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Nitrogen fixation assessment in a legume-dominant alpine community: comparison of different reference species using the ^{15}N isotope dilution technique

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Abstract

In arctic and alpine environments the highest biologically fixed nitrogen inputs are measured in the presence of leguminous plants, with a maximum annual nitrogen input recorded of about 10 kg ha^{-1} year^{-1}. Among the high-elevation legumes, *Trifolium alpinum* spreads over many southern European mountain grasslands as a dominant or eo- dominant species, accounting for 30-40% or more of total above- ground phytomass. We estimated the amount of symbiotic nitrogen fixation in *T. alpinum-dominant* communities using the ^{15}N isotope dilution technique and comparing the calculations derived from different non-fixing reference species. The estimated percentage of nitrogen derived from the atmosphere varied significantly depending on the reference species used and they were comparable to the ones reported in literature. No differences were detected among years. In the community studied, the use of one reference species instead of another can yield differences of more than 10 kg ha^{-1} year^{-1}, which may represent a considerable difference in the estimation of symbiotic nitrogen fixation in alpine ecosystems. Taking a very precautionary approach and using the grass *Nardus stricta* as the reference species, we estimated 21.9 kg ha^{-1} year^{-1} of nitrogen fixation in *T. alpinum-dominant* communities. This quantity of nitrogen is the highest ever estimated in a natural alpine grassland and it is related to the high annual above-ground phytomass of *T. alpinum.*

Keywords

Electronic supplementary material

Introduction

Nitrogen is considered the main limiting nutrient for primary production in many terrestrial ecosystems (Vitousek and Howarth 1991). In arctic and alpine environments, many studies documented that nitrogen supply is affected primarily by mineralization processes (Jacot et al. 2000b; Korner 2003; Yang et al. 2011), but other important sources have been identified in wet deposition and biological fixation (Bowman et al. 1996; Jacot et al. 2000a, b; Yang et al. 2011). In particular, biological fixation is both related to asymbiotic and symbiotic processes, with a decreasing relative contribution of symbiotic nitrogen fixation (largely by legumes) with increasing elevations (Korner 2003). The highest annual nitrogen fixation inputs recorded in high-elevation alpine ecosystems ranged from 8.1 (Southern Rocky Mountains, USA; Bowman et al. 1996) to 10.0 kg ha^{-1} year^{-1} (Tibetan Plateau; Yang et al. 2011), in the presence of a relatively high proportion of leguminous species (11% of cover and 15.7% of above-ground phytomass, respectively).

Among the different methods reported in the literature for estimating symbiotic nitrogen fixation, the ^{15}N isotope dilution technique has been little used in alpine and arctic tundra ecosystems (Arnone 1999; Jacot et al. 2000a; Yang et al. 2011), although this method is commonly used in crop science studies (Chalk and Ladha 1999). To assess the nitrogen fixation with the dilution technique, a little amount of ^{15}N isotope is applied to the soil and the "percent nitrogen derived from the atmosphere" of the legumes (%Ndfa) is calculated by comparison with reference non-fixing plants. The major weakness of the dilution technique is the choice of reference plants (Giller and Witty 1987; Viera-Vargas et al. 1995), an issue discussed extensively in the literature (Pareek et al. 1990; Androsoff et al. 1995; Chalk et al. 1996).

