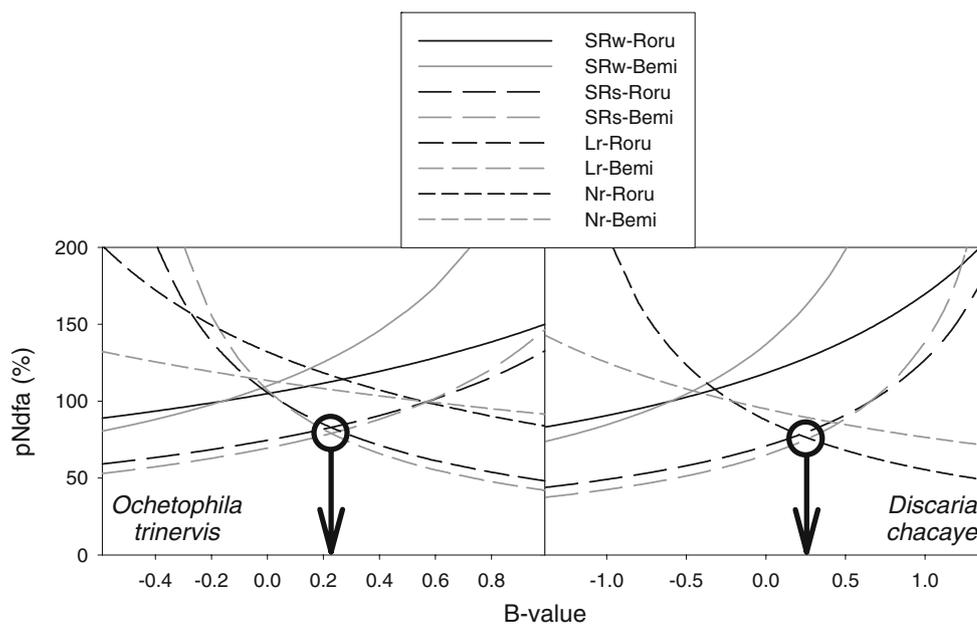
$\delta^{15}\text{N}$ depletion in reference plants with respect to actinorhizal plants. For example this situation was found in plants of *Betula alleghaniensis* and *B. papyrifera*, considered as reference of *Alnus incana* ssp. *rugosa* (Hurd et al. 2001). Reference plants of *Ceanothus*, in the fire prone Chaparral ecosystem in California, had a strong ^{15}N depletion, and it was speculated that it was related to fire cycle (Virginia et al. 1989).

In contrast to the significant relationship between soil and foliage $\delta^{15}\text{N}$ observed for non-actinorhizal plants, the $\delta^{15}\text{N}$ of *O. trinervis* and *D. chacaye* foliage was independent of soil $\delta^{15}\text{N}$ and remained constant at the atmospheric value of 0‰ (Fig. 3, Table 4). $\delta^{15}\text{N}$ values of *O. trinervis* and *D. chacaye* fell within the range of other actinorhizal plants growing in diverse ecosystems (Table 5). In conjunction with the uniformly high foliage N concentra-

Table 5 The $\delta^{15}\text{N}$ (‰) in leaves and nodules of actinorhizal plants under field conditions (range of values or means \pm standard error)

