Seasonal variation in the nitrogen nutrition and carbon assimilation in wild and cultivated *Aspalathus linearis* (rooibos tea)

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Abstract. The Fynbos Biome of southern Africa is a Mediterranean-climate ecosystem with highly infertile soil. It is home to the endemic leguminous shrub *Aspalathus linearis* (rooibos tea), which is both an invaluable wild resource and commercially cultivated plant. Wild rooibos has a narrow geographic range and is confined to mountain ranges of the Cederberg Region. Under projected climate change, warmer and more arid conditions may place additional pressure on these range-restricted plants to survive in an already resource-limited environment. To understand the adaptive strategies that may allow rooibos to persist in its habitat under future climate change, the present study evaluated changes in the photosynthetic activity and nutrient cycling of wild and cultivated *A. linearis*, at the temperature and rainfall extremes of summer and winter. Wild and cultivated rooibos tea had different methods of adapting to nitrogen (N) nutrition and carbon (C) assimilation during wet and dry seasons. In particular, the wild plants were better able to tolerate summer drought by increased water use efficiency and maintaining higher levels of biological N_2 fixation than was the cultivated tea.

Introduction

Aspalathus linearis (Burm.F.) Dahlg., Fabaceae, is an evergreen leguminous shrub indigenous to the Fynbos Biome of the Cape Floristic Region (Dahlgren 1968) and one of the most successful commercially propagated endemic crops of South Africa. The Fynbos Biome is one of five Mediterranean climate regions of the world, which are all recognised as biodiversity hotspots (Cowling *et al*. 1996). There is a particularly remarkable ecological convergence between two of the five, namely the Cape Floristic Region and the kwongan of South-western Australia (Cowling *et al*. 1996; Wisheu *et al*. 2000). Both fynbos and kwongan occur on severely nutrient-impoverished soils, display similar fire regimes, a remarkable convergence in growth forms and species diversity at different spatial scales (Cowling and Wikowski 1994; Cowling *et al*. 1996; Wisheu *et al*. 2000). These areas are particularly species rich, with high levels of rarity and endemism, where 68–75%

of plants are endemic as opposed to 23–50% in other Mediterranean-climate regions (Cowling *et al*. 1996). In addition, several of the fynbos species are restricted to extremely small distribution ranges and face similar threats in terms of habitat fragmentation and climate change (Bomhard *et al*. 2005).

The Mediterranean climate is characterised by a strong seasonal pattern of rainfall, with marked aridity during summer months and most of the annual rainfall falling predominantly during the winter period. The water-deficit conditions during summer months are generally considered to be the main environmental constraint to plant growth and productivity in Mediterranean-type vegetation (Larcher 2000). Low availability of soil nutrients is a further limiting factor in Mediterranean ecosystems (Kruger *et al*. 1983; Sardans *et al*. 2006). Fynbos soils are sandstone-derived and characterised by high acidity and poor nutrient availability

and are especially deficient in nitrogen (N), phosphorus (P) and calcium (Ca) (Kruger *et al*. 1983; Muofhe and Dakora 2000). N and P are known to be limiting nutrients for plant productivity in Mediterranean-type ecosystems, and the concentrations thereof in the ecosystem may also change in response to warming and drought (Lloret *et al*. 1999; Sardans *et al*. 2008). Plant growth and fitness in such an environment depend largely on a species ability to optimally utilise available resources. Many species possess certain eco-physiological traits that enable them to adapt to the existing environmental constraints. One such important trait is biological N_2 fixation. Being a legume, rooibos has a proven ability to fix N_2 and is known to be able to fix well over $100\,kg\,N\,ha^{-1}$ annually (Sprent *et al*. 2009). According to Lamont (1983) and Mitchell *et al*. (1986), several plants growing in Mediterranean ecosystems have developed specialised nutrient-uptake and internal nutrient-cycling strategies. An important mechanism by which critical nutrients are maintained is via nutrient reabsorption, in which nutrients are mobilised from senescing leaves and transported to other plant tissues (Killingbeck 1996). Another functional strategy to deal with restrictive environments is sclerophylly, which is associated with a high ratio of C to N in plant leaves (Rundell 1988). Sclerophylly is a typical characteristic of fynbos vegetation and it has been suggested to be an adaptation to specific stresses such as seasonal water deficits (Bussotti *et al*. 1998) and low-nutrient soils (Gutschick 1999). Apart from nutrition, plants may also adapt via photosynthetic and water-relation adjustments (Chaves *et al*. 2002, 2003; Vitale *et al*. 2012). It has been observed in field-grown plants that as summer drought progresses, leaf photosynthesis is increasingly limited because of a decline in stomatal conductance (Chaves *et al*. 2002). This leads to a reduction in water loss and an increase in water use efficiency (the ratio of water loss to biomass gained).

