Nitrogen nutrition, carbon accumulation and \( \delta^{13}C \) 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 \( ^{15}N \) natural abundance technique was used to determine \( N_2 \) fixation, carbon (C) assimilation and \( \delta^{13}C \) 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 \( N_2 \) 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. \( N_2 \) 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: Leguminoseae, tribe: Podalyrieae) and *Aspalathus* (family: Leguminoseae, tribe: Crotalarieae) 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 linears* subsp. *linears* 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 Rufy 2012). Their acquisition is therefore crucial for plant development and functioning. With legumes, N can be sourced from either the soil, symbiotic N\textsubscript{2} fixation, or both. In the fynbos, *Cyclopia* and *Aspalathus* species meet their N requirements from biological fixation of atmospheric N\textsubscript{2}. Most *Cyclopia* species and *Aspalathus linearis* can obtain over 100 kg N ha\textsuperscript{-1} from symbiotic N\textsubscript{2} fixation for their N nutrition (Löter et al. 2014; Moufhe and Dakora 1999; Spriggs and Dakora 2009). Thus, the taping 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 \( ^{15}\text{N} \) 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. \( ^{15}\text{N} \) and \( ^{14}\text{N} \)) can differ between ecosystems as a result of isotopic fractionation during N cycling (Bai et al. 2009; Robinson 2001). Where N\textsubscript{2}-fixing legumes are concerned, \( ^{15}\text{N} \) composition and N\textsubscript{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 \( ^{13}\text{C} \) and \( ^{15}\text{N} \) 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 \( ^{13}\text{C} \) isotope of carbon in favour of the lighter \( ^{12}\text{C} \) isotope, resulting in a lower ratio of \( ^{13}\text{C}/^{12}\text{C} \) relative to the atmosphere (Condron 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 \( ^{13}\text{C} \) (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; Essenstat 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\textsubscript{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\textsubscript{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 endemicity 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\textsubscript{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 \( ^{15}\text{N} \) and \( ^{13}\text{C} \) natural abundance at Koksrivier, (ii) to compare the symbiotic N nutrition and water-use efficiency of *Aspalathus caleodonensis*, *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 \( ^{15}\text{N} \) and \( ^{13}\text{C} \) 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 Cyclopia tea plants at Koksrivier were grown in natural settings on sandy soils without ploughing, fertilisation 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. longifoilia 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 plants grown along a toposequence in order to assess the 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, Wismar 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, Rivolta, 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):

$$\delta^{15}N = \left( \frac{\text{15N at sample}}{\text{15N at atm}} \right) - 1 \times 1000$$

where the δ15N 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 (%Ndaf) was determined using the equation (Shearer and Kohl 1986):

$$%\text{Ndaf} = [\delta^{15}N_{\text{ref}} - \delta^{15}N_{\text{leg}} / \delta^{15}N_{\text{ref}} - B] \times 100$$

Where δ15Nref is the mean 15N natural abundance of the reference plants, δ15Nleg 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 %Ndaf in Cyclopia was −1.71‰ (Spriggs and Dakora 2009), whilst that used in Aspalathus was −2.00‰ (Moufle 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 δ13C) as (Farquhar et al. 1989):

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

where δ13C is the mean 13C natural abundance of the sample in parts per mill (‰), (13C/12C)sample is the isotopic ratio of plant sample, and (13C/12C)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 \(C. \textit{genistoides}\) 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 \(C. \textit{subternata}\) 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 \(C. \textit{subternata}\) 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 \(C. \textit{subternata}\) 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.

\(\delta^{15}N\) of reference plants

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

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

Isotopic analysis of shoots revealed differences between \(C. \textit{genistoides}\) plants sampled from 10 and 2-year old plantations at Koksrivier. Although N concentrations were similar, the \(\delta^{15}N\) was significantly lower in 10-year old \(C. \textit{genistoides}\) 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 \(\delta^{15}N\) values were also greater in the annually harvested than unharvested \(C. \textit{genistoides}\) plants (Table 3). As a result, %Ndfa was lower in the annually harvested plants (Table 3).

