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Nitrogen nutrition, carbon accumulation and $\delta^{13}\text{C}$ of *Cyclopia* and *Aspalathus* species in different settings of the Cape fynbos, South Africa

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Abstract

Aims

Cyclopia and *Aspalathus* are legumes harvested for production of Honeybush and Rooibos tea, respectively. Farmers grow these species from either seeds or cuttings over several years with continuous annual harvesting. The aims of this study were to assess the effect of plant age, plant species, toposequence, planting material and farmer practice on nitrogen (N) nutrition and water-use efficiency of two *Cyclopia* and *Aspalathus* species in the Cape fynbos.

Methods

The study was conducted using plants from Koksrivier farm located near Gansbaai (33° S 18° E, 39 m.a.s.l), and at Kanetberg farm near Barrydale (33° S 21° E, 830 m.a.s.l). The ¹⁵N natural abundance technique was used to determine N₂ fixation, carbon (C) assimilation and $\delta^{13}\text{C}$ in shoot of *Cyclopia* and *Aspalathus* species.

Important Findings

Older tea plantations of *C. genistoides* and *C. subternata* derived more N from fixation and exhibited greater water-use efficiency than younger plants. At Koksrivier, *Aspalathus caledonensis* and *A. aspalathoides* showed greater water-use efficiency and derived more N from fixation than *Cyclopia genistoides*. Annual harvesting of *C. genistoides* decreased N₂ fixation. At Kanetberg, *C. subternata* plants on the upper and middle slopes derived more N from atmospheric fixation than those on the lower slope. *C. subternata* plants grown from seedlings recorded greater %Ndfa than cuttings. N₂ fixation and water-use efficiency of *Cyclopia* was affected by age, slope and planting material. Further, symbiotic N nutrition and water-use efficiency of *Cyclopia* and *Aspalathus* were related.

Keywords: *Cyclopia subternata*, *Cyclopia genistoides*, Koksrivier, Kanetberg, fynbos

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INTRODUCTION

Species of *Cyclopia* Vent. (family: Leguminosae, tribe: Podalyrieae) and *Aspalathus* (family: Leguminosae, tribe: Crotalariae) are endemic perennial shrub legumes of South Africa's Cape fynbos, which is part of the Cape Floristic Region and a biodiversity hotspot. The soils supporting growth of many plant species in the fynbos are generally sandy, acidic and nutrient-poor, especially in N and P (Goldblatt and Manning 2002). The genus *Cyclopia* has 24 species, of which *C.*

genistoides, *C. subternata* and *C. longifolia* are used as the source of Honeybush tea, a herbal beverage that contributes about 17 million Rands of the South African currency annually to the South African economy (DAFF, Department of Agriculture, Forestry and Fisheries 2013). The genus *Aspalathus* consists of 281 species (Boatwright and Cupido 2011), and is the largest in the fynbos. Of these, only *Aspalathus linearis* subsp. *linearis* is used for making Rooibos tea, another herbal beverage that contributes about R500 million to the South African economy (Joubert and De Beer 2011; <http://www.busrep.co.za>).

Along with water, N and P are the most important mineral nutrients limiting plant growth in both natural and agricultural ecosystems (Graham and Vance 2003; Sinclair and Rufty 2012). Their acquisition is therefore crucial for plant development and functioning. With legumes, N can be sourced from either the soil, symbiotic N_2 fixation, or both. In the fynbos, *Cyclopia* and *Aspalathus* species meet their N requirements from biological fixation of atmospheric N_2 . Most *Cyclopia* species and *Aspalathus linearis* can obtain over 100 kg N ha^{-1} from symbiotic N_2 fixation for their N nutrition (Lötter *et al.* 2014; Moufhe and Dakora 1999; Spriggs and Dakora 2009). Thus, the tapping of fixed-N from symbiotic legumes can significantly reduce the agricultural use of N fertilizers, which are not only costly but also contaminate the environment.

Many factors can affect the ^{15}N isotopic composition of plants. These include plant species or genotypes (Abi-Ghanem *et al.* 2013; Craine *et al.* 2015; Robinson 2001; Reed *et al.* 2008), elevation, toposequence (Cusack *et al.* 2009; Stewart *et al.* 2014; Van der Water *et al.* 2002), plant age (Evans 2001; Moufhe and Dakora 1999) and successional stage (Wang *et al.* 2007). The ratio between the two stable isotopes of N (i.e. ^{15}N and ^{14}N) can differ between ecosystems as a result of isotopic fractionation during N cycling (Bai *et al.* 2009; Robinson 2001). Where N_2 -fixing legumes are concerned, ^{15}N composition and N_2 fixation can vary widely across landscapes, topographies and agroecosystems (Bedard-Haughn *et al.* 2003; Walley *et al.* 2001). N losses via volatilization into the atmosphere from these systems can be low in symbiotic legumes due to reduced rates of chemical conversions (Vance 2001).

The ^{13}C and ^{15}N of higher plants are considered as potential predictors of plant adaptation to a particular environment (Peuke *et al.* 2006; Wang *et al.* 2010). During photosynthesis, plants tend to discriminate against the heavier ^{13}C isotope of carbon in favour of the lighter ^{12}C isotope, resulting in a lower ratio of $^{13}\text{C}/^{12}\text{C}$ relative to the atmosphere (Condon *et al.* 2004). This discrimination is partly due to the slow diffusion of $^{13}\text{CO}_2$ from the atmosphere to the site of carboxylation relative to $^{12}\text{CO}_2$, as well as to the poor reactivity of the heavier $^{13}\text{CO}_2$ with the primary carboxylating enzyme, ribulose-1, 5-bisphosphate carboxylase or Rubisco (Pons *et al.* 2009). Carbon isotope discrimination has been shown to vary with temperature and soil moisture, root growth, soil type (Wittmer *et al.* 2008) and altitude (Casper *et al.* 2012; Sparks and Ehleringer 1997).

Honeybush tea farmers with fields located along the Mountain fynbos, cultivate the *Cyclopia* tea on mountainous slopes. Topography affects symbiotic nitrogen fixation and shoot $\delta^{13}\text{C}$ (or water-use efficiency) by legumes as a result of its heterogeneity in soil moisture, nutrient availability, soil C and N contents (Liu *et al.* 2013; Stewart *et al.* 2014; Yang *et al.* 2013).

For most plants, the efficiency of roots to absorb nutrients declines as plants grow older, leading to differences in shoot nutrient accumulation between young and old plants (Comas *et al.* 2010; Eissenstat and Volder 2010; Volder *et al.* 2005).

Furthermore, roots of younger plants generally have larger nutrient uptake capacities than roots of older plants, and thus the proportion of young roots in the whole root system can strongly affect overall plant nutrient uptake. Knowledge of the dependence of *Cyclopia* on N_2 fixation for its N nutrition as the species grows older is of great importance and has implications for understanding nutrient acquisition, competitive ability and optimal lifespan. This is particularly crucial because *Cyclopia* is grown commercially without supplementation with mineral fertilizers, and this has the risk of depleting nutrients in what is already a low-nutrient environment (Joubert *et al.* 2010; Maseko and Dakora 2013a).