Climate change, which is predicted to cause an increase in aridity in the western coast of South Africa by the end of the century (Hewitson and Crane 2006; IPCC 2007; Engelbrecht *et al*. 2009) may place additional pressure on plants to survive in an already resource-limited environment. Previous research (Archer *et al*. 2008) has suggested that these changing climate conditions might have a severe impact on rooibos tea. It is, therefore, essential to understand the eco-physiological behaviour of these plants in relation to current climatic and soil constraints. However, not much is known about the nutritional and water-relation physiology of *A. linearis* under drought stress in its natural habitat. Local knowledge suggests that wild rooibos shrubs may have higher drought resistance than does the cultivated type (Louw 2006). *A. linearis* is one of the relatively few economically important fynbos species that has successfully made the transition from wild resource to an agriculturally important plant in the 20th century. Wild rooibos was historically used by local inhabitants of the Cederberg Region to produce tea, as well as for its medicinal and health properties (Dahlgren 1968). On the basis of growth form, several wild ecotypes of *A. linearis* have previously been identified (Malgas *et al.* 2007). Preliminary genetic testing has suggested a high level of genetic diversity within *A. linearis* (Van der Bank *et al*. 1995, 1999; Malgas *et al.* 2009). The cultivated type is thought to have originated from a wild type found in the Pakhuis area of the southern Cederberg during the 1930s (Cheney and Scholz 1963; Morton 1983). The entire commercial industry therefore hinge on one *A. linearis* selection made ~80 years ago. This selection was based on qualities such as growth rate, seed production and especially taste.

A useful approach to gain information on plant physiological performance is to study seasonal patterns in nutrient cycling and gas exchange (Llorens *et al*. 2003). Hence, the aim of the present study was to determine how the wet and dry seasons affect the photosynthetic gas exchange, nutrient cycling and water use efficiencies of wild and cultivated *A. linearis*. This will allow us to better understand the ability of *A. linearis* to persist in its habitat under future climate change.

Materials and methods

Site and plant selection

The study was conducted on communal land outside Heuningvlei (32.2°S, 19.13°E, 858 m asl), which is an outpost of the Moravian church village of Wupperthal in the Cederberg mountains, Western Cape, South Africa (Fig. 1). The soils in the area comprise mainly quartzitic sandstones of the Table Mountain Group that are well leached nutrient-poor and acidic soils. Cederberg sandstone fynbos vegetation predominates in this area and contains numerous endemic plant species, of which rooibos is one. The study site included a plantation of cultivated tea and a nearby site with wild rooibos growing among other fynbos plants. Wild populations of *A. linearis* have a narrow geographic range within the fynbos biome and are confined to mountain ranges of the Cedarberg Region (Dahlgren 1968). The wild plants occur naturally between 450-m and 900-m asl, whereas cultivated rooibos is grown over a wider geographical range than is the natural distribution. Cultivated seedlings are normally grown in seedbeds for 6 months under fertilisation and irrigation conditions. Thereafter, the seedlings are transplanted to the field, where they receive no additional fertiliser or irrigation. In the present study, the cultivated plants have been grown for 10 years under field conditions. Similarly, the wild tea plants have been growing in the area for at least 10 years as well. In both sites, there have been no fire events during the past 10 years, and plants have not been subjected to a harvesting cycle during the 2 years of the present study.