The effects of the use of different reference species in the calculation of the %Ndfa of legumes have been studied for many annual crop plants (e.g., soybean, common bean, pea, chickpea), where the choice of the reference species is often justified by comparison with a non-nodulating cultivar or an uninoculated plant of the same species (Chalk and Ladha 1999). In particular, good reference plants should have (1) rooting zones, (2) nitrogen uptake pattern, and (3) growth durations (Danso 1988) similar to the legumes being studied. An appropriate reference species must absorb the same ratio of ^{15}N to soil-derived unlabeled nitrogen during each phase of growth as the legume does (Chalk and Ladha 1999). Another important prerequisite for the ^{15}N dilution technique is the uniform distribution of isotope within the soil profile. Several attempts have been reviewed to minimize isotopic non-uniformity in time and depth (Chalk and Ladha 1999). In non- agricultural ecosystems, where ^{15}N nitrogen cannot be incorporated into the soil (e.g., by plugging), the best solution is to repeat the addition of ^{15}N nitrogen in small amounts over an extended time period. In alpine environments different approaches were used, with a single (Yang et al. 2011), two (Arnone 1999), or two-three ^{15}N additions during the growing season (Jacot et al. 2000a). A better distribution of ^{15}N along the soil profile can be achieved also by repeating the application of ^{15}N in different years over the same experimental areas. Repeated labeling among years could reduce temporal and spatial gradients in ^{15}N concentration in the soil.

Given the difficulties in testing these assumptions, some authors proposed to improve the accuracy of estimates of %Ndfa by averaging the values inferred from several different reference species (e.g., Rennie et al. 1988; Boddey et al. 1990). This approach has been utilized also for wild perennial legumes in arctic and alpine environments (Jacot et al. 2000a, b; Yang et al. 2011). However, there is evidence that the choice of reference species can strongly influence the estimate of the %Ndfa by the legume. Arnone (1999) estimated the %Ndfa in *Trifolium alpinum* L. using a single forb *(Leontodon helveticus* Merat) as the reference species (%Ndfa = 61%), but discouraged the use of *Carex curvula* All. (%Ndfa = 85%), due to its potential to harbor rhizospheric nitrogen fixing bacteria. Yang et al. (2011) found that the use of graminoid plants as reference species significantly decreased the %Ndfa values in alpine meadows (10.0 vs. 9.1

kg ha^{-1} year^{-1} excluding or including graminoids, respectively).

Our work aims to compare the use of different reference species for estimating nitrogen fixation in *T. alpinum-dominant* communities in a 3- year experiment. *T. alpinum* is a leguminous species widespread on different southern European mountain chains (Alps, Pyrenees, North Apennine, and Central Massif) (Aeschimann et al. 2004 ; Lauga et al. 2009). In the alpine belt of the southwestern Alps (Italy and France), *T. alpinum* frequently occurs as a dominant or co-dominant species, accounting for 30--40% or more of total above-ground phytomass (Bassignana and Bomard 2001; Bornard et al. 2007; Lombardi et al. 2007; Falchero et al. 2010). In the Italian southwestern Alps (Piedmont Region), *T. alpinum-dominant* communities (*Caricion curvulae* alliance) spread over about 12% of alpine grass1ands (Cavallero et al. 2007). The specific objectives of our study are: (1) to compare the most frequent co-occurring non-fixing species ofT. *alpinum* communities as reference species for calculating %Ndfa *for T. alpinum;* (2) to assess the effect of repeated ^{15}N labeling among years on the calculation of %Ndfa, and (3) to estimate the actual amount of symbiotic nitrogen fixation from the atmosphere in *T. alpinum-dominant* communities using different reference species.

Materials and methods

Site description

The experiment was carried out from 2005 to 2007 in the Val Troncea Natural Park, in the southwestern Alps (mean coordinates: 6°58'41"E, 44°55'27"N).

Within the *T. alpinum* communities, three sites were delimited within the optimum range of T. *alpinum* communities in the southwestern Alps (Cavallero et al. 2007) (site 1, 2230 m a.s.l.; site 2, 2,350 m; and site 3, 2,550 m). The three sites had similar slope, exposure (on average 18° and 275°N, respectively), and soils. Soils, which were derived from calcareous schist bedrock, were well developed, with organic matter deposition and calcium leaching resulting in acid topsoils (average pH in water 4.5) (IPLA 2007). The climate was typical of inner alpine valleys, with an average temperature of -0.6 °C, total annual precipitation of 994 mm (mainly concentrated in spring and autumn), and snow cover for at least 8 months (Biancotti et al. 1998). Before exclusion from grazing during the experimental period, cattle grazed the sites extensively for several decades.