In cultivating Honeybush tea, farmers in the Cape of South Africa grow the tea over successive years using both seeds and cuttings as planting material. However, farmers prefer to use seeds given the cost of establishing cuttings. Plants established from cuttings show better growth, development and tea yield compared to plants raised from seeds (Maseko and Dakora 2015) because in establishing cuttings, there are nutrients and auxins added to the growing media (Mbangcolo *et al.* 2013). In assessing the P nutrition of *C. subternata* established from cuttings at Kanetberg, Maseko and Dakora (2013a) reported greater rhizosphere phosphatase activity, plant available P and organic P in soils supporting growth of these plants, which led to greater shoot P compared to their counterparts raised from seeds. There is however no information in the literature on whether field-grown *C. subternata* established from cuttings or seeds exhibit differences in N_2 fixation, C concentration and water-use efficiency. Knowledge on possible variation in these parameters is crucial as it would give better understanding on the adaptation of plants from either source of planting material.

The main species of *Cyclopia* currently utilized as a source of tea include *Cyclopia genistoides*, *C. intermedia*, *C. longifolia*, *C. sessilifolia* and *C. subternata*, which are endemic mainly to the mountainous regions and coastal plains of the Western and Eastern Cape Provinces of South Africa. With the background on endemism and commercial species of *Cyclopia*, this study was therefore conducted at Koksrivier using plantations of *C. genistoides* which are located along the coastal fynbos, and at Kanetberg using plantations of *C. subternata* located along the mountain fynbos. The effect that various topographic positions, plant age and planting materials have on N_2 fixation, C concentration and water-use efficiency of *Cyclopia* and *Aspalathus* has not been reported. The aims of this study were: (i) to assess the effect of plant age and farmers' practice (harvested versus unharvested annually) on N nutrition and water-use efficiency of *Cyclopia genistoides* using ^{15}N and ^{13}C natural abundance at Koksrivier, (ii) to compare the symbiotic N nutrition and water-use efficiency of *Aspalathus caledonensis*, *A. aspalathoides* and *C. genistoides* co-occurring in a plantation at Koksrivier and (iii) to evaluate the effect of plant age, soil toposequence and type of planting material (seedling versus cutting) on N nutrition, C concentration and water-use efficiency of *Cyclopia subternata* plants at Kanetberg using ^{15}N and ^{13}C natural abundance.

MATERIALS AND METHODS

Description of study sites

The two study sites where plant sampling was done include Koksrivier and Kanetberg. Koksrivier is a farmer's field located near Gansbaai (33° S 18° E, 39 m.a.s.l.), and receives about 661 mm rainfall annually during the winter from May to September each year. It is a commercial farm of *Cyclopia genistoides* plants grown mainly from seeds. These commercial tea plants at Koksrivier were grown in natural settings on sandy soils without ploughing, fertilization or irrigation.

Kanetberg (33° S 21° E, 830 m.a.s.l) is also a commercial farm which contained mainly *Cyclopia subternata* and some *C. longifolia* plants. These *Cyclopia* species were established from cuttings taken from superior mother plants and grown without fertilization. Kanetberg receives about 564 mm rainfall annually during the winter months (May to September each year). The tea plantation was located at the Kanetberg mountains which are steep and rocky, and characterised by sandy to sandy-loam soils.

Plant and soil sampling/processing

The plants were sampled from two sites, Koksrivier and Kanetberg, in the Cape fynbos of South Africa. Young shoots of *Cyclopia genistoides* were collected from two farmers' fields, one with 10-year old plants and another with 2-year old plants at Koksrivier. Similarly, young shoots of *C. subternata* were sampled from tea plantations at Kanetberg with plants of various ages (namely 8, 7, 6, 5, 4 and 2 years old). The effect of farmer practice of harvesting plant shoots annually for tea was evaluated for N and C metabolism in *C. genistoides* at Koksrivier.

Natural stands of *Aspalathus caledonensis* and *A. aspalathoides* co-existing with *C. genistoides* in the plantation with 2-year old plants at Koksrivier were also sampled and analyzed for comparison with *C. genistoides*.

At Kanetberg, *C. subternata* shoots were collected from plants grown along a toposequence in order to assess the effect of upper, middle and lower slopes on the ¹⁵N and ¹³C composition of plants.

To estimate symbiotic N nutrition, shoots of non-fixing shrub species were sampled concurrently as the legumes from the tea plantations studied. All plant samples (both legumes and non-legumes) were put in labelled paper bags, taken to the laboratory and oven-dried at 60°C for 72 h. The samples were weighed and ground (Hammer mill, Wirsam Scientific and Precision Equipment Pty Ltd, Johannesburg, South Africa) to a fine powder (0.85 mm) for ¹⁵N and ¹³C isotope analysis using mass spectrometry.

At Koksrivier, samples of non-rhizosphere bulk soil were collected from *Cyclopia* fields containing 2-year-old and 10-year-old plants. Bulk soil samples were also collected from the Kanetberg farm. Here, separate soil samples were cored at a depth of 0–30 cm from fields with *C. subternata* grown from seeds and cuttings, and from 8-year-old plants. In the laboratory, the soil samples were air-dried at room temperature, sieved (2.0 mm) and analysed for pH (CaCl₂), total N, P, Fe and K.

Analysis of ¹⁵N/¹⁴N isotopic ratio

To determine the ¹⁵N/¹⁴N ratio, a subsample of finely ground plant material was weighed into tin capsules (3.0 mg of *Cyclopia*, *Aspalathus* or reference plants) and analyzed using a Carlo Erba NA1500 elemental analyzer (Fisons Instruments SpA, Strada, Rivoltana, Italy) coupled to a Finan MAT252 mass spectrometer (Finnigan, MAT CombH, Bremen, Germany) via a Conflo II open-split device.

The isotopic composition of ¹⁵N was measured as (Junk and Svec 1958; Mariotti *et al.* 1981):

$$\delta^{15}\text{N} = \frac{\left(\frac{^{15}\text{N}}{^{14}\text{N}}\right)_{\text{sample}} - \left(\frac{^{15}\text{N}}{^{14}\text{N}}\right)_{\text{atm}}}{\left(\frac{^{15}\text{N}}{^{14}\text{N}}\right)_{\text{atm}}} \times 1000$$

where the $\delta^{15}\text{N}$ value is the ¹⁵N natural abundance of plant sample, expressed in a relative delta (δ) notation, which is the percent atom excess (‰) deviation of the sample from atmospheric N₂ (0.3667 atom % ¹⁵N).