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

Isotopic analysis of \(C. \textit{subternata}\) plants aged 8, 7, 6, 5, 4, and 2 years at Kanetberg revealed significant differences in symbiotic parameters. Although N concentrations were similar for \(C. \textit{subternata}\) plants of all ages (except those aged 6 years), the \(\delta^{15}N\) 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 \(C. \textit{subternata}\) grown at Kanetberg showed that plants from the middle slope had the lowest \(\delta^{15}N\) 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 \(C. \textit{subternata}\) was also assessed at Kanetberg. The data showed that, although cuttings exhibited significantly greater shoot N concentration, the \(\delta^{15}N\) 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 \(\delta^{13}C\) of \(C. \textit{subternata}\) and \(A. \textit{aspalathoides}\) species

\(C. \textit{genistoides}\) 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 \(C. \textit{genistoides}\) plants that were harvested annually for tea also showed higher C concentration compared to those that were not harvested yearly (Table 3). The \(\delta^{13}C\) values of the unharvested \(C. \textit{genistoides}\) 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 \(C. \textit{genistoides}\) and two \(A. \textit{aspalathoides}\) species (\(A. \textit{caledonensis}\) and \(A. \textit{aspalathoides}\)) from Koksrivier revealed greater C accumulation in the tea legume compared to the two \(A. \textit{aspalathoides}\) species (Table 4). However, the C/N ratio was much greater in \(A. \textit{aspalathoides}\) species (Table 4). The two \(A. \textit{aspalathoides}\) species also exhibited less negative \(\delta^{13}C\) values (or greater water-use efficiency) relative to \(C. \textit{genistoides}\) (Table 4). A comparison of \(C. \textit{subternata}\) plants of different ages sampled from Kanetberg revealed slight but significant differences in C concentration. The \(\delta^{13}C\) 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 \(C. \textit{subternata}\) 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 \(\delta^{13}C\) 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 \(\delta^{13}C\) of \(C. \textit{subternata}\) planted at Kanetberg (Table 7). The C/N ratio was however greater in shoots of plants propagated from seed than cuttings (Table 7).
Correlation analysis

The δ¹⁵N of Cyclopia subternata shoots established from cuttings aged 5 years correlated positively \( (r = 0.546, P = 0.053) \) with its δ¹³C at Kanetberg. Mean δ¹⁵N 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 ¹⁵N 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 δ¹⁵N values that enabled good estimates of percent N derived from atmospheric N₂ 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

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**Table 1:** shoot δ¹⁵N (‰) values of reference plants sampled from Koksrivier and Kanetberg farms

| Farm name | Year | Family     | Plant species            | Shoot δ¹⁵N | Mean δ¹⁵N |
|-----------|------|------------|--------------------------|------------|-----------|
| Koksrivier | 10   | Proteaceae | Leucadendron strictum    | 0.73       |           |
|           | 2    | Poaceae    | Pentaschistis curvifolia | 0.72       | 0.73      |
| Kanetberg | 8    | Proteaceae | Leucadendron strictum    | 2.77       |           |
|           | 7    | Proteaceae | Leucadendron strictum    | 0.55       |           |
|           | 6    | Proteaceae | Leucadendron strictum    | 0.27       | 0.41      |
|           | 5 cuttings | Proteaceae | Leucadendron strictum    | 1.17       |           |
|           |      | Proteaceae | Leucadendron strictum    | 2.30       |           |
|           |      | Proteaceae | Protas grandiceps        | 2.90       |           |
|           |      | Restionaceae | Elegia thyrsoidea    | 0.96       |           |
|           |      | Restionaceae | Elegia thyrsoidea    | 1.06       | 1.81      |
|           | 5 seedlings | Proteaceae | Leucadendron strictum    | 1.35       | 2.06      |

**Table 2:** a comparison of N and C nutrition, and δ¹³C of 2- and 10-year old Cyclopia genistoides plants sampled at Koksrivier \( (n = 10) \), near Gansbaai, South Africa, in 2007

| Plant age | N concentration | δ¹⁵N | Ndfa | C concentration | δ¹³C | 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** | 6.44** | 6.58** | 4.60** | 48.50*** | 1.25** |

Values with dissimilar letters in the same column are significantly different at \( P \leq 0.05 \).
Table 3: effect of tea harvesting on N and C nutrition, and δ¹³C, of developing pods of Cyclopia genistoides planted at Koksrivier in 1997 (n = 10)

| Farmers’ practice | N concentration | δ¹³N | Ndfa | C concentration | δ¹³C | C/N ratio |
|-------------------|-----------------|------|------|-----------------|------|-----------|
| Harvested         | 1.78 ± 0.1a     | −1.11 ± 0.1a | 75.37 ± 1.2b | 51.34 ± 0.2a | −27.70 ± 0.1b | 29.04 ± 1.0b |
| Unharvested       | 1.83 ± 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 ≤ 0.05.

