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# DIPLOMARBEIT

Titel der Diplomarbeit

A simple method for in-situ-labelling with $^{15}N$ and $^{13}C$ of native grassland species through foliar brushing

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## 1. ABSTRACT


1. Labelling plants in situ with $^{13}C$ stable isotopes usually requires gas-tight labelling chambers often not available in ecological laboratories. A common method for $^{15}N$ in situ labelling is spraying or watering plants with a heavy isotope enriched solution, which makes a contamination of earth and other plants most likely.
2. In this greenhouse pot experiment, we tested a foliar labelling method where we brushed $^{15}N$ and $^{13}C$ urea solution directly onto leaves of twelve species native to temperate grasslands representing grasses, non-leguminous forbs and legumes. Labelling was first applied for five consecutive days and then sustained once a week over a period of four weeks. Isotopic signals in shoots and roots were measured regularly over four weeks.
3. Across all species, shoots ($^{15}N$: 0.145; $^{13}C$: 0.090 atom percent excess, APE) and roots ($^{15}N$: 0.051; $^{13}C$: 0.023) were significantly enriched after the five-time daily labelling. Generally, $^{15}N$ ($\chi^2 = 26.308$, df = 1, $p < 0.001$) and $^{13}C$ enrichment ($\chi^2 = 24.681$, df = 1, $p < 0.001$) was significantly higher in shoots than in roots. Overall, no clear pattern of isotopic enrichment could be found between plant functional groups. However, grasses showed a more balanced allocation between shoots and roots (averaged shoot-to-root ratio of isotopic enrichment was 1.5 and 1.9 for $^{15}N$ and $^{13}C$, respectively) than forbs (14.6 and 3.2 for $^{15}N$ and $^{13}C$, respectively) and legumes (26.3 and 24.6 for $^{15}N$ and $^{13}C$, respectively).

4. Isotopic enrichment levels after four weeks were either lower, higher or unchanged to that of week one and varied specifically between species or plant part considered.
5. Taking into account the consistent enrichment levels and simple application of this method we suggest a more widespread utilization of this method in ecological research. Tracing studies with plants as the labelled component make possible to investigate below- and aboveground interactions. This basic knowledge of food webs and material flows leads to a better understanding of ecosystems.

**Key-words**: APE, carbon isotope, enrichment studies, foliar labelling, nitrogen isotope, native grassland species, stable isotopes, urea.

## 2. INTRODUCTION

Stable isotope labelling is a wide-spread technique in ecological research to validate and complement studies at natural abundance levels in order to elucidate nutrient cycles and organismic interactions within ecosystems (Michener and Lajtha, 2007). Especially when studying food webs including plants a common approach is to integrate isotopically enriched plant material into the system and trace their paths among the food web partners (Herman et al., 2000; Hood-Nowotny and Knols, 2007; Martens et al., 2001; Seeber et al., 2009; Simard et al., 1997). However, in order to produce isotopically labelled plant material, it is frequently recommended that plants are cultivated in sophisticated labelling chambers for continued release of $^{13}CO_2$ that are often not available in ecological laboratories (Berg et al., 1991). Pulse-labelling, in

which plants are exposed periodically to labelled $CO_2$, circumvents many of the logistical constraints and even allows labelling outside of the laboratory, although airtight labelling chambers are still needed (Bromand et al., 2001; Leake et al., 2006; Subke et al., 2009).

Recently, Hertenberger and Wanek (2004) compared $^{15}N$ labelling efficiencies of the alternative methods root feeding, stem infiltration, leaf tip feeding, vacuum infiltration and surface abrasion on three native species (forbs: *Brassica napus* L., *Centaurea jacea* L.; grass: *Lolium perenne* L.). Generally, their results showed marked differences in plant labelling effectiveness, both with respect to the method applied and the plant species used. Leaf vacuum infiltration and leaf surface abrasion resulted in the lowest $^{15}N$ enrichments of roots and shoots (<1% APE, atom percent excess), while root feeding and stem infiltration (8% APE in shoots and 15% in roots) achieved the best results. Overall, stem infiltration effectively $^{15}N$ labelled plants with thicker stems, while feeding via cut leaf tip was most effective for graminoid plants. Another frequently used alternative, namely spraying isotopically labelled urea onto the plant surface was applied on various crop plants (Schmidt and Scrimgeour, 2001; El-Naggar et al., 2008; Rasmussen et al., 2007; Wichern et al., 2006). However, while spraying generally seems to work well it has the disadvantage that the soil surface needs to be protected in order to avoid contamination with stable isotopes, thus circumventing its use in stands with a dense plant cover where only specific plants need to be labelled. Moreover, it is unclear how foliar labelling would also work for various native species and to what extent the isotopic signal is allocated within the plant.

Thus, the objectives of the current study were to test whether (i) dual isotopic labelling with $^{15}N$ and $^{13}C$ applied directly onto the leaf surface is a suitable method for labelling a variety of native grassland plant species, (ii) isotopic signals differ between plant functional groups and to what extent the isotopic labels are distributed between shoots and roots and (iii) the persistence of the isotopic label differs between tested plant species. We tested this in a pot experiment where 12 plant species comprising three different functional groups (grasses, forbs, legumes) grew in field soil. We hypothesized that labelling efficacy in shoots and special in roots will differ among plant functional groups in correlation with their different performances of shoot- root allocation.

