

Abstract

A longstanding puzzle in isotope studies of C₃ plant species is that heterotrophic plant organs (e.g., stems, roots, seeds, and fruits) tend to be enriched in ¹³C compared to the autotrophic organ (leaves) that provides them with photosynthate. Our inability to explain this puzzle suggests key deficiencies in understanding post-photosynthetic metabolic processes. It also limits the effectiveness of applications of stable carbon isotope analyses in a variety of scientific disciplines ranging from plant physiology to global carbon cycle studies. To gain insight into this puzzle, we excavated whole plant architectures of *Nitraria tangutorum* Bobrov, a C₃ species that has an exceptional capability of fixing sands and building sand dunes, in two deserts in northwestern China. We systematically and simultaneously measured carbon isotope ratios and nitrogen and phosphorous contents of different parts of the excavated plants. We also determined the seasonal variations in leaf carbon isotope ratios on nearby intact plants of *N. tangutorum*. We found, for the first time, that higher nitrogen contents in heterotrophic organs were significantly correlated with increased heterotrophic ¹³C enrichment compared to leaves. However, phosphorous contents had no effect on the enrichment. In addition, new leaves had carbon isotope ratios similar to roots but were progressively depleted in ¹³C as they matured. We concluded that a nitrogen-mediated process, probably the refixation of respiratory CO₂ by phosphoenolpyruvate (PEP) carboxylase, was responsible for the differences in ¹³C enrichment among different heterotrophic organs while processes within leaves or during phloem loading may contribute to the overall autotrophic – heterotrophic difference in carbon isotope compositions.

1 Introduction

The natural abundance analysis of stable carbon isotopes in plants has become an essential tool for studying plant-environmental interactions, plant metabolism, carbon allocation, and biosphere-atmosphere exchanges of carbon fluxes (Dawson et al., 2002;

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Bowling et al., 2008; Tcherkez et al., 2011; Cernusak et al., 2013). Understanding processes and factors controlling carbon isotope compositions in different plant organs, which are not homogenous (Leavitt and Long, 1986), is crucial to the successful applications of this tool. The primary determinant of plant carbon isotope compositions is the photosynthetic discrimination against the heavier carbon isotope ^{13}C . This primary discrimination process has been relatively well understood and detailed theoretical models relating the discrimination to environmental forcing conditions and leaf physiology and biochemistry have been developed (Farquhar et al., 1982; Farquhar and Cernusak, 2012; Gu and Sun, 2014). However, other processes must also influence plant carbon isotope compositions as heterotrophic plant organs (e.g., stems, roots, seeds and fruits) in C_3 plant species have been found to be generally enriched in ^{13}C as compared to the autotrophic organ (leaves) that supplies them with carbohydrates (Craig, 1953; Leavitt and Long, 1982; Ehleringer et al., 1987; Badeck et al., 2005; Cernusak et al., 2009). In contrast to the relatively well-understood photosynthetic carbon isotope discrimination, processes controlling the observed heterotrophic ^{13}C enrichment in C_3 plant species remain unclear even though the phenomenon was first reported sixty years ago (Craig, 1953).

Cernusak et al. (2009) and Ghashghaie and Badeck (2013) summarized more than half a dozen of nonexclusive processes that may explain the heterotrophic ^{13}C enrichment in C_3 plant species. These processes generally belong to two broad groups. Group I processes involve the occurrence of contrasting biochemical and metabolic fractionations between autotrophic and heterotrophic organs, for example, ^{13}C -enriched autotrophic vs. ^{13}C -depleted heterotrophic mitochondrial respirations, low autotrophic vs. high heterotrophic CO_2 fixation by phosphoenolpyruvate (PEP) carboxylase, and low autotrophic vs. high heterotrophic loss rates of ^{13}C -depleted volatile organic compounds, surface waxes and other products from secondary plant metabolism. Group II processes involve the utilization of contrasting organ-building photoassimilates, which in turn may be a result of a number of processes, including fractionations in favor of the heavier isotope during phloem loading/transport,

extensive shoot and root systems (Baas and Nield, 2007; Lang et al., 2013; Li et al., 2013). This characteristic makes it relatively easy to excavate the whole plant including roots for isotope and nutrient analyses, although to our knowledge, this species has never been investigated for heterotrophic ^{13}C enrichment.

We will report, for the first time, that variations in ^{13}C enrichment in different heterotrophic organs strongly depend on their nitrogen contents, indicating a role of a within-organ nitrogen-mediated process in heterotrophic ^{13}C enrichment. We will also show that the observed nitrogen–heterotrophic ^{13}C enrichment relationship is most parsimoniously explained through the respiratory CO_2 refixation by PEP carboxylase. Future studies on heterotrophic ^{13}C enrichment should investigate isotopic effects of nitrogen content and CO_2 refixation in different plant organs.

2 Materials and methods

2.1 Biological and environmental characteristics of *Nitraria tangutorum* Bobrov

Nitraria tangutorum Bobrov (Fig. 1) is a spiny shrub species in the *Nitraria* genus of the Zygophyllaceae family. Species in the *Nitraria* genus are generally xerophytes, widely distributed in the Middle East, Central Asia, and northwestern regions of China. *N. tangutorum*, however, is endemic to the northwestern regions of China, including north-eastern Tibet, Gansu, Qinghai, Xinjiang, western Inner Mongolia, western Ningxia, and northern Shaanxi. It is a pioneer species and has high tolerance for drought, heat, and salts. *N. tangutorum* plays an important ecological role in combating desertification due to its exceptional capabilities in forming phytogenic nebkha dunes which prevent or slow down the movement of sands. According to Li and Jiang (2011) and Li et al. (2013), the process of forming a nebkha typically starts when occasional ample moisture allows a seed to germinate inside clay cracks in dried-up flat beds of previous rivers or lakes. As the resulting ortet grows, it intercepts aeolian sands and the plant enters into a clonal reproductive stage. When branches are buried by sands, layering

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occurs and adventitious roots are formed. Under appropriate sand burial depth and sufficient moisture, ramets are developed from axillary buds in the layering and a clonal colony is formed. If aeolian sand supply is not interrupted, repetitive layering and ramet development will enlarge the colony and further increases its capacity to intercept aeolian sands and a phytogenic nebkha dune is formed (Fig. 1c).