Experimental design

We established six 2 x 1 m permanent plots at each elevation, with a 0.5-m buffer zone to limit border effects. To implement the enriched ^{15}N isotope dilution method (McAuliffe et al. 1958), we added 1,905 mg of $^{15}NH_4SO_3$ (99% ^{15}N enriched) to each plot every year, which corresponds to a nitrogen input of 2 kg ha^{-1} year^{-1}. This amount of nitrogen is low (3- 15 %) compared to the total N yield recorded at similar elevations (Jacot et al. 2000a), and it can be considered negligible in terms of its potential effect as a fertilizer or symbiotic nitrogen fixation inhibitor (Chalk and Ladha 1999).

We dissolved the enriched nitrogen in demineralized water and uniformly distributed it on each of the 18 plots at the beginning of each growing season (the first half of June 2005, 2006, 2007, i.e., each plot was labeled three times during the experimental period). We then watered each plot with demineralized water to improve soil absorption, minimize the adherence of the ^{15}N isotope on plant leaves, and to spatially homogenize the distribution in the soil (Pornon et al. 2007).

Reference species

To implement the enriched ^{15}N isotope dilution method (McAuliffe et al. 1958), four reference species were selected among the most frequently co-occurring non-fixing species of T. *alpinum* communities recorded in the southwestern Alps (Table 1): three grasses *(Nardus stricta* L., *Avenella flexuosa* (L.) Parl., and *Poa alpina* L.) and one sedge *(Carex sempervirens* Vill.). According to the literature (Kutschera and Lichtenegger 1982 and 1992; Oberdorfer 1983; Lichtenegger et al. 1997; Tasser and Tappeiner 2005), the selected reference species differed for rooting zones, considering mean lateral extension and maximum depth of roots, with *N. stricta* as the most similar species to *T. alpinum* (Fig. 1).

Data collection

We sampled the total above-ground phytomass at the ground level using scalpels at the peak *ofT. alpinum* growth (second half of August of each year), over a 30 x 30 cm area randomly distributed within each plot. Afterwards, we separated the green phytomass (i.e., the phytomass produced in the current year, hereafter called "total annual above-ground phytomass") from dead phytomass (i.e., litter and old stems and leaves). Within the total annual above-ground phytomass we sub-sampled all samples by species, separating *T. alpinum* and the four reference species. We then oven dried each single-species sample and the undifferentiated bulk residual sample at 65 °C for 48 h in order to determine the proportion (%) and the annual above-ground phytomass (t ha^{-1} DM) of *T. alpinum.*

To calculate the total nitrogen content (N%to1) and %Ndfa we ground each single-species sample with a Cyclotec mill (Foss Tecator, Hoganas, Sweden, 1 mm screen). The total nitrogen content (N%$_{tot}$) and the ^{15}N-isotope concentration were determined for both *T. alpinum* and the reference species using an elemental analyzer isotope ratio mass spectrometer (EA-IRMS). For EA-lRMS calibrations International Atomic Energy Agency standards were used.

We calculated the %Ndfa of *T. alpinum* for each plot and for each reference species according to the following formula (McAuliffe et al. 1958):

$$\%Ndfa = \left(1 - \frac{15N\%excess\ in\ the\ legume}{15N\%excess\ in\ the\ reference\ plant}\right) x100$$

where the [15]N%excess is the atom percent of 15N in a pooled sample (legume or reference plant) minus 0.3663 % ([15]N% in atmosphere, assumed as constant).

Total symbiotic nitrogen fixation (N$_2$-fix, kg ha^{-1} year^{-1}) was calculated by multiplying T. alpinum annual above-ground phytomass by its N%tot and %Ndfa.