The study site was therefore chosen on the basis of the following three criteria: (1) an area where cultivated and a wild rooibos co-occurred closely, (2) accessibility and (3) known fire history and harvesting practices. Precipitation and air temperatures during the sampling period were obtained from the nearest weather station Mertenhof (32.14°S, 1919°E), which is 10 km from the study site, and meteorological data are reported in Fig. 2. Climate conditions were typical for a Mediterranean-type climate, with hot and dry summers, relatively cold winters, and rainfall periods concentrated in May until September. During the experimental period, summer drought occurred from November to March, when monthly rainfall ranged between 1 and 10 mm, whereas maximum daily temperatures averaged over 30°C. Total rainfall during winter
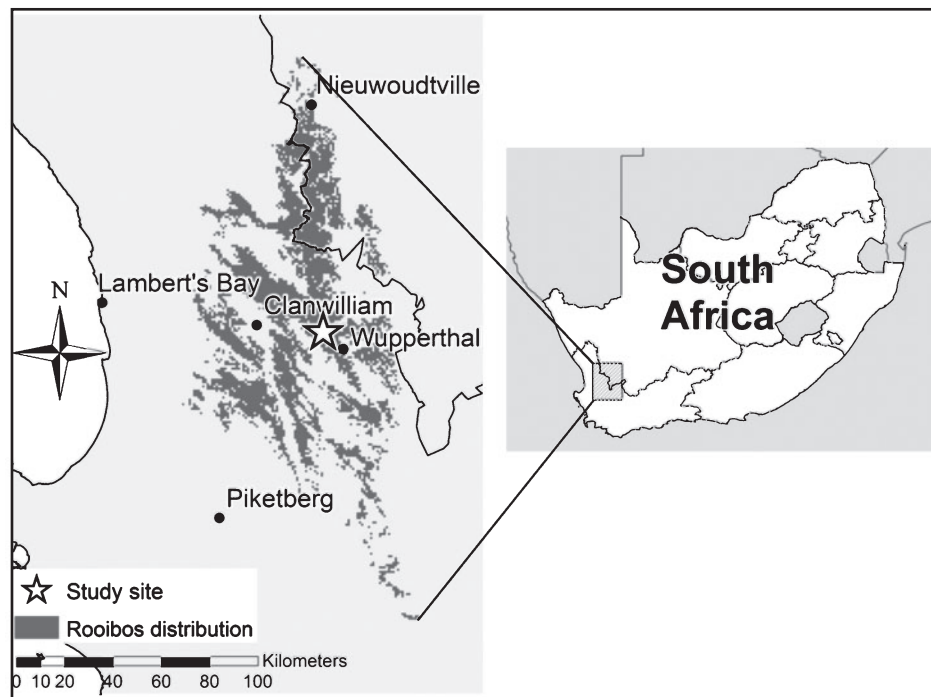
Fig. 1. Map of the Cederberg region, showing the natural distribution of rooibos and location of the study site. The area is located in the north-western part of the Western Cape, South Africa.
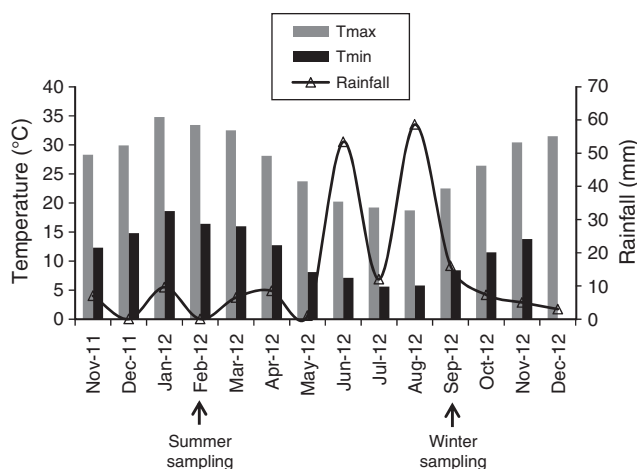

Fig. 2. Monthly mean maximum and minimum temperatures and monthly precipitation from November 2011 until December 2012 in the study area. Arrows indicate the sampling dates.