The percent N derived from N₂ fixation (%Ndfa) was determined using the equation (Shearer and Kohl 1986):

$$\% \text{Ndfa} = \left[\frac{\delta^{15}\text{N}_{\text{ref}} - \delta^{15}\text{N}_{\text{leg}}}{\delta^{15}\text{N}_{\text{ref}} - \text{B}} \right] \times 100$$

Where $\delta^{15}\text{N}_{\text{ref}}$ is the mean ¹⁵N natural abundance of the reference plants, $\delta^{15}\text{N}_{\text{leg}}$ is the ¹⁵N natural abundance of legume, and the B value is the ¹⁵N natural abundance of test legume solely dependent on N₂ fixation for its N nutrition. The B value incorporates the isotopic fractionation associated with N₂ fixation and replaces the value of atmospheric N₂ (Shearer and Kohl 1986). The B value used for estimating %Ndfa in *Cyclopia* was –1.71‰ (Spriggs and Dakora 2009), whilst that used in *Aspalathus* was –2.00‰ (Moufhe and Dakora 1999).

Analysis of ¹³C/¹²C isotopic ratio

The isotopic ratios of C were obtained from the same runs of plant samples used for ¹⁵N/¹⁴N composition. Pee Dee Belemite (PDB) limestone formation from the Cretaceous marine fossil, *Belemnitella americana*, found in South Carolina, was included as a standard after every 5–10 runs. The ratio of ¹³C/¹²C in each shoot sample was used to calculate the ¹³C natural abundance (or $\delta^{13}\text{C}$) as (Farquhar *et al.* 1989):

$$\delta^{13}\text{C} = \left[\frac{\left(\frac{^{13}\text{C}}{^{12}\text{C}}\right)_{\text{sample}}}{\left(\frac{^{13}\text{C}}{^{12}\text{C}}\right)_{\text{standard}}} - 1 \right] \times 1000$$

where $\delta^{13}\text{C}$ is the mean ¹³C natural abundance of the sample in parts per mill (‰), $\left(\frac{^{13}\text{C}}{^{12}\text{C}}\right)_{\text{sample}}$ is the isotopic ratio of plant sample, and $\left(\frac{^{13}\text{C}}{^{12}\text{C}}\right)_{\text{standard}}$ is the isotopic ratio of PDB, a universally accepted standard (Craig 1957).

RESULTS

Chemical properties of bulk soil samples

Bulk soil collected from the 10-year old *Cyclopia genistoides* plantation at Koksrivier had a pH 4.48, and contained

3.6 mg kg⁻¹ P, 46.4 mg kg⁻¹ Fe and 31.4 mg kg⁻¹ K, whilst soil from the 2-year old *C. genistoides* field had pH 4.25, and contained 3.8 mg kg⁻¹ P, 24.5 mg kg⁻¹ Fe and 10.8 mg kg⁻¹ K. Pooled bulk soil from the Koksrivier farm contained 6.4 mg kg⁻¹ N. At Kanetberg, bulk soil collected from the plantation of *Cyclopia subternata* grown from seeds had pH 4.20 and contained 7.8 mg kg⁻¹ P, 97.9 mg kg⁻¹ Fe and 44.8 mg kg⁻¹ K, while the bulk soil from the plantation of *Cyclopia subternata* raised from cuttings recorded pH 4.20, and contained 8.8 mg kg⁻¹ P, 73.6 mg kg⁻¹ Fe and 75.8 mg kg⁻¹ K. The farmer's field with 8-year old *C. subternata* plants at Kanetberg also had pH 4.16, and contained 14.8 mg kg⁻¹ P, 117.6 mg kg⁻¹ Fe and 58.4 mg kg⁻¹ K. The bulk soil collected from Kanetberg farm with 8-year old plants had pH 4.18, and contained 19.6 mg kg⁻¹ N, 10.5 mg kg⁻¹ P, 96.3 mg kg⁻¹ Fe and 59.7 mg kg⁻¹ K.

$\delta^{15}\text{N}$ of reference plants

Where more than one reference plant was sampled at a site, the combined mean $\delta^{15}\text{N}$ value of these plants was used to estimate %Ndfa of the legumes. At Koksrivier, e.g. an average reference plant $\delta^{15}\text{N}$ value of +0.73‰ was used to estimate the %Ndfa of 10- and 2-year old *C. genistoides* plants, as well as the %Ndfa of *Aspalathus caledonensis* and *A. aspalathoides* sampled from the same site. At Kanetberg, the combined mean $\delta^{15}\text{N}$ value used for estimating %Ndfa of the 8, 7, 6, 5, 4 and 2-year old *C. subternata* plants was +1.80‰ (Table 1).

Effect of soil toposequence, plant species, plant age, planting material, and harvesting frequency on shoot N concentration, $\delta^{15}\text{N}$ and %Ndfa of legumes

Isotopic analysis of shoots revealed differences between *C. genistoides* plants sampled from 10 and 2-year old plantations at Koksrivier. Although N concentrations were similar, the $\delta^{15}\text{N}$ was significantly lower in 10-year old *C. genistoides* plants. As a result, the %Ndfa was greater in those plants (Table 2). Shoot regrowth of plants that were harvested annually at Koksrivier showed higher N concentration than those which were never harvested annually (Table 3). The $\delta^{15}\text{N}$ values were also greater in the annually harvested than unharvested *C. genistoides* plants (Table 3). As a result, %Ndfa was lower in the annually harvested plants (Table 3).

A comparison of symbiotic performance between *C. genistoides*, *A. caledonensis* and *A. aspalathoides* sampled from the same plantation at Koksrivier showed marked variation between species. Although N concentration was significantly higher in *C. genistoides*, followed by *A. caledonensis* and *A. aspalathoides*, the $\delta^{15}\text{N}$ value was much lower in *A. aspalathoides*, followed by *A. caledonensis* and *C. genistoides*. As a result, the %Ndfa was markedly higher in *A. aspalathoides*, followed by *A. caledonensis* and *C. genistoides* (Table 4).

Isotopic analysis of *C. subternata* plants aged 8, 7, 6, 5, 4, and 2 years at Kanetberg revealed significant differences in symbiotic parameters. Although N concentrations were similar for *C. subternata* plants of all ages (except those aged 6 years),

the $\delta^{15}\text{N}$ values were lowest for 4- and 7-year old plants, and highest for 8, 5 and 2-year old plants (Table 5). As a result, the %Ndfa was much greater for plants aged 4 years, and lowest for those that were 2 years old (Table 5).

Evaluating the effect of soil toposequence on symbiotic N nutrition of *C. subternata* grown at Kanetberg showed that plants from the middle slope had the lowest $\delta^{15}\text{N}$ value, followed by the upper slope (Table 6). As a result, the %Ndfa was also much higher for the middle slope, followed by the upper slope, and least for the lower slope (Table 6).

The effect of planting material on the symbiotic performance of *C. subternata* was also assessed at Kanetberg. The data showed that, although cuttings exhibited significantly greater shoot N concentration, the $\delta^{15}\text{N}$ values were much lower in plants established from seed than cuttings. As a result, the %Ndfa was significantly higher in plants raised from seedlings than cuttings (Table 7).