Statistical analyses

To analyze differences in total annual above-ground phytomass and *T. alpinum* above-ground phytomass among sites and years, we performed a repeated measures General Linear Model (GLM), with "site" (sites 1, 2, 3) as fixed factor.

We analyzed N%tot' [15]N%excess, %Ndfa, and N$_2$-fix by repeated measures GLM with "reference species" (N. stricta, C. sempervirens, A. flexuosa, and P. alpina) and "site" as fixed factors. We tested significant differences among groups of cases by Tukey's range post hoc test ($p < 0.05$). Percent data were previously tested for homoscedasticity (Levene's test) and for normality (Kolmogorov-Smimov test}, and arcsin-transformed when necessary. We applied a logarithmic transformation (log 10) for the same reason to annual above-ground phytomass and to N$_2$-fix as well.

All the statistical analyses were performed using SPSS v.19 (SPSS 2010).

Results

Total annual above-ground phytomass of the three sites ranged from 2.0 to 2.5 t ha^{-1} and did not significantly differ among sites (Table 2; additional data are given in Online Resource 1). The proportion of T. alpinum ranged from 42 to 57% resulting in a significant difference in annual T. alpinum above-ground phytomass among sites (Table 2). Both total and T. alpinum annual above-ground phytomass significantly varied among years.

As expected, T. alpinum showed higher values of N%tot compared to all the reference species. On average N%tot differed among sites, with a mean gradual reduction at increasing elevations (Table 3; additional data are given in Online Resource 2). The ^{15}N%excess differed remarkably among species, sites and years. The lowest significant value was observed in T. alpinum. Lower values were also observed in N stricta and C. sempervirens, whereas the highest ones were found in P. alpina. The proportion of nitrogen from symbiotic fixation (%Ndfa) in T. alpinum varied significantly depending on the reference species used. The %Ndfa was about 65% using N stricta as the reference species, whereas the highest proportion of nitrogen fixation was estimated by using P. alpina (%Ndfa = 93%). %Ndfa values changed among sites, although the range of variation was limited (ranging 75-82%). The total symbiotic nitrogen fixation of the community ranged from 21.9 kg ha^{-1} year^{-1} using N stricta as the reference species to 32.3 kg ha^{-1} year-1 using P. alpina as the reference species. The estimation of N$_2$-fix was significantly lower using N stricta as reference species when compared with P. alpina, whereas C. sempervirens and A. flexuosa showed intermediate estimates. Differences in total symbiotic nitrogen fixation were observed among sites. The %Ndfa did not change among years and not significant interactions ("species x site" and "species x year") were observed, whereas total symbiotic nitrogen fixation varied among years (Table 3).

Discussion

The estimated percentage of nitrogen fixed from the atmosphere by T. alpinum ranged significantly from 65 to 93% depending on the reference species. This confirms the assumption that the choice of reference species used in the nitrogen dilution method strongly influences the estimation of nitrogen fixation in natural high-elevation grasslands (Yang et al. 2011) as well as in lowland annual crops (Chalk and Ladha 1999).
The reference species studied differed in rooting zone and probably in temporal relative growth rate within the time period between labeling and harvesting compared to T. alpinum. The non-uniform distribution in depth of 15N isotope, which may occur in uncontrolled field conditions, may have been a source of potential errors in the estimation of symbiotic nitrogen fixation (Chalk and Ladha 1999). Although no data about seasonal differences in mineral nitrogen uptake patterns are available for the reference species used, with a single labeling event every year it is likely that early-season species may have used a higher proportion of ^{15}N isotope compared to late-season species due to the higher concentration of ^{15}N isotope in the soil at the beginning of the growing season. Moreover, a portion of the enriched ^{15}N isotope could have been rapidly immobilized by the soil microbial community at the beginning of the growing season (Lipson et al. 1999), introducing another possible error in the estimation of symbiotic nitrogen fixation. For all these reasons, the limitations of the enriched ^{15}N isotope dilution method should be taken into account when analyzing our results.
Given similar species rooting zone, we suggest the grass N. stricta as the best reference species or the estimation of %Ndfa in T. alpinum swards. Using this species, we assessed a %Ndfa of 65 % and this value is consistent with the data reported by Arnone (1999) (%Ndfa in T. alpinum = 61%) who used the forb L. helveticus as the reference species. On the contrary, P. alpina, which showed the highest value of ^{15}N%excess and %Ndfa, was capable of rapidly absorbing the nitrogen