The height of a *N. tangutorum* nebkha ranges from 1 to 3 m and some can reach 5 m. The base of a nebkha often has the shape of an ellipse with the major axis parallel to the local prevailing wind direction. The formation of nebkhas alters local microenvironments and provides habitats for other desert species. Li and Jiang (2011) described in detail the biological and environmental characteristics of species in the *Nitraria* genus with a focus on *N. tangutorum*.

2.2 Study sites

The field work was carried out at two desert locations. The first study site was within an experimental area (40°24' N, 106°43' E) managed by the Experimental Center of Desert Forestry of the Chinese Academy of Forestry. This site is located in Dengkou County, Inner Mongolia Autonomous Region, China. Dengkou County is at the junction between the Hetao Plain and Ulan Buh Desert of the Mongolian Plateau in the middle reaches of the Yellow River. The mean annual temperature is 8.84 °C and the mean annual precipitation is 147 mm with 77.5 % of annual rainfall occurring from June to September (1983–2012 averages). The mean annual potential evaporation is 2381 mm (Li et al., 2013). The soil in the study region in general is sandy soil and gray-brown desert soil (Cambic Arenosols and Luvic Gypsisols in FAO taxonomy). The *N. tangutorum* nebkhas at the study site are formed on clay soils deposited by the Yellow River. Although the plant community is dominated by *N. tangutorum*, xerophytic species such as semi-shrub *Artemisia ordosica*, perennial grass *Psammochloa villosa*, and annual species *Agriophyllum squarrosum* and *Corispermum mongolicum* can so be found.

The second study site was the Gansu Minqin Desert Ecosystem Research Station (38°34' N, 102°58' E), Minqin County, Gansu Province, China. Minqin County is located

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in the lower reaches of Shiyang River, surrounded by the Badain Jaran Desert in the west and north and the Tengger Desert in the east. The mean annual temperature is 8.87 °C and the mean annual precipitation is 117 mm with 73.1 % of annual rainfall occurring from June to September (1983–2012 averages). The mean annual potential evaporation is 2643 mm (Du et al., 2010). Thus the second study site is somewhat drier than the first site but with similar annual mean temperatures. The soil at the Minqin site is similar to that at the Dengkou site with sandy soil in the nebkhas and gray-brown desert soil between nebkhas. The native vegetation in the study area is usually dominated by shrubs and semi-shrubs with species such as *N. tangutorum* and *Calligonum mongolicum*. Experimental plots used in this study contained semi-fixed nebkha dunes developed by the growth of *N. tangutorum*. Typically in dry years, *N. tangutorum* is the only species growing in the nebkhas although in wet years, annual species such as *Agriophyllum squarrosum* and *Corispermum mongolicum* can also be found. Because the Minqin site is drier than the Dengkou site, the nebkhas at the Minqin site are generally smaller and less populated with plants than at the Dengkou site. The rooting depth is deeper at the Minqin site than at the Dengkou site (Table 1).

2.3 Excavation of *Nitraria tangutorum* nebkhas

In August 2012, we excavated three nebkhas at each study site. The geometrical and biometrical characteristics of the six nebkhas were summarized in Table 1. At the Dengkou site, the three nebkhas were excavated in a sampling area of 40 m × 40 m. At the Minqin site, nebkhas were generally much smaller. To ensure availability for analyses of sufficient biomass materials at this site, particularly fine roots (see below), three sampling areas each with a dimension of 30 m × 30 m were established and three nebkhas from each sampling area were tentatively excavated. Two nebkhas from one sampling area and one from another were determined to have sufficient amount of fine roots for analyses and were therefore excavated fully.

We excavated the nebkhas by carefully teasing away the sands from the mounds to expose the root architecture of *N. tangutorum* with particular attention paid to the

preservation of fine roots. The roots of a *N. tangutorum* can be found inside the sand mounds as well as inside the clay layer that generally forms a plain on which the sand mounds rest. We therefore also excavated any roots inside the clay layer to a depth until no more roots could be found.

We separated the whole plant biomass into leaves, stems, in-sand roots and below-plain roots. The in-sand roots, which were roots found inside the nebkha sands but above the plain formed by the underlying clay layer, were further separated into in-sand fine roots (diameter ≤ 2 mm) and in-sand coarse roots (diameter > 2 mm). The same root diameter threshold was used to separate the below-plain roots, which were found inside the clay layer under the nebkha sands. Furthermore, the below-plain fine and coarse roots were grouped in a 20 cm depth increment from the plain surface. We did not separate the in-sand fine and coarse roots into layers as a nebkha has a cone shape on top, making a layer hard to define. Also we did not use a simple “below-ground” group because “ground” is not well defined in a nebkha-populated landscape and because there are large physical and chemical differences between sands and clay which may affect the isotope compositions of roots growing in them. Litter was rarely found on the nebkhas, presumably because strong winds at the study sites can easily blow away any litter produced. However, woody debris from dead ramets was present inside the sand mounds and was collected during excavation. Thus for each nebkha, we differentiated the following categories of *N. tangutorum* biomass: the autotrophic organ of leaves, the heterotrophic organs of stems, in-sand fine roots (ISFR), in-sand coarse roots (ISCR), below-plain fine roots (BPFR) in 20 cm depth increments, and below-plain coarse roots (BPCR) in 20 cm increments, and the heterotrophic woody debris (WD). Nutrient contents and carbon isotope compositions were measured separately for each category.