## 3. MATERIAL AND METHODS

### 3.1. Plant and soil material

The experiment was conducted in an unheated greenhouse at the University of Natural Resources and Applied Life Sciences, Vienna, from March to June 2008. Foliar isotopic labelling was tested on 12 different plant species commonly inhabiting Central European low-fertile grasslands. Test plants included the graminoids - *Arrhenatherum elatius* L., *Briza media* L., *Bromus erectus* Huds., *Dactylis glomerata* L.; the non-legume forbs - *Leucanthemum vulgare* Lam., *Plantago lanceolata* L., *Rumex obtusifolius* L., *Salvia pratensis* L., *Knautia arvensis* Coult.; and the herbaceous legumes - *Lotus corniculatus* L., *Medicago lupulina* L., *Trifolium pratense* L. (seeds were obtained from a commercial provider; Rieger Hofmann, Blaufelden-Raboldshausen, Germany). We seeded one individual of one plant species per pot (three litre volume, 14.5

cm x 14.5 cm side length, 22 cm height); to ensure regular germination we initially place three seeds on the soil surface but later reduced it to one plant per pot. Pots were filled with a 2:1 field soil quartz sand (quartz sand particle size 1.4 – 2.2 mm) mixture (pH = 7.6, N = 0.092%, P = 64.5 mg $kg^{-1}$, K = 113.6 mg $kg^{-1}$). The field soil was obtained from an arable field of the Experimental Farm of the University of Natural Resources and Applied Life Sciences Vienna, Groß-Enzersdorf, sieved through a 1 cm sieve and sterilized at 120 ℃ for 12 hours before filled into the pots.

Plants were watered with a constant amount of deionised water when needed. The pots were randomly arranged on a greenhouse table and randomised once a week. We started with 24 replicates from each plant species and harvested three replicates of labelled and not labelled plant species once a week over a period of four weeks after the initial labelling period (see below).

### 3.2. Foliar labelling, harvest and isotopic analyses

For foliar labelling we prepared a 97 atom% $^{13}C$ and 2 atom% $^{15}N$ urea solution by dissolving 100 mg 99 atom% $^{13}C$ urea and 2 mg 98 atom% $^{15}N$ urea (Sigma Aldrich, Vienna, Austria) in 50 ml distilled water. To ensure good contact of the labelling solution with the leaf surface, 12.5 µl wetting agent (Neo-Wett, Kwizda, Vienna, Austria) was added. The control solution consisted of 50 ml distilled water, 102 mg unlabelled urea and 12.5 µl wetting agent. The chosen concentrations are similar to the ones Schmidt and Scrimgeour, (2001) used. Labelling started after the plants developed two leaves by applying the solution with a small paint-brush on the upper and lower leaf surfaces (cotyledons were not labelled); during brushing leaves were held with forceps. Only small

amounts of the solution were carefully applied to avoid contamination of the soil. Leaves treated with adequate solution became a shiny leaf surface, making it easy to see which leaves were already treated. Labelling was applied once a day over five consecutive days.

Three replicate pots per plant species and treatment were harvested at day six after the beginning of labelling by carefully excavating the plants separating roots and shoots. In the following three weeks the remaining replicates were only labelled once a week. After each following week three replicates per plant species and treatment were harvested two days after the last labelling. Roots were immediately washed free of soil and dried at 65℃ for at least 24 hours. At all four harvesting dates, roots of all legume species showed rhizobia nodules. Shoots were carefully washed, scanned on a flatbed scanner (300 dpi) and afterwards dried at 65 ℃ for at least 24 hours. Leaf area was measured using image analysing software (ImageJ for Windows, Institute of Health, Washington D.C., USA). The dried plant material was ground to a fine powder in a ball mill and weighed in tin capsules for isotopic analyses. Samples were analysed by Continuous-flow IRMS (EA 1110; CE Instruments, Milano, Italy) connected to the gas isotope ratio mass spectrometer (Delta Plus; Finnigan MAT, Bremen, Germany). The natural abundance was calculated against the V-PDB standard for $^{13}C$ and against air for $^{15}N$. Enrichment of plants after labelling was calculated by subtracting at% of the control plants from at% of the labelled plants, yielding atom% enrichment values (APE).

### 3.3. Statistical analyses

Since data were not distributed normally, we analysed them using non-parametric tests: Kruskal-Wallis-Tests were used to test for significance of differences between means of two or more groups, Mann-Whitney-U-tests were used for pairwise comparisons. Spearman correlations were calculated for testing the relationships between total plant dry mass and leaf area and $^{15}N$ enrichment and $^{13}C$ enrichment. All statistical analyses were performed using SPSS 15.0 software (SPSS Inc. Headquarters, Chicago, Illinois, USA).

## 4. RESULTS

### 4.1. Plant biomass production and C and N concentrations

With the exception of the grass *B. media*, the test plants grew well during the experiment increasing their biomass on average by 255% from week one to week four (Table 1). Species varied greatly in biomass and C and N concentrations without a clear difference between functional groups (Table 1). At the start of the experiment, non-leguminous forbs had the highest biomass followed by grasses and legumes.