available in the environment (i.e., ^{15}N added during the experiment), probably due to its shallow rooting system and high nitrogen-use efficiency, i.e., the proportion of applied N taken up by the species (Korner 2003). The use of P. alpina as the reference species resulted in the highest %Ndfa for T. alpinum (93%), with an increase in estimation of about 30% as compared to N. stricta. Based on this result we discourage the use of P. alpina as a reference species in alpine environments. Moreover, several authors have suggested selecting appropriate reference plants based on similar nitrogen- use efficiency compared to the legume (Rennie 1984; Rennie and Dubetz 1986; Kucey et al. 1988; Kucey 1989). Also C. sempervirens has a shallow rooting system, but its ^{15}N%excess value was more similar to the one of N. stricta. As observed by other authors, many alpine Carex species harbor rhizospheric N$_2$ fixing bacteria (Arnone 1999; Korner 2003) and in our study this could have led to a lower ^{15}N%excess compared to other species. For the same reason, the use of C. curvula as a reference species for T. alpinum was discouraged by Arnone (1999). Although with a similar root depth, A. flexuosa showed a higher ^{15}N%excess and %Ndfa compared to N. stricta, probably due to the higher mean lateral root extension, which could have permitted a higher ^{15}N isotope uptake from the topsoil layer.

Average %Ndfa calculated from several references species, with the purpose of improving the estimate (Rennie et al. 1988; Boddey et al. 1990), should be applied with caution under our study conditions. Jacot et al. (2000a) estimated very high values of %Ndfa (ranging 88-93%) for T. alpinum, using average values calculated from three, five, or seven reference species on three different sites. Using this approach of including all reference species in the calculation, we would assign an average %Ndfa of 79%, with an increase in estimation of nitrogen fixation of about 14% compared to N. stricta.

Within the optimal altitudinal range of T. alpinum communities, the %Ndfa varied among sites, confirming that symbiotic N2 fixation could vary upon different site conditions, e.g., for different temperature and soil pH (Kessler et al. 1990; Graham 1991). However, the not significant interaction "species x site" showed that all reference plants acted in a similar way regardless of site conditions, a desirable feature for reference species. Our data highlighted that each reference species could be probably used in different environments within the optimal range of T. alpinum without introducing site-dependent source of variations in the estimation of %Ndfa.

The repeated ^{15}N labeling among years did not affect the calculation of %Ndfa, although the ^{15}N%excess increased in the third year of the experiment, meaning that the ratio between the ^{15}N%excess in the legume and in the reference species was constant among years regardless of annual climatic variations. Moreover, this result was also confirmed by the not significant "species x year" interaction on %Ndfa. According to our results, Jacot et al. (2000a) detected no significant annual effects on %Ndfa for different legumes at similar elevations, in a 2-year study with two labeling events every year. Based on our results, the ^{15}N isotope dilution technique with a single labeling event could be justified in high-elevation sites, probably due to short growing season.

In the community that was studied, the increase in T. alpinum annual above-ground phytomass throughout years was probably related to different climatic conditions, as confirmed by a similar increasing trend in total annual above-ground phytomass.