(JJA) was 145 mm and minimum temperatures ranged between 3.5°C and 4.4°C.

Plant and soil sampling

Data were collected from two sites where wild and cultivated *A. linearis* populations occurred separately. The first site included cultivated rooibos tea, where plants were grown in rows, set 1.5 m apart on an area of ~50 × 60 m. The second site featured wild rooibos plants growing among other fynbos species, and was located ~100 m from the first site.

Ten mature shrubs of similar age and size were randomly selected in both sites; thus, 20 shrubs in total were sampled. Sampling was conducted during late summer months (dry season) as well as during the late winter months (wet season). The shrubs were numbered and the same shrubs were sampled in each season.

For the plant samples, a 20-cm portion of disease-free and undamaged branch was cut off the top of each shrub and placed in paper bags. Recent leaf litter was collected at the base of each selected plant (litter deposited most recently according to the local farmers was taken). Plant material was clearly marked and dried in an oven at 60°C for 72 h. After drying, the plant material was finely ground for C and N stable isotope analysis. For the soil samples, three samples (each 10 cm deep) were taken from underneath each shrub, equally spaced from each other. These three samples were pooled to form one bulk sample for each shrub. Another three soil samples (each 10 cm deep) were taken 1 m away from each shrub, equally spaced from each other. The samples were once again combined to form a single sample for each plant. Soil samples were kept at 15°C until analysed.

Calculations of N-reabsorption efficiency, $\delta^{15}N$ and $\delta^{13}C$

Nitrogen-reabsorption efficiency was calculated as the difference between maximum N pools in green leaves and N pools in senescent leaves, as described by Killingbeck (1996), as follows:

$$(X_{Gr} - X_{Sen})/X_{Gr} \times 100, \qquad (1)$$

where X_{Gr} is the N concentration of green foliage and X_{Sen} is the N concentration of senescent leaves. Calculations were made separately for each plant before statistical analysis.

The stable isotope analyses were carried out at the Archaeology Department, University of Cape Town. Values were expressed relative to the Pee-Dee Belemnite (PDB) standard for $\delta^{13}C$ and relative to atmospheric N for $\delta^{15}N$, as (%), according to the following equation:

$$\delta Z = (R_{sample}/R_{standard} - 1) \times 1000, \qquad (2)$$

where Z is the heavy isotope of either N or C, and R is the ratio of heavier to lighter isotope for the sample and standard ($^{13}C/^{12}C$ or $^{15}N/^{14}N$).

The oven-dried plant components were milled in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA), after which the samples were combusted in a Flash 2000 organic elemental analyser. Three in-house standards (Merck Gel, Lentil, *Acacia saligna*) were used and calibrated against the standards of International Atomic Energy Agency (IAEA). N is expressed in terms of its value relative to atmospheric N, whereas C is expressed in terms of its value relative to Pee-Dee Belemnite.

Gas-exchange measurements

Maximum photosynthetic rates (Pn), stomatal conductance (Gs) and transpiration rates (E) were measured using a using a LI-6400 portable infrared gas analyser (Li-Cor, Lincoln, NE, USA). The reference CO_2 concentration was maintained at 0.04% CO_2 in air, flow rate was $500\,\mu mol\,s^{-1}$, whereas light intensity inside the chamber was set at $1500\,\mu mol$ photons $m^{-2}\,s^{-1}$ (light-saturated value derived from light-saturation curves). Measurements were made during mid-morning on clear, sunny days. The measured leave material was removed, oven-dried and weighed to express gas-exchange rates on a leaf dry-mass (g) basis.