**Table 1**. Dry mass, N and C concentration of shoots and roots of 12 native grassland species comprising the functional groups grasses, non-leguminous forbs and leguminous forbs after one week and after four weeks of foliar labelling. See Materials and Methods. Means, n=3.

| | | | Week one | | | | | | Week four | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Dry mass (mg) | | N conc (%) | | C conc (%) | | Dry mass (mg) | | N conc (%) | | C conc (%) | |
| Functional group | Plant species | Plant part | Control | Labelled | Control | Labelled | Control | Labelled | Control | Labelled | Control | Labelled | Control | Labelled |
| Grasses | | | | | | | | | | | | | | |
| | *A. elatior* | Shoot | 74.2 | 94.9 | 5.2 | 4.7 | 42.0 | 42.1 | 154.2 | 208.1 | 4.3 | 4.3 | 46.0 | 42.4 |
| | | Root | 28.6 | 21.7 | 2.7 | 1.8 | 38.9 | 37.7 | 29.5 | 115.4 | 1.6 | 1.2 | 46.2 | 43.2 |
| | *B. media* | Shoot | 57.2 | 78.3 | 3.0 | 2.8 | 49.1 | 39.7 | | | | | | |
| | | Root | 2.3 | 13.5 | 1.2 | 1.7 | 49.2 | 42.2 | | | | | | |
| | *B. erectus* | Shoot | 60.1 | 36.2 | 4.7 | 4.2 | 41.5 | 40.3 | 240.7 | 259.3 | 3.3 | 3.7 | 47.0 | 41.3 |
| | | Root | 37.5 | 16.8 | 1.5 | 2.1 | 31.5 | 36.4 | 159.9 | 161.4 | 1.5 | 1.7 | 42.5 | 37.9 |
| | *D. glomerata* | Shoot | 64.6 | 74.2 | 4.4 | 4.5 | 40.6 | 40.8 | 220.2 | 214.8 | 4.0 | 4.2 | 46.8 | 41.3 |
| | | Root | 31.0 | 28.5 | 1.4 | 1.8 | 42.4 | 39.0 | 154.2 | 80.7 | 1.4 | 2.1 | 47.8 | 41.9 |
| Forbs | *K. arvensis* | Shoot | 23.0 | 21.3 | 3.3 | 4.2 | 43.0 | 45.4 | 26.1 | 31.8 | 2.8 | 4.4 | 38.9 | 39.3 |
| | | Root | 11.1 | 19.1 | 2.7 | 3.9 | 46.2 | 42.9 | 18.5 | 17.7 | 1.6 | 2.9 | 48.7 | 46.1 |
| | *L. inuitianum* | Shoot | 30.6 | 69.8 | 3.4 | 3.5 | 45.7 | 39.9 | | 107.6 | | 3.5 | | 40.2 |
| | | Root | 7.3 | 19.7 | 2.8 | 2.2 | 46.6 | 41.3 | | 63.0 | | 3.0 | | 40.2 |
| | *P. lanceolata* | Shoot | 18.3 | 22.9 | 4.5 | 3.9 | 41.4 | 42.2 | 84.4 | 97.4 | 3.4 | 3.7 | 46.2 | 41.5 |
| | | Root | 22.2 | 28.7 | 3.0 | 5.1 | 36.1 | 40.4 | 55.7 | 74.5 | 1.8 | 2.5 | 46.4 | 41.3 |
| | *R. obtusifolius* | Shoot | 235.5 | 408.8 | 3.8 | 4.6 | 44.3 | 40.2 | 356.3 | 619.8 | 3.5 | 3.4 | 42.8 | 38.3 |
| | | Root | 350.8 | 288.1 | 2.1 | 2.4 | 46.0 | 41.3 | 115.2 | 569.9 | 1.7 | 2.2 | 46.3 | 40.3 |
| | *S. pratense* | Shoot | 52.7 | 21.2 | 5.0 | 4.5 | 41.5 | 41.1 | 20.3 | 37.0 | 3.5 | 3.6 | 40.8 | 37.3 |
| | | Root | 16.6 | 17.0 | 3.2 | 3.9 | 42.3 | 40.7 | 17.3 | 25.9 | 3.1 | 3.0 | 46.5 | 45.2 |
| Legumes | *L. corniculatus* | Shoot | 5.8 | 8.1 | 5.0 | 7.6 | 47.1 | 42.1 | 8.2 | 5.6 | 5.8 | 7.1 | 43.7 | 46.1 |
| | | Root | 3.6 | 9.9 | 3.8 | 6.5 | 44.3 | 38.6 | 6.4 | 4.5 | 1.6 | 3.6 | 40.4 | 45.3 |
| | *M. lupulina* | Shoot | 19.8 | 11.3 | 4.1 | 3.1 | 46.3 | 39.6 | 91.1 | 67.6 | 3.1 | 3.6 | 46.4 | 42.1 |
| | | Root | 4.7 | 6.8 | 2.1 | 3.2 | 49.9 | 41.7 | 60.2 | 33.0 | 3.0 | 4.1 | 46.5 | 41.1 |
| | *T. pratense* | Shoot | 3.3 | 7.6 | 6.1 | 7.3 | 46.1 | 42.8 | 13.5 | 13.6 | 5.7 | 3.6 | 48.6 | 33.5 |
| | | Root | 3.5 | 7.2 | 2.0 | 3.4 | 45.1 | 40.0 | 6.5 | 6.7 | 2.8 | 2.6 | 50.0 | 44.5 |