The high quantity of annual above-ground phytomass of T. alpinum, ranging from 0.8 to 1.2 t ha^{-1},resulted in a very high annual nitrogen input, (21.9 kg ha^{-1} year^{-1}), even using a precautionary approach, i.e., N stricta as the reference species. This value is related to the high annual above-ground phytomass of T. alpinum and not to abnormally high values of %Ndfa. This quantity of nitrogen is about 2- to 3-fold the maximum input previously reported for alpine environments (Bowman et al. 1996; Yang et al. 2011) and about 20-fold the input estimated in similar high-elevation grasslands in the Swiss Alps (Arnone 1999; Jacot et al. 2000b). In our legume-dominant community, the inclusion of all reference species in the calculation of average %Ndfa, as discussed in literature, would have led to an average nitrogen fixation of more than 27 kg ha^{-1} year^{-1} (with an increase in estimation of nitrogen fixation of about 5.5 kg ha^{-1} year^{-1} more than N. stricta), which represents an important difference in the estimation of symbiotic nitrogen fixation. This finding

highlights that the annual input derived from biological nitrogen fixation by legumes can be a large contribution of the annual N flux in the system on an annual scale, above all in legume-dominated grasslands. In similar environments, wet deposition ranges from 1.6 to 2.5 kg ha^{-1} year^{-1} and mineralization reaches about 4.7 kg ha^{-1} year^{-1} (Jacot et al. 2000b).

A reliable estimation of symbiotic nitrogen fixation can have important implications for understanding the ecology of T. alpinum-dominant communities. Nitrogen released from the legumes in the soil probably affects local species composition and vegetation dynamic succession, as observed in similar environments by others authors (Thomas and Bowman, 1998; Lonati et al. 2011; Olsen et al. 2013). Cattle and sheep have extensively grazed T. alpinum communities in the southwestern Alps for centuries (Probo et al. 2013; Tocco et al. 2013), which is probably a key factor in their ecology. Extensive grazing may have a positive effect on nutrient balances for leguminous species if herbage removal (and related nitrogen removal) exceeds urine and dung (and related nitrogen) deposition, wet deposition, and nitrogen release from mineralization processes (Probo et al. 2014). This decrease in nutrient availability may offset the effect of the amount of nitrogen annually fixed by T. alpinum.

Conclusions

In alpine environments estimation of the total nitrogen fixation in leguminous species using the 15N isotope dilution technique requires care in the choice of reference species. In T. alpinum-dominant communities, the use of one reference species instead of another (i.e., N. stricta vs. P. alpina) can give differences of more than 10 kg ha^{-1} year^{-1},which represents an important difference in the estimation of symbiotic nitrogen fixation. In high-elevation ecosystems this amount is about twice the total nitrogen derived by wet deposition and mineralization processes. Moreover, it is comparable to the amount of the highest annual symbiotic nitrogen fixation previously recorded in high-elevation alpine ecosystems. No differences in the "percent nitrogen derived from atmosphere" were detected among years, meaning that the ratio between the ^{15}N%excess in the legume and in the reference species was constant among years regardless of annual climatic variations. For these reasons, the use of the enriched ^{15}N isotope dilution method for the estimation of symbiotic nitrogen fixation for T. alpinum appears highly species-dependent but not year-dependent. Taking a very precautionary approach and using the grass N stricta as the reference species, we estimated 21.9 kg ha^{-1} year^{-1} of nitrogen fixation in T. alpinum-dominant communities. This quantity of nitrogen is the highest ever estimated in a natural alpine grassland and it is related to the high annual above-ground phytomass of T. alpinum.