Effect of soil toposequence, plant species, plant age, planting material and harvesting frequency on C accumulation, C/N ratio and $\delta^{13}\text{C}$ of *Cyclopia* and *Aspalathus* species

Cyclopia genistoides shoots from plants aged 10 and 2 years at Koksrivier showed greater C concentration in the younger plants but greater water-use efficiency in the older plants (Table 2). However, there was no effect of plant age on C/N ratio. Shoot re-growth of *C. genistoides* plants that were harvested annually for tea also showed higher C concentration compared to those that were not harvested yearly (Table 3). The $\delta^{13}\text{C}$ values of the unharvested *C. genistoides* plants were significantly higher than their annually harvested counterparts (Table 3). The C/N ratio was similarly greater for the unharvested tea plants (Table 3).

Isotopic analysis of *C. genistoides* and two *Aspalathus* species (*A. caledonensis* and *A. aspalathoides*) from Koksrivier revealed greater C accumulation in the tea legume compared to the two *Aspalathus* species (Table 4). However, the C/N ratio was much greater in *A. aspalathoides* (Table 4). The two *Aspalathus* species also exhibited less negative $\delta^{13}\text{C}$ values (or greater water-use efficiency) relative to *C. genistoides* (Table 4). A comparison of *C. subternata* plants of different ages sampled from Kanetberg revealed slight but significant differences in C concentration. The $\delta^{13}\text{C}$ was lowest in the youngest plants and much greater in their older counterparts. The C/N ratios were also lower in younger plants and in 6-year-old plants (Table 5).

At Kanetberg, soil toposequence also had an effect on C accumulation in *C. subternata* plants. As shown in Table 6, shoot C concentration was slightly higher in plants from the lower toposequence relative to middle or upper toposequence. There were however no differences in $\delta^{13}\text{C}$ and C/N ratio of plants from the different toposequences. Similarly, the type of planting material used (seed versus cuttings) had no effect on the C concentration and $\delta^{13}\text{C}$ of *C. subternata* planted at Kanetberg (Table 7). The C/N ratio was however greater in shoots of plants propagated from seed than cuttings (Table 7).

Table 1: shoot $\delta^{15}\text{N}$ (‰) values of reference plants sampled from Koksrivier and Kanetberg farms

Farm name	Year	Family	Plant species	Shoot $\delta^{15}\text{N}$	Mean $\delta^{15}\text{N}$	
Koksrivier	10	Proteaceae	<i>Leucadendron strictum</i>	0.73		
	2	Poaceae	<i>Pentaschistis curvifolia</i>	0.72	0.73	
Kanetberg	8	Proteaceae	<i>Leucadendron strictum</i>	2.77		
		Proteaceae	<i>Leucadendron strictum</i>	2.58		
		Proteaceae	<i>Leucadendron strictum</i>	2.73		
		Proteaceae	<i>Leucadendron strictum</i>	2.63		
		Proteaceae	<i>Leucadendron strictum</i>	2.31		
		Proteaceae	<i>Leucadendron strictum</i>	3.35		
	7	Proteaceae	<i>Leucadendron strictum</i>	3.99	2.91	
		Proteaceae	<i>Leucadendron strictum</i>	0.55		
	6	Proteaceae	<i>Leucadendron strictum</i>	0.27	0.41	
		Proteaceae	<i>Leucadendron strictum</i>	1.17		
		5 cuttings	Proteaceae	<i>Leucadendron strictum</i>	2.30	
			Proteaceae	<i>Protea grandiceps</i>	2.90	
		5 seedlings	Restionaceae	<i>Eligia thyrsoidea</i>	0.96	
			Restionaceae	<i>Eligia thyrsoidea</i>	1.06	1.81
Proteaceae	<i>Protea longifolia</i>		2.68			
Proteaceae	<i>Leucadendron strictum</i>		1.79			
	Proteaceae	<i>Leucadendron strictum</i>	2.43			
	Proteaceae	<i>Leucadendron strictum</i>	1.35	2.06		

Table 2: a comparison of N and C nutrition, and $\delta^{13}\text{C}$ of 2- and 10-year old *Cyclopia genistoides* plants sampled at Koksrivier ($n = 10$), near Gansbaai, South Africa, in 2007

Plant age	N concentration	$\delta^{15}\text{N}$	Ndfa	C concentration	$\delta^{13}\text{C}$	C/N ratio
Years	%	‰	%	%	‰	g/g
10	1.72 ± 0.04a	-1.30 ± 0.08b	83.29 ± 3.20a	50.89 ± 0.17b	-26.87 ± 0.11a	29.62 ± 0.56a
2	1.81 ± 0.05a	-1.07 ± 0.05a	73.71 ± 2.00b	51.49 ± 0.23a	-27.75 ± 0.07b	28.57 ± 0.75a
F-Statistics	2.34 ^{ns}	6.44 ^{**}	6.58 ^{**}	4.60 ^{**}	48.50 ^{***}	1.25 ^{ns}

Values with dissimilar letters in the same column are significantly different at $P \leq 0.05$.

Correlation analysis

The $\delta^{15}\text{N}$ of *Cyclopia subternata* shoots established from cuttings aged 5 years correlated positively ($r = 0.546$, $P = 0.053$) with its $\delta^{13}\text{C}$ at Kanetberg. Mean $\delta^{15}\text{N}$ also correlated positively with annual rainfall at Kanetberg ($r = 0.897$, $P = 0.039$) and Koksrivier ($r = 0.883$, $P = 0.047$).

DISCUSSION

Nitrogen nutrition in native legumes of the Cape fynbos was assessed using the ^{15}N natural abundance technique. The reference plant used in this study at different sites was *Leucadendron strictum* belonging to the family Proteaceae. *Pentaschistis curvifolia* (Poaceae) and *Elegia thyrsoidea* (Restionaceae) were also included as reference plants at the two sites. Relative to the test legumes, these reference plants provided much greater $\delta^{15}\text{N}$ values that enabled good estimates of percent N derived

from atmospheric N_2 fixation (Tables 2–7). Independent of the experimental treatments assessed in this study, *C. subternata* obtained 82–93% of its N nutrition from symbiotic fixation at Kanetberg, *C. genistoides* 74–83% at Koksrivier, *A. caledonensis* and *A. aspalathoides* 86% and 91%, respectively, at Koksrivier. Clearly, these levels of symbiotic N nutrition by the test fynbos legumes are quite high and suggest functional adaptation to the very low N concentrations of the Cape fynbos soils, estimated to be 0.001–0.002% N or (1–2 mg N g⁻¹ soil, Cramer 2010).