### 4.2. Isotopic enrichment after one-week foliar labeling

Overall, $^{15}N$ isotopic enrichment after 5 times labelling was significantly higher in shoots (0.145 APE) than in roots (0.051 APE; $\chi^2 = 26.308$, df = 1, $p < 0.001$; Fig. 1). Across species, $^{15}N$ enrichment differed marginally significantly in shoots ($\chi^2 = 18.199$, df = 11, $p = 0.077$) and significantly in roots ($\chi^2 = 24.237$, df = 11, $p = 0.012$; Fig. 1). Plant functional groups did not differ in their shoot $^{15}N$ enrichment ($\chi^2 = 1.636$, df = 2, $p = 0.441$), however grass roots showed a significantly higher mean $^{15}N$ enrichment (0.091 APE) than non-leguminous forbs (0.038 APE; U = 33.000, n = 12; 14, $p = 0.008$) and legumes (0.009 APE; U = 5.000, n = 12; 6, $p = 0.002$). Legume root $^{15}N$ enrichment was significantly lower than $^{15}N$ enrichment in roots of non-leguminous forbs (U = 15.000, n = 14; 6, $p = 0.026$) or grasses (U = 5.000, n = 12; 6, $p = 0.002$). Generally, grasses showed a more balanced allocation of $^{15}N$ enrichment between shoots and roots (shoots average: 0.125 APE; roots average: 0.091 APE) than the other functional groups where more $^{15}N$ was allocated towards shoots (Fig. 1). Average $^{15}N$ enrichment in shoots of non-leguminous forbs was 0.142 APE vs. 0.038 APE in roots. Legumes showed the most unbalanced allocation of $^{15}N$ enrichment between shoots (0.163 APE) and roots (0.009 APE).

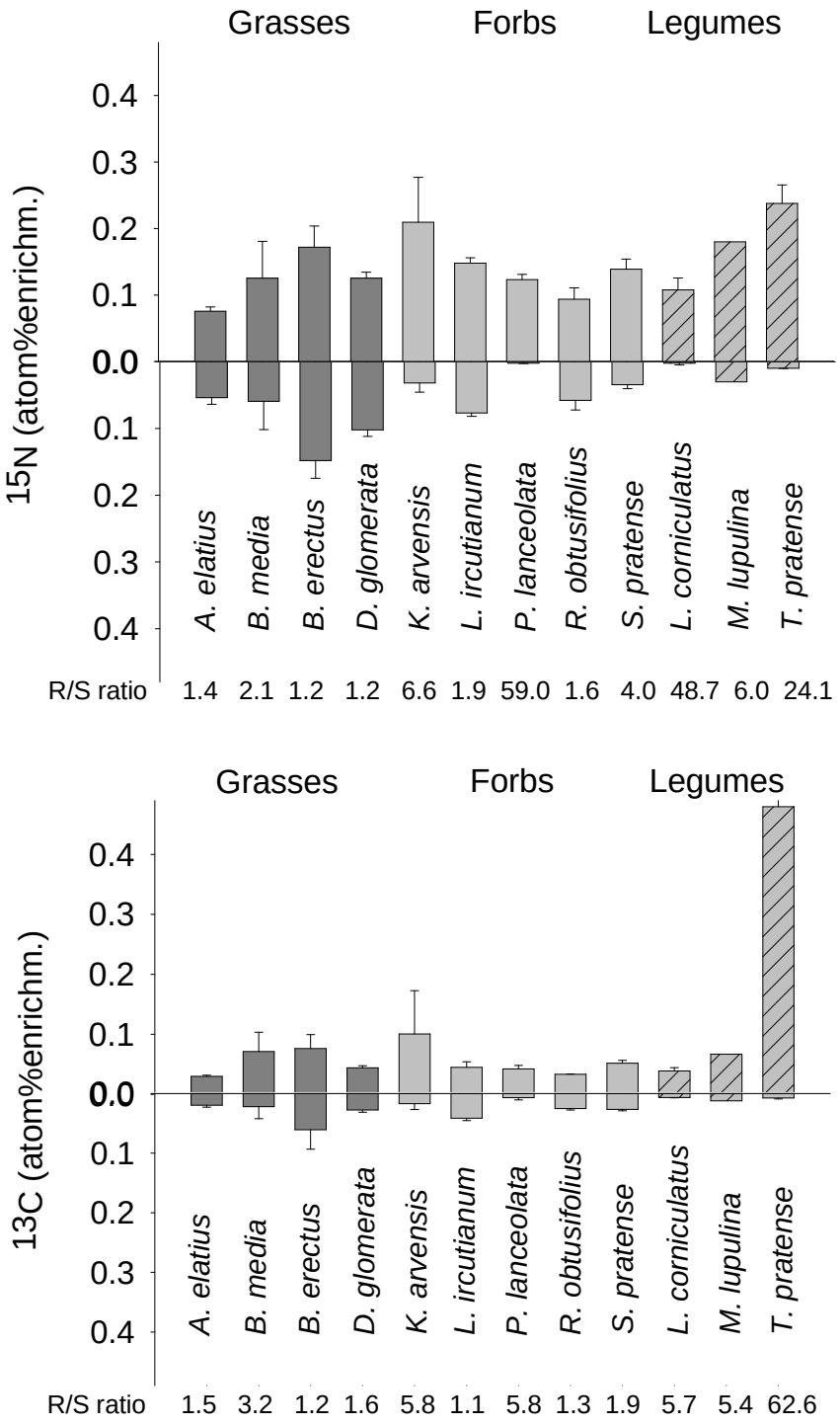


**Figure 1**. Enrichment of $^{15}N$ and $^{13}C$ in shoots and roots of 12 native grassland species comprising the functional groups grasses, non-leguminous forbs and leguminous forbs after daily foliar labelling during 5 days. See Materials and Methods for species abbreviations. Means ± SE, n = 3.