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2.4 Measurements of nutrient contents and carbon isotope compositions with excavated biomass

All categories of *N. tangutorum* biomass (leaves, stems, ISFR, ISCR, BPFR in 20 cm increments, BPCR in 20 cm increments, and WD) from each excavated nebkha were dried to constant weight (60 °C, 48 h). The dry weight of biomass was determined with 0.01 g accuracy on an analytical scale. The biomass carbon stocks were expressed relative to the base area of the nebkha which was assumed to be an ellipse. The fraction of each component was also calculated.

Dried materials were randomly selected from each biomass category and ground to 80 mesh. The resultant powder was separated into six duplicates. Three duplicates were analyzed for carbon (C), nitrogen (N) and phosphorous (P) contents and the remaining three for isotope compositions. The C, N and P contents were measured in the Environmental Chemistry Analysis Laboratory in the Institute of Geographic Sciences and Natural Resources Research, the Chinese Academy of Sciences, Beijing, China. Total sample carbon and nitrogen were measured with the vario MACRO cube (Elementar Company, Germany). The analytical precision was better than 0.5% Relative Standard Deviation (RSD). Total phosphorus was measured with the ICP-OES OPTIMA 5300DV (PE, USA). The analytical precision was better than 2% RSD.

The carbon isotope compositions were analyzed at the Stable Isotope Ratio Mass Spectrometer Laboratory of the Chinese Academy of Forestry (SIRMSL, CAF), Beijing, China. The instrument used was a Delta V Advantage Mass Spectrometer (Thermo Fisher Scientific, Inc., USA) coupled with an elemental analyzer (FlashEA 1112; HT Instruments, Inc., USA) in the continuous flow mode. Isotope compositions were expressed using the delta notation (δ) in parts per thousand (‰): $\delta^{13}\text{C}$ (‰) = $[(R_{\text{sample}})/(R_{\text{standard}}) - 1] \times 1000$, where R is the ratio of ^{13}C to ^{12}C . The measurement applied the IAEA-600 standard (Caffeine) relative to V-PDB (Vienna PeeDee Formation Belemnite Limestone). The analytical precision was better than 0.1‰ based on replicate measurements of the reference standard.

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2.5 Measurements of seasonal variations in leaf $\delta^{13}\text{C}$ and C_i/C_a ratio

Photosynthetic carbon isotope discrimination depends on environmental conditions (Farquhar et al., 1982; Farquhar and Cernusak, 2012; Gu and Sun, 2014); consequently, leaf carbon isotope ratio $\delta^{13}\text{C}$ may change seasonally, potentially making the autotrophic–heterotrophic differences in carbon isotope compositions time dependent. Thus in addition to the isotopic and nutrient analyses for samples from the excavated plant materials, we also measured seasonal variations in leaf carbon isotope compositions and ratios of leaf intercellular airspace (C_i) to ambient (C_a) CO_2 concentrations on nearby un-excavated nebkhas at both the Dengkou and Minqin study sites. Four samples of leaves were taken in each month from May to September of 2012 at both sites and analyzed for carbon isotope ratios at the SIRMSL of CAF. The seasonal variations in C_i/C_a ratios were measured with a Li-6400 portable photosynthetic system (LiCor Environmental Sciences, Lincoln, NE, USA) each month from June to September of 2012 at the Dengkou site with 24–28 samples per month and from July to September of 2011 at the Minqin site with 16 samples per month. The chamber environment (temperature, light, and relative humidity) was kept close to ambient conditions at the time of measurement. Seasonal variations in leaf nutrient contents were not measured. The measurements of seasonal variations in leaf $\delta^{13}\text{C}$ provide a dynamic reference framework for examining the $\delta^{13}\text{C}$ values of heterotrophic organs while the independent measurements of seasonal variations in C_i/C_a ratios allow us to determine whether the seasonal patterns in leaf $\delta^{13}\text{C}$ are consistent with our current understanding of the photosynthetic carbon isotope discrimination (Farquhar et al., 1982).

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2.6 Quantification of heterotrophic ^{13}C enrichment and statistical analyses

We quantified the difference in carbon isotope composition between the leaves (autotrophic) and a heterotrophic organ with the following expression:

$$\Delta^{13}\text{C}_{\text{organ}} = \left(\frac{R_{\text{leaf}}}{R_{\text{organ}}} - 1 \right) \times 1000 = \left(\frac{\delta^{13}\text{C}_{\text{leaf}}/1000 + 1}{\delta^{13}\text{C}_{\text{organ}}/1000 + 1} - 1 \right) \times 1000 = \frac{\delta^{13}\text{C}_{\text{leaf}} - \delta^{13}\text{C}_{\text{organ}}}{1 + \delta^{13}\text{C}_{\text{organ}}/1000} \quad (1)$$

Thus a value of $\Delta^{13}\text{C}_{\text{organ}} < 0$ indicates an enrichment of ^{13}C in a heterotrophic organ relative to the leaves while $\Delta^{13}\text{C}_{\text{organ}} > 0$ indicates heterotrophic depletion. The values of $\delta^{13}\text{C}_{\text{Leaf}}$ used to calculate $\Delta^{13}\text{C}_{\text{organ}}$ came from leaves harvested from *N. tangutorum* of the excavated nebkhas, not from those for seasonal patterns. The use of Δ in Eq. (1) makes the relationship between autotrophic and heterotrophic organs analogous to that between reactants and products (Farquhar et al., 1989), which is appropriate for the purpose of this study. A great advantage of introducing $\Delta^{13}\text{C}_{\text{organ}}$ is that heterotrophic ^{13}C enrichment can be compared not only among the organs of the same plant but also across different plants at the same site or at different sites which may differ in autotrophic isotopic signatures. Thus the use of $\Delta^{13}\text{C}_{\text{organ}}$ facilitates the identification of general patterns.