Whether at Koksrivier or Kanetberg, symbiotic N nutrition of both *C. genistoides* and *C. subternata* were altered by plant age. As found for *A. linearis* (Moufhe and Dakora 1999), plants from the older tea plantations derived more N from fixation than their younger counterparts, with the exception of *C. subternata* plants aged 4 years (Tables 2 and 5). Data obtained by Huss-Danell *et al.* (2007) also showed increased

Table 3: effect of tea harvesting on N and C nutrition, and $\delta^{13}\text{C}$, of developing pods of *Cyclopia genistoides* planted at Koksrivier in 1997 ($n = 10$)

Farmers' practice	N concentration %	$\delta^{15}\text{N}$ ‰	Ndfa %	C concentration %	$\delta^{13}\text{C}$ ‰	C/N ratio g/g
Harvested	1.78±0.1a	-1.11±0.0a	75.37±1.2b	51.34±0.2a	-27.70±0.1b	29.04±1.0b
Unharvested	1.38±0.0b	-1.29±0.0b	82.80±1.5a	49.30±0.1b	-25.69±0.1a	36.03±0.8a
F-Statistics	16.91***	15.00***	15.00***	89.50***	155.50***	29.37***

Values with dissimilar letters in the same column are significantly different at $P \leq 0.05$.

Table 4: N and C nutrition, and $\delta^{13}\text{C}$ of *Cyclopia* and *Aspalathus* species sampled from Koksrivier farm established in 2005 ($n = 10$)

Legume species	N concentration %	$\delta^{15}\text{N}$ ‰	Ndfa %	C concentration %	$\delta^{13}\text{C}$ ‰	C/N ratio g/g
<i>Cyclopia genistoides</i>	1.81±0.1a	-1.07±0.1a	73.71±2.0b	51.50±0.2a	-27.67±0.1c	28.57±0.8b
<i>Aspalathus caledonensis</i>	1.65±0.1b	-1.62±0.1b	86.24±2.7a	47.19±0.6c	-26.12±0.1a	28.91±1.0b
<i>Aspalathus aspalathoides</i>	1.40±0.0c	-1.76±0.1b	91.06±3.0a	49.43±0.4b	-26.76±0.1b	35.36±0.6a
F-Statistics	23.50***	28.24***	12.16***	28.50***	90.50***	23.44***

Values with dissimilar letters in the same column are significantly different at $P \leq 0.05$.

Table 5: N and C nutrition, and $\delta^{13}\text{C}$ of *Cyclopia subternata* plants of different ages sampled from Kanetberg ($n = 10$)

Plant age Years	N concentration %	$\delta^{15}\text{N}$ ‰	Ndfa %	C concentration %	$\delta^{13}\text{C}$ ‰	C/N ratio g/g
8	1.77±0.0b	-1.15±0.1a	83.92±1.5c	49.79±0.3b	-27.82±0.1b	28.06±0.3a
7	1.84±0.0b	-1.44±0.0c	92.40±1.2a	50.78±0.3a	-27.76±0.1b	27.67±0.5a
6	2.94±0.4a	-1.29±0.1b	88.17±1.7b	50.63±0.3a	-27.67±0.1b	20.12±2.6c
5	1.94±0.0b	-1.14±0.1a	83.96±1.6c	50.38±0.1b	-27.89±0.1b	26.12±0.6b
4	1.65±0.0b	-1.47±0.0c	93.27±1.2a	49.61±0.1b	-27.42±0.1a	30.09±0.4a
2	2.04±0.0b	-1.08±0.0a	82.12±1.0c	49.98±0.2b	-28.16±0.1c	25.56±0.3b
F-Statistics	8.99***	11.57***	11.57***	5.20***	7.70***	9.78***

Values with dissimilar letters in the same column are significantly different at $P \leq 0.05$.

Table 6: N and C nutrition, and $\delta^{13}\text{C}$ of 5-year old *Cyclopia subternata* plants sampled at Kanetberg along a soil toposequence ($n = 10$)

Topography	N concentration %	$\delta^{15}\text{N}$ ‰	Ndfa %	C concentration %	$\delta^{13}\text{C}$ ‰	C/N ratio g/g
Upper	1.94±0.04a	-1.15±0.1ab	84.00±1.6ab	50.38±0.1b	-27.89±0.1a	26.12±0.6a
Middle	1.87±0.03a	-1.31±0.1b	88.70±2.1a	50.22±0.3b	-27.77±0.1a	26.85±0.3a
Lower	1.92±0.04a	-0.98±0.1a	79.23±1.6b	51.35±0.1a	-27.60±0.1a	26.85±0.5a
F-Statistics	0.78 ^{ns}	7.08***	7.08***	8.30***	3.30 ^{ns}	0.79 ^{ns}

Values with dissimilar letters in the same column are significantly different at $P \leq 0.05$.

N_2 fixation with plant age. In this study, plants from the older plantations were associated with greater acid and alkaline phosphatase activity, high rhizosphere P and increased P in plant shoots (Maseko and Dakora 2013a), suggesting that

the generally improved P nutrition of older plants probably increased N_2 fixation. Additionally, the greater concentration of K and Fe in soil from the older 10-year plantation (31.4 mg kg^{-1} K and 46.4 mg kg^{-1} Fe) when compared to that from the

Table 7: N and C nutrition, and $\delta^{13}\text{C}$ of 5-year old *Cyclopia subternata* plants raised from cuttings and seedlings at Kanetberg ($n = 10$)

Planting material	N concentration %	$\delta^{15}\text{N}$ ‰	Ndfa %	C concentration %	$\delta^{13}\text{C}$ ‰	C/N ratio g/g
Seed	1.70 ± 0.04b	-1.41 ± 0.0b	91.48 ± 1.6a	50.76 ± 0.3a	-27.73 ± 0.1a	29.97 ± 0.7a
Cuttings	1.94 ± 0.04a	-1.15 ± 0.1a	83.96 ± 1.6b	50.38 ± 0.1a	-27.89 ± 0.1a	26.12 ± 0.6b
F-Statistics	17.24***	11.37***	11.37***	1.37 ^{ns}	1.70 ^{ns}	18.69***

Values with dissimilar letters in the same column are significantly different at $P \leq 0.05$.

younger plantation (10.8 mg kg⁻¹ K and 24.5 mg kg⁻¹ Fe mg kg⁻¹) could have also stimulated greater plant growth and N₂ fixation in older than younger plants, as both nutrients are reported to enhance N₂ fixation in legumes (Santiago *et al.* 2012; Singh and Kataria 2012; Tang *et al.* 1999).

Annual harvesting of plant shoots for making tea was found to decrease N₂ fixation, and hence %Ndfa, in *C. genistoides* (Table 3), a finding consistent with the data of Vance *et al.* (1979), which showed 88% decrease in nodule activity in harvested alfalfa relative to control. In an experiment where a woody perennial plant, *Gliricidia sepium*, was partially or completely defoliated, Nygren *et al.* (2000) found 10–60% decrease in nitrogenase activity. So the lower %Ndfa recorded in annually harvested *C. genistoides* plants in this study could be due to reduced nodule functioning. Decapitation of shoots of nodulated legumes apparently results in reduced gaseous permeability and O₂ diffusion to N₂-fixing nodules, leading to decreased nitrogenase activity, and hence N₂ fixation (Dakora and Atkins 1989; Nygren *et al.* 2000; Schmitt *et al.* 2013). At Kanetberg, soil topequence was found to affect symbiotic functioning of *C. subternata* nodules, with plants on the upper and middle slopes deriving more N from atmospheric N₂ fixation than those on the lower slope (Table 6). This was probably due to better nodule development and greater nodule functioning in the well-drained soils of the upper and middle topequence. But whether soil ¹⁵N values along the topequence were affected by denitrification (especially in the wetter lower slope) was not assessed in this study, even though plant uptake of the accumulated heavier ¹⁵N isotope from soil can affect estimates of N₂ fixation using the ¹⁵N natural abundance technique.