Table 4: N and C nutrition, and δ¹³C of Cyclopia and Aspalathus species sampled from Koksrivier farm established in 2005 (n = 10)

| Legume species          | N concentration | δ¹³N | Ndfa | C concentration | δ¹³C | C/N ratio |
|-------------------------|-----------------|------|------|-----------------|------|-----------|
| Cyclopia genistoides    | 1.81 ± 0.1a     | −1.07 ± 0.1a | 73.71 ± 2.0b | 51.50 ± 0.2a | −27.67 ± 0.1c | 28.57 ± 0.8b |
| Aspalathus caledensis    | 1.65 ± 0.1b     | −1.62 ± 0.1b | 86.24 ± 2.7a | 47.19 ± 0.6c | −26.12 ± 0.1a | 28.91 ± 1.0b |
| Aspalathus aspalathoides| 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 ≤ 0.05.

Table 5: N and C nutrition, and δ¹³C of Cyclopia subternata plants of different ages sampled from Kanetberg (n = 10)

| Plant age | N concentration | δ¹³N | Ndfa | C concentration | δ¹³C | C/N ratio |
|-----------|-----------------|------|------|-----------------|------|-----------|
| Years     | %               | %    | %    | %               | %    | 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 ≤ 0.05.

Table 6: N and C nutrition, and δ¹³C of 5-year old Cyclopia subternata plants sampled at Kanetberg along a soil toposequence (n = 10)

| Topography | N concentration | δ¹³N | Ndfa | C concentration | δ¹³C | 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***       | 7.08***        | 7.08***       | 8.30***        | 3.30***        | 0.79***        |

Values with dissimilar letters in the same column are significantly different at P ≤ 0.05.

N₂ 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 N₂ 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
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 N\(_2\) fixation in older than younger plants, as both nutrients are reported to enhance N\(_2\) 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 N\(_2\) 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 O\(_2\) diffusion to N\(_2\)-fixing nodules, leading to decreased nitrogenase activity, and hence N\(_2\) fixation (Dakora and Atkins 1989; Nygren et al. 2000; Schmitt et al. 2013). At Kanetberg, soil toposequence was found to affect symbiotic functioning of C. subtertata nodules, with plants on the upper and middle slopes deriving more N from atmospheric N\(_2\) 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 toposequence. But whether soil \(^{15}\)N values along the toposequence were affected by denitrification (especially in the wetter lower slope) was not assessed in this study, even though plant uptake of the accumulated heavier \(^{15}\)N isotope from soil can affect estimates of N\(_2\) fixation using the \(^{15}\)N natural abundance technique.

Although N derived from symbiotic fixation by C. subtertata 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 subtertata 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; Psoraleae 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 N\(_2\) fixation for their N nutrition.

### \(^{13}\)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. subtertata, 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 Cesalpinioide and Mimosoide legumes, with about 50–55% in Chamaecrista and Styphnolobium species. Furthermore, Yoneyama and Ohtani (1983) and Sprent et al. (1996) found that C ≥ 49% was associated with more negative \(^{13}\)C values, just as in this study higher shoot %C was associated with more negative \(^{13}\)C values (Tables 2–4). As a result, there was a positive correlation between %C and \(^{13}\)C of C. subtertata cuttings aged 5 years (r = 0.48, P = 0.009), and of C. subtertata plants growing on the lower toposequence (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 N\(_2\)-fixing

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

| Planting material | N concentration | \(^{15}\)N | Ndfa | C concentration | \(^{13}\)C | C/N ratio |
|-------------------|-----------------|---------|------|-----------------|-------|----------|
| 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***        | 1.70*** | 18.69*** |

Values with dissimilar letters in the same column are significantly different at P ≤ 0.05.

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ability, symbiotic legumes tend to have C/N values < 24 g g⁻¹, while non-legumes exhibit C/N ratios > 24 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 25–30 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 ¹³C values of C₃ plants have been used as an indicator of water-use efficiency (Farquhar et al. 1989). Theoretically speaking, plant species discriminate against ¹³C during photosynthetic CO₂ fixation by Rubisco. This is because the diffusion of ¹³CO₂ across the stomatal pore is 4.4-fold lower than ¹²CO₂ (Farquhar et al. 1982). However, less ¹³C discrimination occurs when there is partial closure of stomata due to water deficit. Thus, low ¹³C discrimination (less negative δ¹³C values) implies greater water-use efficiency during photosynthesis, while high ¹³C discrimination indicates more negative δ¹³C values and lower water-use efficiency during photosynthesis. In this study, the δ¹³C 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 δ¹³C 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 δ¹⁵N values or symbiotic N nutrition, and water-use efficiency of fynbos legumes from different experimental settings.

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