Two-way ANOVA analyses (organ by site) were performed with SPSS (Ver.17.0). Carbon, nitrogen, and phosphorus contents, $\delta^{13}\text{C}$, $\Delta^{13}\text{C}_{\text{organ}}$, C/N ratios, N/P ratios and C/P ratios were analyzed for differences between organs and between study sites. Tukey post-hoc tests were used to determine pairwise differences for significant effects ($P < 0.05$). Regression analyses were used to determine the relationship between the heterotrophic ^{13}C enrichment and nutrient contents.

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3 Results

3.1 Variations in $\Delta^{13}\text{C}_{\text{organ}}$ among plant organs and between study sites

At both the Dengkou and Minqin study sites, the values of $\Delta^{13}\text{C}_{\text{organ}}$ for all heterotrophic organs examined were significantly smaller than zero, indicating that without any exception, the heterotrophic organs were enriched in ^{13}C compared to the leaves (Fig. 2). However, there were considerable variations in $\Delta^{13}\text{C}_{\text{organ}}$ among the heterotrophic organs at both study sites and between the heterotrophic organs across the study sites. Stems were less enriched (closer to zero) than roots at both sites. At the Dengkou site, the most enriched organ was the coarse roots inside the nebkha sands. At the Minqin site, the most enriched part was the fine roots inside the negkha sands although the difference between the coarse and fine roots inside the sands was not significant. At the Dengkou site, the coarse roots were consistently more enriched than the corresponding fine roots both inside the nebkha sands and below the plains. In contrast at the Minqin site, the coarse roots were less enriched than the corresponding fine roots except for the roots deep into the plains (40–80 cm) where the coarse roots were more enriched. However at both sites, the statistical power of the coarse – fine root isotope differences were low as they were not significant at the significance level of 0.05. At the Dengkou site, the woody debris was more enriched than the stems but less enriched than the roots while at the Minqin site, it was less enriched than either the stems or the roots. In all biomass categories investigated, the Dengkou site was more enriched than the Minqin site, particularly in below-plain roots and in woody debris.

3.2 Variations in nutrient concentrations among plant organs and between sites

There are considerable variations in nutrient contents among plant organs and between sites (Fig. 3). At both the Dengkou and Minqin sites, leaves appeared to have the lowest C (Fig. 3a) but highest N (Fig. 3b) and P (Fig. 3c) contents. At both sites, stems tended to have lower N contents than roots either inside the sand dunes or below the plains

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under the sand dunes; in contrast, P contents in stems were within the variations of P contents in roots. At the Denkou site, roots inside the sand dunes had lower N contents than roots below the plain; at the Minqin site, the coarse roots inside the sand dunes had lower N than either coarse or fine roots below the plain while the fine roots inside the sand dunes had N within the variations of those of coarse and fine roots below the plain. At the Dengkou site, the fine roots appeared to have higher P than coarse roots but the differences diminished from inside sands to below plain. There were no clear patterns on root P at the Minqin site. Woody debris had N contents similar to stems at both sites and tended to have significantly less P contents than leaves, stems or roots. Between the two study sites, the leaves had lower C but higher N and P contents at the Dengkou site than at the Minqin site, but the difference is not significant at the significance level of 0.05. In contrast, heterotrophic organs at the Dengkou site tended to have significantly higher N and P contents than at the Minqin site. This contrast suggests that *N. tangutorum* may be able to maintain nutrient contents in leaves for photosynthesis at the expense of stems and roots.

Consistent with the variations in C, N and P contents, there were also substantial variations in the ratios of C/N (Fig. 4a), N/P (Fig. 4b) and C/P (Fig. 4c) among plant organs and between sites. For the live biomass (leaves, stems, and roots), the ratios of C/N ranged from about 11 to 30, N/P from 20 to 40 and C/P from 300 to 700. As expected, leaves had the lowest C/N and C/P ratios at both sites. Leaves also had the lowest N/P ratios except for stems. Overall, the Dengkou site had lower ratios of C/N and C/P but higher ratios of N/P than the Minqin site, particularly for roots below the plain.

3.3 Relationships between ^{13}C enrichment and nutrient contents

The observed large variations in ^{13}C enrichment and nutrient contents among heterotrophic organs and between study sites give us an opportunity to examine whether ^{13}C enrichment in heterotrophic organs relative to leaves could be affected by their nutrient contents. We found that across the two study sites and across the heterotrophic

site. If the C_i/C_a ratios were the only factor controlling the leaf $\delta^{13}\text{C}$, then the Dengkou site should have consistently lower leaf $\delta^{13}\text{C}$ (higher C_i/C_a ratios increase discrimination against ^{13}C during photosynthesis). To the contrary, the Dengkou site had higher leaf $\delta^{13}\text{C}$ than the Minqin site in May, June and July; only in August and September, the difference in leaf $\delta^{13}\text{C}$ was consistent with the effect of the difference in C_i/C_a ratios between the two sites (although the difference in leaf $\delta^{13}\text{C}$ between the two sites were still not significant).

Interestingly, the leaf $\delta^{13}\text{C}$ in May and June was close to the biomass-weighted average of root $\delta^{13}\text{C}$ at both study sites, suggesting that the initial building materials of new leaves might have largely come from stored carbon in roots.