Across species, the enrichment of $^{13}C$ was generally higher in shoots (0.090 APE) than in roots (0.023 APE; $\chi^2 = 24.681$, df = 1, $p < 0.001$; Fig. 1). Comparing all species, the $^{13}C$ enrichment in shoots did not differ among species, however differed marginally among species in roots ($\chi^2 = 18.176$, df = 11, p = 0.078; Fig. 1). Functional groups did not differ in their $^{13}C$ shoot enrichment but in their $^{13}C$ root enrichment ($\chi^2 = 6.623$, df = 2, p = 0.036). With an average of 0.033 APE, grasses had similar $^{13}C$ root enrichment than non-leguminous forbs (mean = 0.022 APE, U = 78.000, n = 12, p = 0.758), however $^{13}C$ root enrichment in grasses was significantly higher than that of legumes (mean = 0.008 APE, U = 11.000, n = 12, p = 0.019). Legumes had significantly lower $^{13}C$ root enrichment than non-leguminous forbs (U = 14.000, n = 6; p = 0.021) and grasses (U = 11.000, n = 6; p = 0.019). Similar to $^{15}N$ the allocation of $^{13}C$ in shoots and roots was more balanced in grasses than in non-leguminous forbs and legumes with generally more $^{13}C$ enrichment in shoots than in roots (Fig.1).

### 4.3. Time courses of isotopic enrichments

Overall, there was considerable $^{15}N$ and $^{13}C$ enrichment both in shoots and roots even four weeks after the first labelling (Fig. 2, 3). Averaged across species, grass shoots showed a significant decrease of $^{15}N$ enrichment over the four weeks ($\chi^2 = 6.102$, df = 2, p = 0.047; Fig. 2) while the $^{15}N$ enrichment in grass roots remained unchanged over the four weeks ($\chi^2 = 3.032$, df = 2, p = 0.220; Fig. 2). Grass $^{13}C$ enrichment significantly decreased over the four weeks in shoots ($\chi^2 = 12.541$, df = 2, p = 0.002; Fig. 3) and roots ($\chi^2 = 6.061$, df = 2, p = 0.048; Fig. 3). Non-leguminous forb $^{15}N$ enrichment in shoots and roots

remained unchanged over the four weeks ($\chi^2$ = 3.443, df = 2, p = 0.179 for shoots and $\chi^2$ = 1.698, df = 2, p = 0.428 for roots; Fig. 2). Non-leguminous forb $^{13}$C enrichment in shoots decreased significantly ($\chi^2$ = 10.822, df = 2, p = 0.004; Fig. 3) but remained unchanged in forb roots ($\chi^2$ = 3.488, df = 2, p = 0.175; Fig. 3). Legume $^{15}$N enrichment significantly increased in shoots ($\chi^2$ = 6.045, df = 2, p = 0.049; Fig. 2) and roots ($\chi^2$ = 12.123, df = 2, p = 0.002; Fig.2) during the four weeks. Legume $^{13}$C remained unchanged in shoots ($\chi^2$ = 2.224, df = 2, p = 0.329; Fig. 3) but significantly increased in roots over the experimental period ($\chi^2$ = 13.228, df = 2, p = 0.001; Fig.3).