Actinorhizal species	Leaves	Nodules	Geographic location	Site description	Reference
<i>Alnus incana</i> ssp. <i>rugosa</i>	-1.2 (0.1)		Adirondack Mountains, U.S.A.	Shrub wetland	Hurd et al. 2001
<i>Alnus incana</i> ssp. <i>incana</i>	-1.1		Sweden	Forest tundra	Michelsen et al. 1998
<i>Alnus incana</i>	-2.3 (0.3)		France	Alpine forest	Domenach and Kurdali 1989
<i>Alnus incana</i>	-0.22 to -1.25		Sweden	Degraded forest soil	Myrold and Huss-Danell 2003
<i>Alnus glutinosa</i>	-0.14 to -2.6		France, Germany, Poland	Sandy soils	Domenach and Kurdali 1989
<i>Alnus rubra</i>	-1.26 to -1.06		British Columbia, Canada	Forest	Markham and Chanway 1999
<i>Alnus rubra</i>	-2.8 to 4.5				Binkley et al. 1985
<i>Alnus glutinosa</i> , <i>A. cordata</i> , <i>A. rubra</i>	-1.3 to -0.14		France	Sandy soil	Kurdali et al. 1993
<i>Elaeagnus angustifolia</i>	-0.87 (0.11)	-2.90 (0.29)	New Mexico		Tjepkema et al. 2000
<i>Shepherdia argentea</i>	-0.30 (0.13)	-3.45 (0.24)	New Mexico		Tjepkema et al. 2000
<i>Coriaria arborea</i>	-1.05 (0.19)	-2.62 (0.27)	New Zealand		Tjepkema et al. 2000
<i>Purshia tridentata</i>	-1.7 (0.2)		Northern California, USA	Mixed pine forest	Busse et al. 2007
<i>Alnus firma</i>	-3.9	+5.5	Japan	garden	Yoneyama and Sasakawa 1991
<i>Myrica rubra</i>	-2.1	+0.7	Japan	garden	Yoneyama and Sasakawa 1991
<i>Elaeagnus pungens</i>	-2.5	-3.3	Japan	garden	Yoneyama and Sasakawa 1991
<i>Coriaria japonica</i>	-1.8	-1.6	Japan	garden	Yoneyama and Sasakawa 1991
<i>Ceanothus prostratus</i>	-2.7		Northern California, USA	Mixed pine forest	Busse et al. 2007
<i>Ochetophila trinervis</i>	-1.23 to -0.72		Northwest Patagonia	Scrubland	Chaia and Myrold, unpublished
<i>Ochetophila trinervis</i>	-0.16 to +0.58	-3.89 to -2.51	Northwest Patagonia	Steppe and transitional scrubland	this study
<i>Discaria chacaye</i>	-1.18 to +0.66	-2.68 to -1.89	Northwest Patagonia	Steppe and transitional scrubland	this study

Fig. 4 Estimated B-values for *Ochetophila trinervis* and *Discaria chacaye* based on observed ranges of $\delta^{15}\text{N}$ in foliage of actinorhizal and common reference plants. SRw, San Ramón wetland, SRs San Ramón scrubland, Lr, Limay riverbank, Nr, Ñirihuau riverbank, Roru, *Rosa rubiginosa*, Beni, *Berberis microphylla*



tion of these two actinorhizal plants and the universal presence of vesicles observed in root nodules, these data strongly suggest that *O. trinervis* and *D. chacaye* obtain a significant amount of their N from N₂ fixation.

The standard approach for quantifying the proportion of N₂ fixed relies on the use of a suitable reference plant and a B-value, which represents the fractionation that occurs during the N₂ fixation process (Högberg 1997). Ideally, reference plants are ecophysiologicaly similar to the fixing plant (e.g., similar rooting patterns, phenology, N preferences, etc.); pragmatically, foliar $\delta^{15}\text{N}$ values for the reference plant must be significantly different than those of the fixing plant, which in part reflects sampling variability (Busse et al. 2007). High field variability in our study meant that quantitative estimates

could be made only at the San Ramón wetland and Ñirihuau riverbank sites.

Plots of pNdfa as a function of B-value are hyperbolas that vary in slope depending upon whether the $\delta^{15}\text{N}$ of the fixing plant is greater or less than that of the reference plant (Fig. 4). The intersections of the hyperbolas define an envelope within which the correct B-values must lie. Based on this analysis, the optimal B-values were 0.23 for *O. trinervis* and 0.27 for *D. chacaye*. Because we selected a single value it is not possible to provide an estimate of its variation; however, the standard error based on all reasonable B-values for a given plant was about 0.1. The theoretical B-values we estimated for *O. trinervis* and *D. chacaye* were close to that used for *Alnus rubra*

Table 6 Estimates of the proportion of N derived from N₂-fixation (pNdfa) by *Ochetophila trinervis* and *Discaria chacaye*, using estimated B-values (Fig. 4) and common reference species. Values are means±95% confidence interval