4 Discussion

A major finding from this study is that the nitrogen content of a heterotrophic organ, expressed either as a fraction of total dry biomass or as a ratio of C to N or N to P, is strongly correlated with this organ's enrichment in ^{13}C relative to leaves with higher nitrogen concentrations corresponding to larger enrichment. Because this relationship is caused by variations among heterotrophic organs and because normalizing the heterotrophic N content by the corresponding leaf N content did not improve or even worsened this relationship, the process responsible for it must reside inside the heterotrophic organs themselves. Further, this process must be mediated by nitrogen.

What nitrogen-mediated process could be responsible for the positive nitrogen- ^{13}C enrichment relationship among heterotrophic organs? A parsimonious candidate is the respiratory CO_2 refixation by PEP carboxylase. CO_2 from the respiration of heterotrophic organs may dissolve into water and be hydrated into HCO_3^- which is then fixed by PEP carboxylase into oxaloacetate. Both the dissolution of CO_2 into water and the fixation of HCO_3^- by PEP carboxylase discriminate slightly against ^{13}C . However, the hydration process fractionates strongly in favor of ^{13}C and causes it to concentrate

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in HCO_3^- . Consequently, the CO_2 refixation by PEP carboxylase has a net fractionation of 5.7‰ in favor of ^{13}C relative to the gaseous CO_2 (Farquhar, 1983; Melzer and O'Leary, 1987; Farquhar et al., 1989). Thus the respiratory CO_2 refixation by PEP carboxylase should lead to a depletion of ^{13}C in CO_2 escaped to outside compared to the original substrates for respiration while heterotrophic organs should be ^{13}C -enriched due to the addition of organic materials from PEP carboxylase activities. Previous studies have reported high PEP carboxylase activities in heterotrophic organs of a variety of C_3 plant species (Melzer and O'Leary, 1987; Berveiller and Damesin, 2008; Gessler et al., 2014). If increased N content increases the respiratory CO_2 refixation in heterotrophic organs, then it should also increase ^{13}C enrichment in these organs. Berveiller et al. (2010) showed that CO_2 refixation rates of *Fagus sylvatica* stems increased as stem nitrogen content increased, which provides a direct support for the hypothesis that CO_2 refixation by PEP carboxylase is a process responsible for our observed positive relationship between nitrogen and ^{13}C enrichment in heterotrophic organs.

Observed respiration rates of leaves, stems and roots tend to increase with increased nitrogen contents (Reich et al., 2008). This does not necessarily contradict the PEP carboxylase hypothesis suggested above. The actual respiration rates of these organs may increase so much with increased nitrogen contents that the increase cannot be offset by the increased refixation rates by PEP carboxylase. Consequently, the observed rates of CO_2 evolved from heterotrophic organs may still increase even though the refixation rates have increased with increased nitrogen contents.

The PEP carboxylase hypothesis does imply that the CO_2 escaped to outside from the heterotrophic organs are depleted in ^{13}C compared to the substrates utilized for respiration. As shown in the review of Ghashghaie and Badeck (2013), most isotopic studies on root respiration have found that CO_2 evolved from roots are depleted in ^{13}C compared with bulk root material, in contrast to leaf dark respiration which is generally enriched. For stem respiration, however, more contradictory results have been

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reported. Wingate et al. (2010) showed that CO_2 evolved from stems of *Pinus pinaster* was depleted in ^{13}C compared with the currently measured net CO_2 flux by photosynthetic branches or with the phloem water-soluble organic matter and wood cellulose. Gessler et al. (2009) also found that stem respiration of *Ricinus communis* was depleted in ^{13}C relative to the assumed respiratory substrates. In contrast to these studies, Damesin and Lelarge (2003) reported that stem respiration of *Fagus sylvatica* was enriched in ^{13}C compared with the total organic matter while Kodama et al. (2008) showed that CO_2 evolved from the stem of *Pinus sylvestris* had higher or similar $\delta^{13}\text{C}$ values compared to that of phloem exudate organic matter, depending on respiration rates. More studies are needed to determine whether carbon isotope fractionations of stem respiration depend on species, ages, or environments. Also, the dissolution and hydration of respiratory CO_2 may decouple in location from the fixation of HCO_3^- by PEP carboxylase if there is a strong transpiration stream in xylem, with isotopic consequences. For example, respiratory CO_2 can be dissolved and hydrated in roots and stems but the HCO_3^- molecules formed can be carried up in xylem transpiration streams (Aubrey and Teskey, 2009; Angert and Sherer, 2011; Bloemen et al., 2013, Trumbore et al., 2013) and fixed by PEP carboxylase in branches, which will serve to redistribute isotope signatures among different parts of the plant body.

Additional researches are also needed to determine whether there are other causes for the observed heterotrophic N– ^{13}C enrichment relationship. For example, if different organ N contents are associated with chemical compounds with different isotope signatures or different “fragmentation fractionation” (enzymatic reaction of substrate molecules with heterogeneous ^{13}C distribution, Tcherkez et al., 2004), one may expect organ N contents to be correlated with organ isotope signatures, potentially leading to the observed relationship. Another possibility to consider is that atmospheric $\delta^{13}\text{C}$ has been decreasing since the Industrial Revolution due to the emission of ^{13}C -depleted fossil CO_2 . If a heterotrophic organ contains a higher fraction of carbon with an old age, then its bulk $\delta^{13}\text{C}$ would be higher. Stems and roots should contain more old carbon than leaves do. We do not have data to quantify this possibility. However, a qualitative

reasoning led us to doubt that a general decreasing trend in atmospheric $\delta^{13}\text{C}$ can explain the observed heterotrophic N– ^{13}C enrichment relationship. Although we do not know the ages of the six nebkhas excavated, atmospheric N deposition has probably been increasing during the life time of these nebkhas. Therefore younger tissues should contain lower $\delta^{13}\text{C}$ and higher N, which would imply a negative N– ^{13}C enrichment relationship, opposite to what we observed. Therefore the positive heterotrophic N– ^{13}C enrichment relationship probably has a phytogetic, rather than an atmospheric, origin.