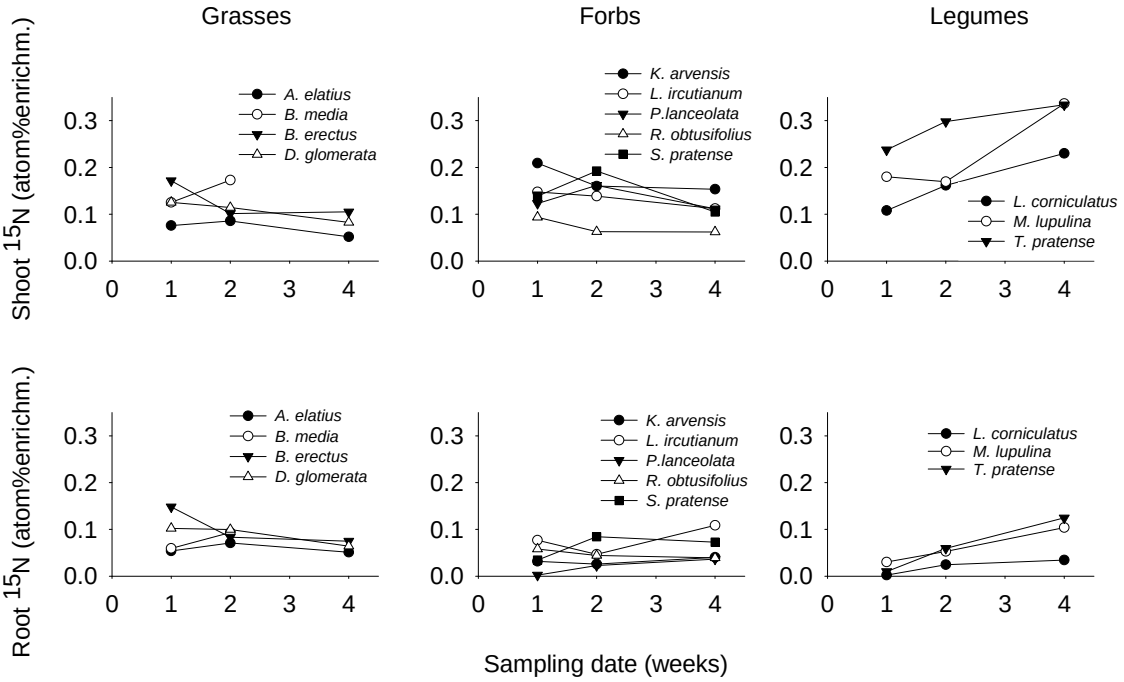


**Figure 2**. Time course of enrichment of $^{15}$N in shoots and roots of 12 native grassland species comprising the functional groups grasses, non-leguminous forbs and leguminous forbs during four weeks of foliar labelling. See Materials and Methods for species abbreviations. Means, n = 3.

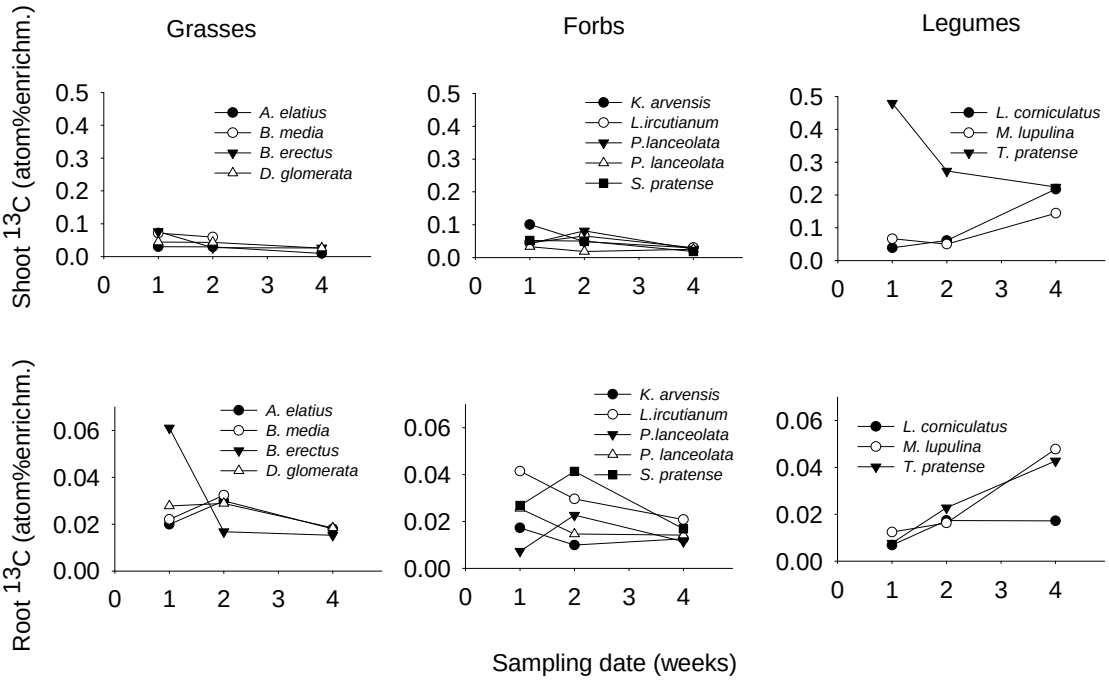


**Figure 3**. Time course of enrichment of $^{15}N$ in shoots and roots of 12 native grassland species comprising the functional groups grasses, non-leguminous forbs and leguminous forbs during four weeks of foliar labelling. See Materials and Methods for species abbreviations. Means, n=3.

### 4.4. Correlations between isotopic enrichments and plant characteristics

Across all species and dates, $^{15}N$ enrichment of shoots and roots was significantly negatively correlated with either shoot or root dry mass, respectively (Table 2). Only shoot $^{13}C$ enrichment was significantly correlated with either shoot dry mass or leaf area while root $^{13}C$ was unrelated to root dry mass. Among functional groups only non-leguminous forbs showed statistically significant correlations between isotope ($^{13}C$ or $^{15}N$) enrichment and either dry mass or leaf area while isotope enrichments in grasses and legumes were unrelated to the studied parameters (Table 2).