Acknowledgments

Electronic supplementary material

Below is the link to the electronic supplementary material.
Online Resource 1: Mean ± standard error of total annual above-ground phytomass and T. alpinum annual above-ground phytomass in the study sites during three years of measurements (PDF 8 kb)

Online Resource 2: Mean ± standard error of total nitrogen (N%tot), nitrogen excess (15N%excess}, percent nitrogen derived from the atmosphere (%Ndfa), and total symbiotic nitrogen fixation (N_2-fix) for different species in the study sites during three years of measurements (PDF 26 kb)

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Table 1 Frequency, minimum, and maximum cover (%) of the species recorded within *T. alpinum*-dominant communities based on 271 surveys in the southwestern Alps (Cavallero et al. 2007). Species collected in the study are shown in in bold. LS = leguminous species, RS = reference species

Species	Frequency (%)	Cover (%) minimum	maximum
Trifolium alpinum (**LS**)	98	3.6	52.3
Carex sempervirens (**RS**)	97	1.6	24.8
Nardus stricta (**RS**)	76	0.7	16.7
Poa alpina (**RS**)	65	0.6	17.4
Anthoxanthum alpinum	63	0.2	14.5
Avenella flexuosa (**RS**)	59	0.1	18.7

Table 2 Mean ± standard error of the mean of total annual above-ground phytomass and *T. alpinum* annual above-ground phytomass in the study sites during three years of measurements. ns = not significant (p > 0.05). Sites and years with no letters in common were significantly different (p ≤ 0.05). +++ = p ≤ 0.001; ++ = p ≤ 0.01; ns: not significant

Variables	Total annual above-ground phytomass (t ha^{-1})		*T. alpinum* annual above-ground phytomass (t ha^{-1})	
Site				
p	ns		++	
Site 1 (2230 m a.s.l.)	2.23±0.208		1.20±0.097	b
Site 2 (2350 m a.s.l.)	2.03±0.102		0.83±0.042	a
Site 3 (2550 m a.s.l.)	2.51±0.169		1.01±0.084	ab
Year				
p	+++		++	
Year 2005	1.86±0.136	a	0.82±0.041	a
Year 2006	2.04±0.095	a	1.04±0.068	b
Year 2007	2.87±0.165	b	1.18±0.110	b
Site x Year	++		ns	

Table 3 Differences among species, sites and years in total nitrogen ($N\%_{tot}$), nitrogen excess ($^{15}N\%excess$), percent nitrogen derived from the atmosphere ($\%Ndfa$), and total symbiotic nitrogen fixation (N_2-fix). Species, sites and years with no letters in common were significantly different ($p \leq 0.05$). +++ = $p \leq 0.001$; ++ = $p \leq 0.01$; ns: not significant

Variables	$N\%_{tot}$ (%)	$^{15}N\%excess$ (%)	%Ndfa (%)	N_2-fix (kg ha^{-1} year^{-1})
Species				
p	+++	+++	+++	+++
Trifolium alpinum	3.30±0.056 c	0.02±0.002 a	-	-
Nardus stricta	1.78±0.027 a	0.07±0.004 b	65±2.1 a	21.9±1.44 a
Carex sempervirens	1.88±0.029 b	0.10±0.006 c	73±1.7 b	25.3±1.48 ab
Avenella flexuosa	1.75±0.034 a	0.19±0.014 d	84±1.5 c	30.4±1.69 bc
Poa alpina	1.88±0.062 b	0.37±0.018 e	93±0.7 d	32.3±1.82 c
Sites				
p	+++	+++	++	++
Site 1	2.22±0.071 c	0.12±0.013 a	75±2.3 a	32.7±2.13 b
Site 2	2.14±0.070 b	0.19±0.016 c	82±1.7 b	22.9±0.87 a
Site 3	2.03±0.079 a	0.14±0.016 b	79±1.7 b	26.9±1.20 b
Year				
p	+++	+++	ns	+++
Year 2005	1.91±0.060 a	0.13±0.016 a	78±2.2	17.9±0.73 a
Year 2006	2.35±0.073 c	0.13±0.012 a	78±1.9	28.5±0.96 b
Year 2007	2.00±0.078 b	0.18±0.018 b	80±1.6	33.0±1.71 b
Species x Site	+++	+++	ns	ns
Species x Year	+++	ns	ns	ns

Fig. 1 Root characteristics (mean lateral extension and maximum depth) of *T. alpinum* and the four reference species