It is important to clarify that our suggestion that the process responsible for the positive heterotrophic N– ^{13}C enrichment relationship resides in heterotrophic organs does not imply that the cause(s) for heterotrophic enrichment of ^{13}C relative to leaves resides entirely in heterotrophic organs. In fact, to explain the full magnitude of the observed heterotrophic enrichment (2‰), about 35% ($100 \times 2/5.7$) of the carbon of heterotrophic organs has to have cycled through PEP carboxylase once, which appears to be surprisingly large for C_3 plants (Hobbie et al., 2003). Also, our finding that the $\delta^{13}\text{C}$ of leaves in the early growing season was close to the mean isotope ratio of roots but decreased as the season progressed indicates that processes inside leaves must also contribute to the overall isotope differences between leaves and heterotrophic organs if the leaf samples for reference are from middle to late growing seasons. The reference leaf samples in our calculation of $\Delta^{13}\text{C}_{\text{organ}}$ were from middle growing seasons (August). Therefore, the progressive seasonal depletion in foliar ^{13}C increased the magnitude of the obtained $\Delta^{13}\text{C}_{\text{organ}}$. Furthermore, any fractionations during phloem loading should also affect the overall autotrophic–heterotrophic isotope differences (Cernusak et al., 2009; Ghashghaie and Badeck, 2013). While these exo-heterotrophic processes may boost the overall magnitude of heterotrophic ^{13}C enrichment, they cannot explain its relationship with nitrogen content among heterotrophic organs.

It is likely that leaf nitrogen also plays an important role in determining ^{13}C enrichment in heterotrophic organs relative to leaves. We do not have enough leaf-level data to examine this issue in depth but findings from previous studies allow us to speculate

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about what this role might be. Sparks and Ehleringer (1997) and Livingston et al. (1999) found that leaf nitrogen content is positively correlated with leaf $\delta^{13}\text{C}$. This positive correlation exists probably because higher leaf nitrogen increases leaf photosynthetic capacity, which results in decreased C_i/C_a ratios and thus reduced discrimination against ^{13}C during photosynthesis. However, a positive relationship between leaf N and leaf $\delta^{13}\text{C}$ does not necessarily mean that higher leaf N will reduce the degree of heterotrophic enrichment in ^{13}C compared to leaves as heterotrophic organs use photosynthetic products from leaves. An interesting pathway for leaf N to influence heterotrophic ^{13}C enrichment may lie in the relationship between leaf N and dark respiration. It is known that leaf dark respiration scales with leaf nitrogen (Reich et al., 2008). It is also known that leaf dark respiration is enriched in ^{13}C , contrary to respirations of stems and roots (Ghashghaie and Badeck, 2013). Thus higher leaf nitrogen may actually increase the depletion of ^{13}C in leaves relative to heterotrophic organs. Consequently one may expect that nitrogen in autotrophic and heterotrophic organs of plants contributes to the isotope difference between these two types of organs in the same direction but through fundamentally different mechanisms.

5 Conclusions

We conclude that heterotrophic ^{13}C enrichment is affected jointly by fractionation processes occurring within heterotrophic organs and those within leaves. Processes taking place between heterotrophic organs and leaves (e.g., phloem loading if fractionation occurs) may also contribute to this phenomenon. A nitrogen mediated process, probably CO_2 refixation by PEP carboxylation, may be responsible for variations in ^{13}C enrichment within heterotrophic organs while processes within leaves or between leaves and heterotrophic organs may determine the overall magnitude of heterotrophic ^{13}C enrichment. We suggest that future efforts should focus on the roles of nitrogen and refixation of respiratory CO_2 by PEP carboxylase in carbon isotope fractionation processes both within leaves and within heterotrophic organs as well as in between

them. The findings of this study may have implications beyond isotope ecology. There has been a general lack of studies of refixation of respiratory CO₂ by PEP carboxylase in C₃ plant species. To our knowledge, no current terrestrial carbon cycle models consider this post-photosynthetic process. If PEP carboxylase can significantly affect carbon isotope compositions in heterotrophic organs of C₃ plant species, it may very well have strong influence on post-photosynthetic plant carbon budget and therefore terrestrial ecosystem carbon balance.

The Supplement related to this article is available online at doi:10.5194/bgd-11-13127-2014-supplement.

Acknowledgements. Field work, data acquisition and analyses were conducted at the Institute of Desertification Studies, Chinese Academy of Forestry with support in part by grants from the Forestry Public Welfare Scientific Research Funding (201104077), the Program of Introducing Advanced Forestry Technologies from Other Countries (2008-4-47), the Science and Technology Foundation (CAFYBB2007008) and the Lecture and Study Program for Outstanding Scholars from Home and Abroad of the Chinese Academy of Forestry, and the National Key Technology R&D Program of the Ministry of Science and Technology of China (2012BAD16B01). Data analyses and manuscript writing were partly carried out at Oak Ridge National Laboratory (ORNL) with support from US Department of Energy, Office of Science, Biological and Environmental Research Program, Climate and Environmental Sciences Division. It also received support from the ORNL's Laboratory Directed Research and Development Program. ORNL is managed by UT-Battelle, LLC, for the US Department of Energy under contract DE-AC05-00OR22725.