Site	Reference plant	<i>Ochetophila trinervis</i> (%)	<i>Discaria chacaye</i>
San Ramón wetland	<i>Berberis microphylla</i>	120±14	124±14
	<i>Rosa rubiginosa</i>	106±21 ^a	121±14 ^a
San Ramón scrubland	<i>Berberis microphylla</i>	70±32	73±12
	<i>Rosa rubiginosa</i>	75±32	68±16
Limay riverbank	<i>Berberis microphylla</i>	58±7	- ^b
	<i>Rosa rubiginosa</i>	71±38	—
Ñirihuau riverbank	<i>Berberis microphylla</i>	107±5 ^a	90±6 ^a
	<i>Rosa rubiginosa</i>	113±10 ^a	74±14

^a Foliage $\delta^{15}\text{N}$ was statistically different ($p < 0.05$) between fixing and reference plants; in other all other cases differences in foliar $\delta^{15}\text{N}$ were not statistically different

^b pNdfa could not be determined for *D. chacaye* because its foliage $\delta^{15}\text{N}$ was farther from atmospheric N₂ than that of the reference plants at this site, yielding negative pNdfa values

(Binkley et al. 1985), although slightly more positive than those reported for various other actinorhizal species, including *A. glutinosa*, *A. incana*, *Casuarina cunninghamiana*, *Datisca glomerata*, and *Myrica gale* (Domenach et al. 1988; Tjepkema et al. 2000).

Using these optimal B-values, we were able to calculate the pNdfa for *O. trinervis* and *D. chacaye* at most sites (Table 6). In all cases where the $\delta^{15}\text{N}$ of fixing and reference foliage were significantly different, *O. trinervis* and *D. chacaye* obtained almost all of their N from N_2 fixation. Overall, *Ochetophila trinervis* was more effective at fixing N_2 compared to *Discaria chacaye*. This was most distinct at the San Ramón scrubland site.

The nodules of both actinorhizal species were depleted in ^{15}N , with respect to leaves, as most actinorhizal species surveyed to the moment (Fig. 2, Table 5). Conversely, nodules of some actinorhizal plants, like *Alnus*, *Casuarina* (Tjepkema et al. 2000) and *Myrica rubra* (Yoneyama and Sasakawa 1991), and of most legume species are usually found to be enriched in ^{15}N when compared to leaves or other plant organs (reviewed by Boddey et al. 2000). Differences in ^{15}N enrichment among N_2 -fixing nodules are interesting from the standpoint of nodule metabolism (Shearer and Kohl 1989). It was suggested that ^{15}N isotope fractionation is associated with reactions such as deamination and transamination (Högberg 1997). ^{15}N enrichment in legume nodules has been associated with production of polyamines (Yoneyama et al. 1998). In contrast, *O. trinervis* and members of the Elaeagnaceae, which have been found to have nodules depleted in ^{15}N , export N to the xylem as asparagine (Valverde and Wall 2003). It would be of interest to establish if N compounds in nodules of members of Rhamnaceae are also depleted in ^{15}N .

Until this study, the only estimates of N_2 fixed under natural conditions by members of the family Rhamnaceae came from species of the North American genus *Ceanothus* (tribe Rhamneae) (Busse 2000a; Busse 2000b; Busse et al. 2007). Regarding plants of the tribe Colletieae, mostly distributed in South America (Aagesen 1999), it was only known that nodules of *Retanilla ephedra*, *R. stricta*, *Colletia spinosissima*, and *Talguenea quinquenervia*, from the Chilean matorral, were able to uptake significant amount of $^{15}\text{N}_2$ and to reduce acetylene, indicating N_2 fixation capabilities (Silvester et al. 1985), and that the acetylene reduction activity in nodules of *T. quinquenervia* exhibit seasonal changes (Balboa 1989). This study provides evidence that other rhamnaceous species, *Discaria chacaye* and *Ochetophila trinervis*, offer a potential to increase the content of soil N due to their N_2 fixation capacity. The use of these species for restoration purposes has not been evaluated; however, it would be beneficial to evaluate the use of rhamnaceous actinorhizal plants in northern Patagonia in locations where soil degradation is severe.

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