Although N derived from symbiotic fixation by *C. subternata* plants raised from seed was high, actual amounts of N-fixed were much greater in cuttings than seedlings (908 versus 473 mg plant⁻¹) (Maseko and Dakora 2015) probably due to enhanced P nutrition from higher rhizosphere acid phosphatase activity, which led to greater P availability in the rhizosphere, and hence increased P accumulation in shoots (see Maseko and Dakora 2013a). However, the higher P and K concentration in soils planted to *Cyclopia subternata* cuttings could have promoted plant growth and increased N contribution by cuttings relative to seedlings. It was also interesting to note that *A. aspalathoides* growing naturally in the same environment as *A. caledonensis* and *C. genistoides* at Koksrivier could obtain as much as 91% of its N nutrition from symbiosis, as

opposed to 86% by *A. caledonensis* and 74% by *C. genistoides* (Table 4). Whether this was due to differences in the microsymbionts nodulating these species, remains to be determined. Results of a 16S rDNA gene sequencing by Kanu and Dakora (2012) showed that eight test species of *Psoralea* formed root nodules with *Rhizobium*, *Mesorhizobium* and *Burkholderia* species, an indication of nodulation promiscuity. Lemaire *et al.* (2015) also found that members of the tribes Crotalarieae and Indigofereae nodulated with both alpha- and beta-rhizobia; Psoraleeae generally preferred *Mesorhizobium* symbionts whilst Podalyrieae nodulated with *Burkholderia*. The reported nodulation of native legumes in the Cape fynbos by a wide variety of microsymbionts could be an adaptation for their survival in a low-N environment. However, *A. aspalathoides* was also found to differentially accumulate more Ca, Mg, S, Na, Fe and Mn in shoots for growth than *A. caledonensis* and *C. genistoides* (Maseko 2013), and this could explain its superior symbiotic performance. Whatever the case, the legume species sampled from the various fields in the fynbos revealed high dependency on symbiotic N₂ fixation for their N nutrition.

$\delta^{13}\text{C}$ and C metabolism

Carbon accumulation in plants represents a direct measure of photosynthetic activity. In this study, shoot C concentration of fynbos legumes differed only slightly, though sometimes significantly. They ranged from 50% to 51% for *C. subternata*, 49% to 52% for *C. genistoides*, 49% for *A. aspalathoides* and 47% for *A. caledonensis*. These differences in shoot C levels could be indicative of the types of storage compounds used by each species. In fact, Post *et al.* (2007) have suggested that shoot C concentration greater than 40% is an indication of high-lipid distribution. Sprent *et al.* (1996) also detected variations in shoot C concentration of nodulated Ceasalpinoid and Mimosoid legumes, with about 50–55% in *Chamaecrista* and *Stryphnodendron* species. Furthermore, Yoneyama and Ohtani (1983) and Sprent *et al.* (1996) found that C ≥ 49% was associated with more negative $\delta^{13}\text{C}$ values, just as in this study higher shoot %C was associated with more negative $\delta^{13}\text{C}$ values (Tables 2–4). As a result, there was a positive correlation between %C and $\delta^{13}\text{C}$ of *C. subternata* cuttings aged 5 years ($r = 0.48$, $P = 0.009$), and of *C. subternata* plants growing on the lower topequence ($r = 0.63$, $P = 0.050$).

Because photosynthetic C accumulation in plants is regulated by N nutrition, the C/N ratio is generally regarded as a good indicator of the N status of plants. Due to their N₂-fixing

ability, symbiotic legumes tend to have C/N values $< 24\text{ g g}^{-1}$, while non-legumes exhibit C/N ratios $> 24\text{ g g}^{-1}$ (Hobbie 1992). In this study, estimates of C/N ratios ranged from 29 to 36 g g^{-1} for *C. genistoides* at Koksrivier to $25\text{--}30\text{ g g}^{-1}$ for *C. subternata* at Kanetberg. However, of the *Aspalathus* species, *A. aspalathoides*, had the highest C/N ratio of 35 g g^{-1} , indicating elevated C accumulation in response to its high symbiotic functioning (91 %Ndfa). However, if surplus C accumulated by these legumes was stored as N-free compounds, then the C/N ratio would be expected to be high. Coincidentally, Honeybush tea prepared from *Cyclopia* species as well as Rooibos tea beverage produced from *A. linearis* are reportedly very rich in phenolic compounds (De Beer and Joubert 2010; Iswaldi *et al.* 2011). So, at the very least, phenolics are likely the major N-free storage compounds of these legumes, and their confirmed presence would account for the unusually high C/N ratios in shoots. Given the low N concentration in fynbos soils (Cramer 2010), the ability of these legumes to switch from N-containing to N-free storage molecules (Raven *et al.* 2004) would be an ecological advantage in overcoming nutrient stress.

The ^{13}C values of C_3 plants have been used as an indicator of water-use efficiency (Farquhar *et al.* 1989). Theoretically speaking, plant species discriminate against ^{13}C during photosynthetic CO_2 fixation by Rubisco. This is because the diffusion of $^{13}\text{CO}_2$ across the stomatal pore is 4.4-fold lower than $^{12}\text{CO}_2$ (Farquhar *et al.* 1982). However, less ^{13}C discrimination occurs when there is partial closure of stomata due to water deficit. Thus, low ^{13}C discrimination (less negative $\delta^{13}\text{C}$ values) implies greater water-use efficiency during photosynthesis, while high ^{13}C discrimination indicates more negative $\delta^{13}\text{C}$ values and low water-use efficiency during photosynthesis. In this study, the $\delta^{13}\text{C}$ varied from -27.7‰ to -25.7‰ for *C. genistoides* and -28.2‰ to -25.7‰ for *C. subternata*, while *A. caledonensis* and *A. aspalathoides* had $\delta^{13}\text{C}$ values of -26.1‰ and -26.8‰ , respectively (Table 4). These data suggest that older *Cyclopia* plants exhibited greater water-use efficiency, and hence enhanced N nutrition from symbiosis (Table 2).