**Table 2**. Spearman correlations between $^{15}N$ and $^{13}C$ APE isotopic enrichments and plant characteristics across species and sampling dates. Significant correlations in **bold**.

| | Across species | | Grasses | | Forbs | | Legumes | |
|---|---|---|---|---|---|---|---|---|
| Variable | $r_s$ | p | $r_s$ | p | $r_s$ | p | $r_s$ | p |
| *Shoot $^{15}N$* | | | | | | | | |
| vs. shoot $^{13}C$ | **0.823** | **< 0.001** | ?? | | ?? | | ?? | |
| vs. shoot dry mass | **-0.498** | **< 0.001** | -0.122 | 0.500 | **-0.588** | **<0.001** | -0.073 | 0.740 |
| vs. leaf area | **-0.597** | **<0.001** | -0.231 | 0.195 | **-0.597** | **<0.001** | -0.080 | 0.717 |
| *Shoot $^{13}C$* | | | | | | | | |
| vs. shoot dry mass | **-0.466** | **< 0.001** | -0.236 | 0.187 | **-0.403** | **0.008** | 0.197 | 0.368 |
| vs. leaf area | **-0.487** | **< 0.001** | -0.301 | 0.088 | **-0.420** | **0.006** | -0.197 | 0.368 |
| *Root $^{15}N$* | | | | | | | | |
| vs. root $^{13}C$ | **0.672** | **< 0.001** | ?? | | ?? | | ?? | |
| vs. root dry mass | **0.294** | **0.003** | -0.080 | 0.660 | -0.131 | 0.409 | 0.186 | 0.394 |
| *Root $^{13}C$* | | | | | | | | |
| vs. root dry mass | -0.012 | 0.908 | -0.221 | 0.217 | -0.225 | 0.153 | -0.110 | 0.959 |

## 5. DISCUSSION

To the best of our knowledge this is the first study demonstrating that brushing $^{15}N$ and $^{13}C$ urea onto the leaf surface of a dozen of grass, non-leguminous forb and legume species is a feasible method for *in-situ* dual-labelling native plant species. Moreover, the positive correlation between $^{15}N$ and $^{13}C$ enrichment for both shoots and roots indicates that the tested species can successfully be labelled with the two isotopes. In contrast to other studies who sprayed stable isotopes onto the leaf surface (Below et al., 1985; Palta et al., 1991; Schmidt and Scrimgeour, 2001; Yasmin et al., 2006), our brushing method has the advantage of being more precise as certain plant species in rather dense stands can be labelled without unintentionally contaminating other plant individuals or soil. Because most of the previous studies on foliar labelling were

conducted on crop species (maize: Below et al., 1985, Schmidt and Scrimgeour, 2001; wheat: Palta et al., 1991; chickpea: Yasmin et al., 2006) it was interesting to see that also native plant species with much lower growth rates could be successfully labelled via foliar feeding.

Our expectation that functional groups would significantly differ in their isotopic enrichments were only partly met. While isotopic enrichments in shoots were similar between functional groups, the allocation of $^{15}N$ into roots was highest in grasses followed by forbs and legumes, while $^{13}C$ enrichment was similar in grasses and forbs though higher than in legumes. This indicates that functional groups mainly differed regarding their allocation of the isotopic label within the plants. The four grass species have a higher $^{15}N$ and $^{13}C$ allocation into the root systems compared with the non-leguminous forbs and legumes, which is caused by the higher root shoot ratios of grasses, which means a greater nutrient and energy allocation to the roots (Craine, 2002, Fitter, 1987; Wilsey, 2006). Moreover, grass root systems usually also have less ligneous structures and higher turnover rates than other root systems leading to a more rapid incorporation of carbon and nitrogen in root systems (Gross et al. 1992; Eissenstat 2000). This is also confirmed by the study of Hertenberger and Wanek (2004) showing that the $^{15}N$ signal reached the roots of the grass *L. perenne* after commenced labelling with stem infiltration after 20 hours, while in the forb *C. jacea* not until 26 hours. Despite higher root biomass than shoot biomasses, the forb *P. lanceolata* and the legume *L. corniculatus* allocated only very small amounts of $^{15}N$ and $^{13}C$ into their roots indicating very little turnover rates of the rather course root systems of these species.

Considering the more general patterns of $^{15}N$ enrichment, species allocated on average 74% $^{15}N$ into shoots simultaneously indicating that a

substantial amount was transported into their root systems. The 26% isotope allocation into roots is in contrast to other, mainly crop, studies, suggesting that only a small fraction of the label taken up by leaves was transferred to roots (Palta et al 1991; Schmidt and Scrimgeour, 2001; Khan et al., 2002; McNeill et al., 1997; Russell and Fillery, 1996). These contrasting findings perhaps reflect morphological and physiological differences between native plants and crops. Native species are in permanent competition for water and nutrients and have to allocate their biomass into root systems. Crops are breed for fast growth which is achieved by above-ground biomass allocation and they do not invest much in their root system. These different growth pattern leads to a better incorporation of the isotopes into the root system of native plants. Our finding of a considerably lower $^{13}C$ enrichment than a $^{15}N$ enrichment in shoots and roots across is consistent with other studies (Schmidt and Scrimgeour, 2001), reflecting that carbon enters leaves mainly in gaseous form as $CO_2$ (Larcher 2003). Nevertheless, it was still interesting to see that also liquid $^{13}C$ applied onto the leaf surface can enter the plant tissue and is incorporated into the root system. Another reason for restricted $^{13}C$ enrichment discussed is the over-supply of N to the plant, which leads to reduced carbohydrate accumulation (Gooding and Davies, 1992), however this is not very likely in our system as the soil nutrient concentrations were only moderate. Among legumes, only 7% $^{15}N$ and 11% $^{13}C$ of the whole-plant isotopic signal were allocated into roots suggesting that rhizobia associated with all tested legume species might have diluted the isotopic signal in roots. An exception to the $^{13}C$ enrichment patterns is the legume *T. pratense* which showed a four-times higher $^{13}C$ enrichment in shoot than all other species. We explain this pattern by the small biomass increase of this species, leading to an accumulation of the isotopic label in the

shoots with only little transfer to the roots. Moreover, in stable isotope studies there is always the possibility of sample contamination, however we find it unlikely that all three sample replicates were contaminated.