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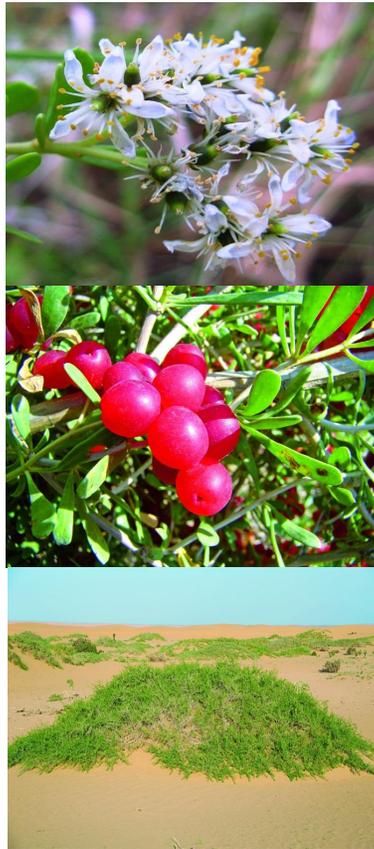


Figure 1. Flowers (top, 10 June 2009, Minqin), fruits (middle, 18 July 2009, Minqin) and nebkha (bottom, 3 August 2010, Dengkou) of *Nitraria tangutorum* Bobrov. Pictures courtesy of Jianmin Chu, Research Institute of Forestry, Chinese Academy of Forestry.

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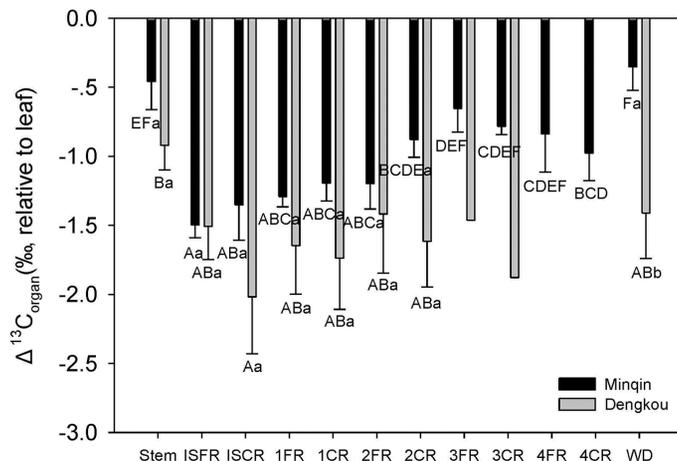


Figure 2. The difference in carbon isotope compositions between leaves and heterotrophic organs of *Nitraria tangutorum* Bobrov, which is measured by $\Delta^{13}\text{C}_{\text{organ}}$ in Eq. (1) and averaged across the nebkhas excavated at the same study site (Dengkou or Minqin). Negative values indicate ^{13}C enrichment in heterotrophic organs compared to leaves. Upper-case letters denote ANOVA results within a study site (i.e., comparing $\Delta^{13}\text{C}_{\text{organ}}$ among different organs at the same site) and lower case letters between the two sites (i.e., comparing $\Delta^{13}\text{C}_{\text{organ}}$ of the same organ between the two sites). IS stands for in-sand, FR fine root and CR coarse root. 1, 2, 3 and 4 in front of FR or CR stand for 0–20, 20–40, 40–60 and 60–80 cm below the plains on which nebkhas rest. Woody debris (WD) from dead ramets is also included in the figure. No ANOVA results for 3FR and 3CR at the Dengkou site as there was only one nebkha having roots between 40 to 60 cm. No roots were found below 60 cm at the Dengkou site.

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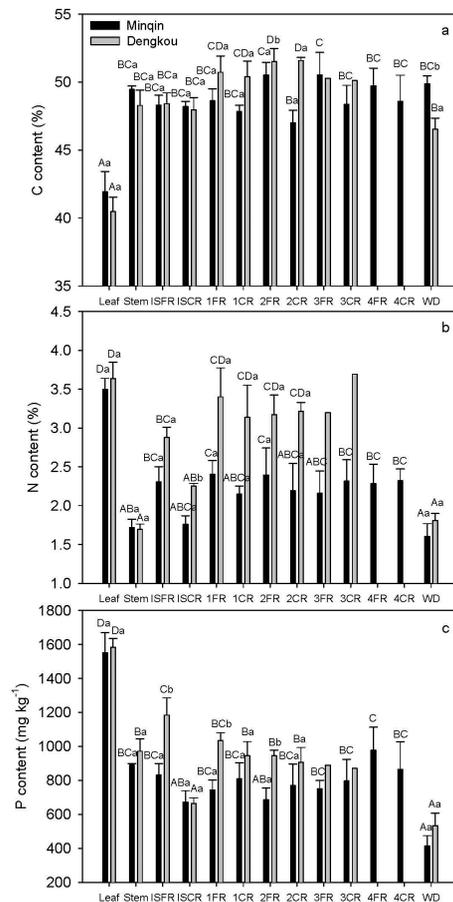


Figure 3. Carbon (a), nitrogen (b) and phosphorous content (c) of different organs of *Nitraria tangutorum* Bobrov, at the Dengkou and Minqin study sites. Symbols and letters denoting ANOVA results are explained in Fig. 2.