Furthermore, the decrease in symbiotic functioning of *C. genistoides* caused by annual harvesting of shoots for tea was probably due to poor water-use efficiency (-27.7‰) when compared with the unharvested control (-25.7‰). The same could be said of *A. caledonensis* and *A. aspalathoides*, which showed greater water-use efficiency and therefore derived a higher proportion of their N nutrition from symbiosis than *C. genistoides*, which co-occurred in the same habitat and showed lower water-use efficiency and less N derived from symbiosis (Table 4). The fact that the more water-use efficient legumes obtained the most N from atmospheric fixation was again confirmed for *C. subternata* plants aged 4 years (Table 5).

In summary, this study has shown that plants from the older tea plantations of both *C. genistoides* and *C. subternata* derived more N from fixation than their younger counterparts. Older *Cyclopia genistoides* plants were also more water-use efficient than younger plants. With *C. subternata*, however, the juvenile

plants were more water-use efficient. *Cyclopia genistoides* plants that were not harvested annually for tea derived greater N from fixation and were also the most water-use efficient. Between the co-occurring *Aspalathus* and *Cyclopia* species at Koksrivier, the former derived more N from fixation and was more water-use efficient. Furthermore, although *Cyclopia subternata* plants established from cuttings showed greater shoot N concentration, those from seedlings derived higher N from fixation. Taken together, these data show a close relationship between $\delta^{15}\text{N}$ values or symbiotic N nutrition, and water-use efficiency of fynbos legumes from different experimental settings.

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REFERENCES

- Abi-Ghanem R, Bodah ET, Wood M, *et al.* (2013) Potential breeding for high nitrogen fixation in *Pisum sativum* L.: germplasm phenotypic characterization and genetic investigation. *Am J Plant Sci* **4**:1597–600.
- Bai E, Boutton TW, Liu F, *et al.* (2009) Spatial variation of the stable nitrogen isotope ratio of woody plants along a topoedaphic gradient in a subtropical savannah. *Oecologia* **159**:493–503.
- Bedard-Haughn A, van Groenigen JW, van Kessel C (2003) Tracing ^{15}N through landscapes: potential uses precautions. *J Hydrol* **272**:175–90.
- Boatwright JS, Cupido CN (2011) *Aspalathus crewiana* sp. nov. (Crotalariaeae, Fabaceae) from the Western Cape Province, South Africa. *Nordic J Bot* **29**:513–7.
- Casper BB, Goldman R, Lkhagva A, *et al.* (2012) Legumes mitigate ecological consequences of a topographic gradient in a northern Mongolian steppe. *Oecologia* **169**:85–94.
- Comas LH, Bauerle TL, Eissenstat DM (2010) Biological and environmental factors controlling root dynamics and function: effects of root ageing and soil moisture. *Austr J Grape Wine Res* **16**:131–7.
- Condon AG, Richards RA, Rebetzke GJ, *et al.* (2004) Breeding for high water-use efficiency. *J Exp Bot* **55**:2447–60.
- Craig H (1957) Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analysis of carbon dioxide. *Geochim Cosmochim* **12**:133–49.
- Craine JM, Brookshire ENJ, Cramer MD, *et al.* (2015) Ecological interpretations of nitrogen isotope ratios of terrestrial plants and soils. Marchner Review. *Plant Soil* 1–26, <http://dx.doi.org/10.1007/s11104-015-2542-1>
- Cramer MD (2010) Phosphate as a limiting resource: introduction. *Plant Soil* **334**:1–10.

- Cusack DF, Silver W, McDowell WH (2009) Biological nitrogen fixation in two tropical forests: ecosystem-level patterns and effects of nitrogen fertilization. *Ecosystems* **12**:1299–315.
- Department of Agriculture, Forestry and Fisheries (DAFF) (2013) *Report, Honeybush Tea, Honeybush Tea Industry Profile*. <http://www.nda.agric.za> (15 September 2013, date last accessed).
- Dakora FD, Atkins CA (1989) Diffusion of oxygen in relation to structure and function in legume root nodules. *Austr J Plant Physiol* **16**:131–40.
- De Beer D, Joubert E (2010) Development of HPLC method for *Cyclopia subternata* phenolic compound analysis and application to other *Cyclopia* spp. *J Food Comp Anal* **23**:289–97.
- Eissenstat DM, Volder A (2010) The efficiency of nutrient acquisition over the life of a root. In BassiriRad H (ed). *Nutrient Acquisition by Plants An Ecological Perspective*, Vol. **181**, Ecological Studies. Berlin: Springer, 185–220.
- Evans RD (2001) Physiological mechanisms influencing plant nitrogen isotope composition. *Trends Plant Sci* **6**:121–6.
- Farquhar GD, O'Leary MH, Berry JA (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Austra J Plant Physiol* **9**:121–37.
- Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* **40**:503–37.
- Goldblatt P, Manning JC (2002) Plant diversity of the Cape region of Southern Africa. *Ann Missouri Bot Garden* **89**:281–302.
- Graham PH, Vance CP (2003) Legumes: importance and constraints to greater use. *J Plant Physiol* **131**:872–7.
- Hobbie SE (1992) Effects of plant species on nutrient cycling. *Trends Ecol Evol* **7**:336–9.
- Huss-Danell K, Chaia E, Carlsson G (2007) N₂ fixation and nitrogen allocation to above and below ground plant parts in red clover-grasslands. *Plant Soil* **299**:215–26.
- Iswaldi I, Arraez-Roman D, Rodriguez-Medina I, et al. (2011) Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (*Aspalathus linearis*) by HPLC-ESI-MS (TOF/IT). *Anal Bioanal Chem* **400**:3643–54.
- Joubert ME, Wooldridge J, Booyse M (2010) Tissue element concentrations and mineral removal rates in honeybush tea I: *Cyclopia genistoides*. *J Plant Nutr* **33**:1789–800.
- Joubert E, De Beer D (2011) Rooibos (*Aspalathus linearis*) beyond the farm gate: from herbal tea to potential phytopharmaceutical. *South Afr J Bot* **77**:869–86.
- Junk G, Svec H (1958) The absolute abundance of the nitrogen isotopes in the atmosphere and compressed gas from various sources. *Geochimica et Cosmochimica Acta* **14**:234–43.
- Lemaire B, Dlodlo O, Chimphango S, et al. (2015) Symbiotic diversity, specificity and distribution of rhizobia in native legumes of the Core Cape Subregion (South Africa). *FEMS Microbiol Ecol* **91**:1–11.
- Liu Y, Niu H, Xu X (2013) Foliar $\delta^{13}\text{C}$ response patterns along a moisture gradient arising from genetic variation and phenotypic plasticity in grassland species of Inner Mongolia. *Ecol Evol* **3**:262–7.
- Kanu SA, Dakora FD (2012) Symbiotic nitrogen contribution and biodiversity of root-nodule bacteria nodulating *Psoralea* species in the Cape Fynbos, South Africa. *Soil Biol Biochem* **54**:68–76.
- Lötter D, van Garderen EA, Tadross M, et al. (2014) Seasonal variation in the nitrogen nutrition and carbon assimilation in wild and cultivated *Aspalathus linearis* (rooibos tea). *Austr J Bot* **62**:65–73.
- Mariotti A, Germon JC, Hubert P, et al. (1981) Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustrations for the denitrification and nitrification processes. *Plant Soil* **62**:413–30.
- Maseko ST (2013) N₂ fixation, P nutrition, C and mineral accumulation, and $\delta^{13}\text{C}$ of nodulated natural stands of legumes in the Cape fynbos, South Africa. Doctoral thesis, Tshwane University of Technology, South Africa.
- Maseko ST, Dakora FD (2013a) Rhizosphere acid and alkaline phosphatase activity as a marker of P nutrition in nodulated *Cyclopia* and *Aspalathus* species in the Cape fynbos of South Africa. *South Afr J Bot* **89**:289–95.
- Maseko ST, Dakora FD (2013b) Plant enzymes, root exudates, cluster roots and mycorrhizal symbiosis are the drivers of P nutrition in native legumes growing in P deficient soil of the Cape fynbos in South Africa. *J Agric Sci Technol* **A3**:331–40.
- Maseko ST, Dakora FD (2015) Vegetatively-propagated *Cyclopia subternata* plants adopt mechanisms that enhance N and P availability better than seed-established plants. *South Afr J Bot* **98**:188.
- Mbangcolo MM, Reinten EY, Agenbag GA (2013) Effect of an organic plant fertiliser on the establishment of rooted cuttings of two species of *Cyclopia* (honeybush). *South Afr J Plant Soil* **30**:57–60.
- Moufhe ML, Dakora FD (1999) Nitrogen nutrition in nodulated field plants of the shrub tea legume *Aspalathus linearis* assessed using ¹⁵N natural abundance. *Plant Soil* **209**:181–6.
- Nygren P, Cruz P, Domenach AM, et al. (2000) Influence of forage harvesting regimes on dynamics of biological dinitrogen fixation of a tropical woody legume. *Tree Physiol* **20**:41–8.
- Peuke AD, Gessler A, Rennenberg H (2006) The effect of drought on C and N stable isotopes in different fractions of leaves, stems and roots of sensitive and tolerant beech ecotypes. *Plant Cell Environ* **29**:823–35.
- Pons TL, Flexas J, von Caemmerer S, et al. (2009) Estimating mesophyll conductance to CO₂: methodology, potential errors, and recommendations. *J Exp Bot* **60**:2217–34.
- Post DM, Layman CA, Arrington DA, et al. (2007) Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. *Oecologia* **152**:179–89.
- Raven JA, Handley LL, Andrews M (2004) Global aspects of C/N interactions determining plant-environment interactions. *J Exp Bot* **55**:11–25.
- Reed SC, Cleveland CC, Townsend AR (2008) Tree species rates of free-living nitrogen fixation in a tropical rain forest. *Ecology* **89**:2924–34.
- Robinson D (2001) $\delta^{15}\text{N}$ as an integrator of the nitrogen cycle. *Trends Ecol Evol* **16**:153–62.
- Santiago LS, Wright SJ, Harms KE, et al. (2012) Tropical tree seedling growth responses to nitrogen, phosphorus and potassium addition. *J Ecol* **100**:309–16.
- Schmitt A, Pausch J, Kuzyakov Y (2013) C and N allocation in soil under ryegrass and alfalfa estimated by ¹³C and ¹⁵N labelling. *Plant Soil* **368**:581–90.
- Shearer G, Kohl DH (1986) N₂-fixation in field settings: estimations based on natural ¹⁵N abundance. *Austr J Plant Physiol* **13**:699–756.