### 5.1. Persistence of the labelling signal

No data on the persistence of the labelling signal after foliar feeding are available for native plant species. With the proposed method isotopic enrichment levels in shoots and roots were generally low for both $^{15}N$ and $^{13}C$ (<1% APE) and these levels either decreased (e.g. grass shoots), increased (e.g. legume shoots) or remained unchanged (e.g. forb shoots) over four weeks with a one-time re-labelling a week. Our current enrichment levels are similar to those achieved on the native forb *C. jacea* or the grass *L. perenne* using the leaf tipping method measured 48 hours after labelling (Hertenberger and Wanek, 2004). In order to increase the isotopic signal in the tested plant species, a higher concentration of the urea solution would be necessary, however this would also involve the risk of causing leaf burning damage (Bremner 1995). This need to use low urea concentrations also limits the maximum $^{13}C$ enrichment achievable with urea leaf-feeding. More than one labelling a week would probably sustain a higher isotopic signal over a longer period as indicated by the negative correlation between isotopic signal and shoot mass or leaf area, however whether this varies among species requires further testing.

Taken together, the simple dual-labelling method tested in the current study appears to be a feasible alternative to growing plants in gas-tight laboratory chambers or using portable labelling enclosures. The convenient procedure opens new avenues for studying ecological interactions *in-situ* in

plant communities and can help disentangling interrelationships between below- and aboveground food webs.

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# 8. APPENDIX

## 8.1. Zusammenfassung

1. Die *in situ* Markierung von Pflanzen mit $^{13}C$, welches über angereichertes $CO^2$ in die Pflanzen gelangt, erfordert speziell dafür vorgesehene Klimakammern, die nicht allen Laboratorien zur Verfügung stehen. Bei einer häufig angewandten Methode Pflanzen *in situ* mit $^{15}N$ zu markieren, wird die angereicherte Lösung über den Pflanzen versprüht, oder sie damit gegossen. Beides verursacht eine Verunreinigung von Boden und anderen Pflanzen.
2. In diesem Glashausversuch testeten wir eine neue Methode für die *in situ* Markierung von Pflanzen. Wir pinselten eine $^{15}N$ und $^{13}C$ Urea Lösung direkt auf die Blattoberfläche von zwölf Wildpflanzen, die für temperate Grasländer typisch sind. Darunter befanden sich Gräser, nicht-leguminose Krautige und Leguminosen. Die Markierung erfolgte an fünf folgenden Tagen, danach einmal wöchentlich innerhalb einer Zeitspanne von vier Wochen. Die Isotopensignale von Wurzel und Spross der unterschiedlichen Arten wurden regelmäßig gemessen.
3. Von allen Pflanzenarten waren Spross ($^{15}N$: 0,145; $^{13}C$: 0,090 atom percent excess, APE) und Wurzel ($^{15}N$: 0,051; $^{13}C$: 0,023) nach der Markierung an fünf folgenden Tagen signifikant angereichert. Generell war die Anreicherung von $^{15}N$ ($\chi^2 = 26{,}308$, $df = 1$, $p < 0{,}001$) und $^{13}C$ ($\chi^2 = 24{,}681$, $df = 1$, $p < 0{,}001$) im Spross höher als in der Wurzel. Es konnte kein Muster bei der Anreicherung innerhalb der drei funktionellen Gruppen erkannt werden. Gräser zeigten eine gleichmäßigere Verteilung

des Signals zwischen Sproß und Wurzel (durchschnittliches Spross-Wurzel Verhältnis der Isotopen Anreicherung: 1,5 für $^{15}N$ und 1,9 für $^{13}C$) als nicht-leguminose Krautige (14,6 für $^{15}N$ und 3,2 für $^{13}C$) und Leguminosen (26,3 für $^{15}N$ und 24,6 für $^{13}C$).

4. Die Isotopensignale während der vierwöchigen Markierung nahmen entweder ab, zu oder blieben gleich gegenüber den Signalen nach der einwöchigen Markierung und variierten zwischen den Arten und Pflanzenteilen.
5. Zieht man die beständigen Isotopensignale und die einfache Durchführbarkeit in Betracht, ist unsere Methode ideal zur Untersuchung von ökologischen Fragestellungen. Sogenannte tracing studies mit Pflanzen, als die markierte Komponente, machen es möglich ober- und unterirdische Interaktionen leichter nachzuvollziehen. Aus solchen Studien können neue Erkenntnisse über Nahrungsnetze und Materialkreisläufe gewonnen werden und dies führt zu einem besseren Verständnis von Ökosystemen.

### 8.2. Lebenslauf


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