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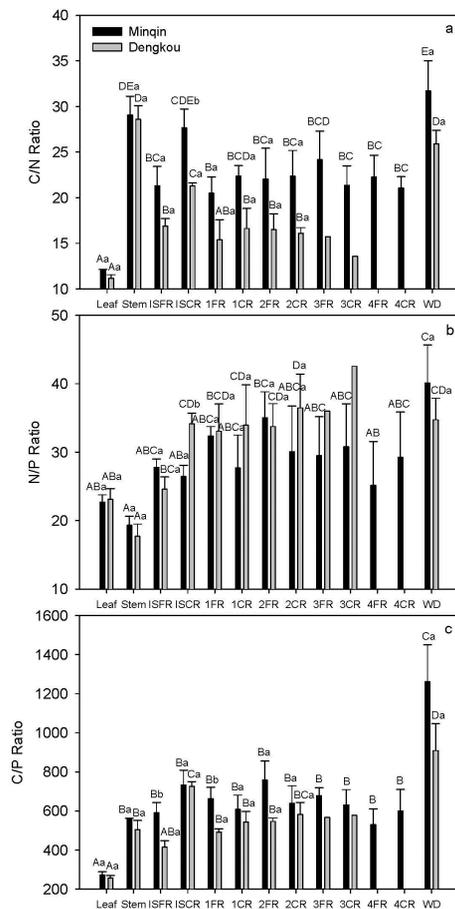


Figure 4. Carbon to nitrogen (a), nitrogen to phosphorous (b) and carbon to phosphorous ratios (c) of different organs of *Nitraria tangutorum* Bobrov, at the Dengkou and Minqin study sites. Symbols and letters denoting ANOVA results are explained in Fig. 2.

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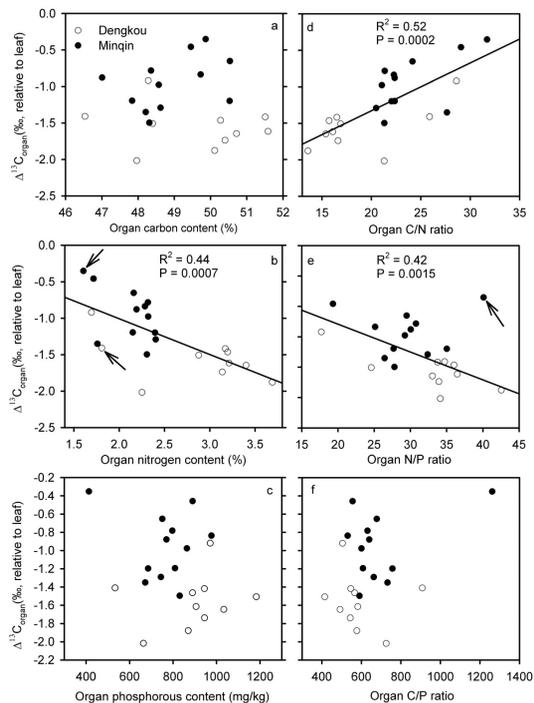


Figure 5. Nutrient dependence of the difference in carbon isotope compositions between leaves and heterotrophic organs of *Nitraria tangutorum* Bobrov, which is measured by $\Delta^{13}\text{C}_{\text{organ}}$ in Eq. (1) and averaged across the nekhas excavated at the same study site. Negative values indicate ^{13}C enrichment in heterotrophic organs compared to leaves. Changes of $\Delta^{13}\text{C}_{\text{organ}}$ as a function of organ contents of carbon (a), nitrogen (b) and phosphorous (c) and of organ ratios of carbon to nitrogen (d), nitrogen to phosphorous (e), and carbon to phosphorus (f). The two arrows in (b) indicate values for woody debris from dead ramets at each study site while in (d) indicates an outlier caused by measurements in phosphorous content (see the outlier in c and f).

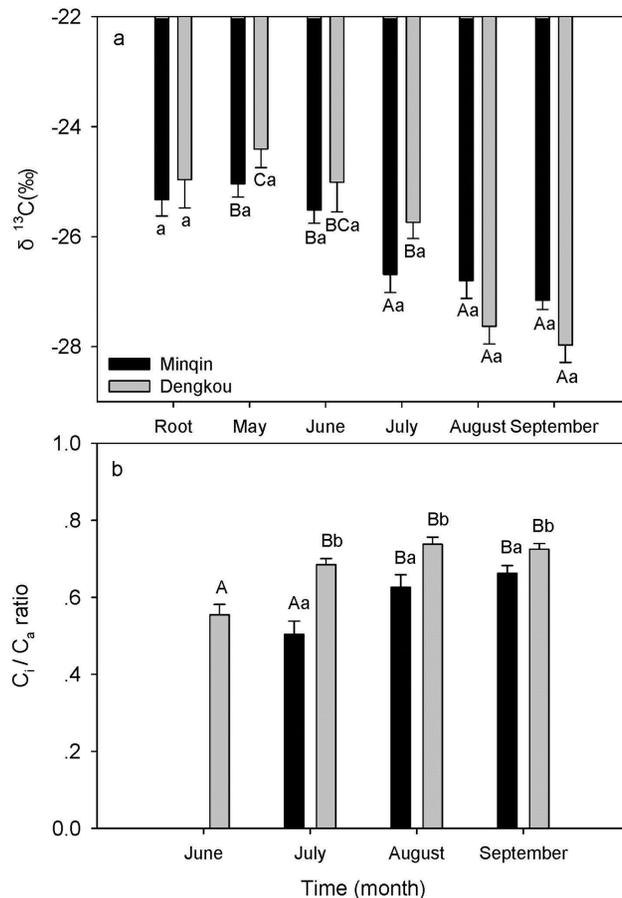


Figure 6. Seasonal changes in the ratios of leaf carbon isotopes (**a**) and intercellular (C_i) to ambient (C_a) CO_2 concentrations of *Nitraria tangutorum* Bobrov at the Dengkou and Minqin study sites. For comparison, the biomass-averaged isotope ratios of roots from the excavated nebkhas are also shown in (**a**).