- Sinclair TR, Rufty TW (2012) Nitrogen and water resources commonly limit crop yield increases, not necessarily plant genetics. *Glob Food Secur* **1**:94–8.
- Singh N, Kataria N (2012) Role of potassium fertilizer on nitrogen fixation in chickpea (*Cicer arietinum* L.) under quantified water stress. *J Agric Technol* **8**:377–92.
- Sparks JP, Ehleringer JR (1997) Leaf carbon isotope discrimination and nitrogen content for riparian trees along elevational transects. *Oecologia* **109**:362–7.
- Sprent JJ, Geoghegan IE, Whitty PW, et al. (1996) Natural abundance of ^{15}N and ^{13}C in nodulated legumes and other plants in the cerrado and neighbouring regions of Brazil. *Oecologia* **105**:440–6.
- Spriggs AC, Dakora FD (2009) Field assessment of symbiotic N_2 fixation in wild and cultivated *Cyclopia* species in the South African fynbos by ^{15}N natural abundance. *Tree Physiol* **29**:239–47.
- Spriggs AC, Dakora FD (2009) Symbiotic performance of selected *Cyclopia* Vent (honeybush) rhizobia under nursery and field conditions. *Symbiosis* **48**:143–53.
- Stewart KJ, Grogan P, Coxson DS, et al. (2014) Topography as a key factor driving atmospheric nitrogen exchanges in arctic terrestrial ecosystems. *Soil Biol Biochem* **70**:96–112.
- Tang C, Robson AD, Dilworth MJ (1999) A split-root experiment shows that iron is required for nodule initiation in *Lupinus angustifolius* L. *New Phytol* **115**:61–7.
- Van der Water PK, Leavitt SW, Betancourt JL (2002) Leaf $\delta^{13}\text{C}$ variability with elevation, slope aspect, and precipitation in the southwest United States. *Oecologia* **132**:332–43.
- Vance CP (2001) Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. *Plant Physiol* **127**:390–7.
- Vance CP, Heichel GH, Barnes DK, et al. (1979) Nitrogen fixation, nodule development, and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. *Plant Physiol* **64**:1–8.
- Volder A, Smart DR, Bloom AJ, et al. (2005) Rapid decline in nitrate uptake and respiration with age in fine lateral roots of grape: implications for root efficiency and competitive effectiveness. *New Phytol* **165**:493–502.
- Walley F, Fu G, van Groening JW, et al. (2001) Short-range spatial variability of nitrogen fixation by field-grown chickpea. *Soil Sci Soc Am J* **65**:1717–22.
- Wang L, Shaner PJL, Macko S (2007) Foliar $\delta^{15}\text{N}$ patterns along successional gradients at plant community and species levels. *Geophys Res Lett* **34**: L16403.
- Wang L, D'Odorico P, Ries L, et al. (2010) Patterns and implications of plant-soil $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in African savanna ecosystems. *Quater Res* **73**:77–83.
- Wittmer MHOM, Auerswald K, Tungalag R, et al. (2008) Carbon isotope discrimination of C_3 vegetation in Central Asian grassland as related to long-term and short-term precipitation patterns. *Biogeosciences* **5**:913–24.
- Yoneyama T, Ohtani T (1983) Variations of natural ^{13}C abundances in leguminous plants. *Plant Cell Physiol* **24**:971–7.
- Yang YG, Shen Y, Shao SG, et al. (2013) Spatial distribution in forest soil nutrients and its relationship with ecological factors on the up stream of Taihu lake basin. *Adv Mater Res* **652**:1660–3.