Instantaneous and integrated water-use efficiency

Instantaneous (photosynthetic) water use efficiency (WUE_i) was calculated from gas exchange, to assess the efficiency of water usage during photosynthesis. The WUE_i was estimated by dividing the photosynthetic rate (Pn) by the transpiration rate (E), at the same light level, and is expressed as net photosynthetic rate/transpiration rate. Integrated WUE describes leaf WUE (based on stomatal behaviour) over the whole growing season and can be estimated using C isotope discrimination, whereby plants discriminate against ^{13}C during photosynthesis.

Statistical analysis

A two-way ANOVA with season and type (wild and cultivated rooibos) as a fixed factor was performed on gas exchange, stable isotope composition and N reabsorption, to detect differences in responses within seasons and across types. The normality of data and homogeneity of variance were tested by the Shapiro–Wilk and Levene tests, respectively, before ANOVA, to ensure that the assumptions of ANOVA were met. The different variables and their interactions were tested to detect significant factors (Kaleidagraph, Synergy Software, Reading, PA, USA). Where the ANOVA revealed significant differences among treatments, the means ($n = 10$) were separated using a *post hoc* Tukey's HSD, a multiple-range test based on a significance level of $P = 0.05$.

Results

Plant nutrient and isotope analysis

Leaf N concentrations displayed a marked difference between wild and cultivated rooibos, being significantly lower for wild tea. These differences were observed in both summer and winter. Both wild and cultivated rooibos types displayed the highest leaf N concentrations during winter, with values ranging between 0.91% and 1.53% (Fig. 3).

The nutrient reabsorption of N followed a seasonal trend similar to that for the foliar N concentration. Absorption of N from senescing material was highest during winter when 32% of cultivated- and 23% of wild-plant N was reabsorbed (Fig. 3). Cultivated tea displayed a significantly higher N reabsorption than did wild tea in both seasons. The greatest difference between wild and cultivated rooibos was observed during the summer period.

In contrast to the N concentration, the leaf C values were higher in wild tea than in the cultivated tea, although this was significant only during the winter period. Seasonally, leaf C content was significantly lower during winter than in summer in cultivated tea. The C : N ratio was significantly higher in the wild plants than in cultivated plants. During summer, wild plants showed a comparatively higher (10%) C : N ratio (Fig. 3). The C : N ratio followed a similar trend for both wild and cultivated plants, with the highest values during summer and lowest in winter.

There was a distinct difference in the ability of wild and cultivated rooibos to fix atmospheric N via biological N_2 fixation (BNF). On average, BNF in wild rooibos plants was 2.3 times higher than that in the cultivated plants (Fig. 4). Differences between wild and cultivated rooibos were most prominent during summer when BNF in wild tea was approximately nine times higher. However, the lower BNF rates in the cultivated plants were not accompanied by similar decreases in foliar N concentrations. Both types displayed the same seasonal pattern, with significantly higher BNF during winter.

Wild and cultivated rooibos revealed substantial differences in $\delta^{13}C$ values, with wild tea showing significantly lower values. On average, wild tea had 1.04 times lower $\delta^{13}C$ values than did cultivated tea, with the most significant difference being observed during summer (Fig. 4). Both wild and cultivated plants displayed the same pattern, with the lowest $\delta^{13}C$ values occurring during winter and the maximum in summer.

Photosynthesis and water relations

There was a significant difference in photosynthetic rates between seasons and between types (wild and cultivated *A. linearis*). The lowest photosynthetic assimilation rates were recorded during the summer period for both wild and cultivated plants (Fig. 5). The wild type, however, had a higher photosynthetic rate than did the cultivated type during the summer period, whereas this was not the case during the winter. Cultivated tea had higher photosynthetic and
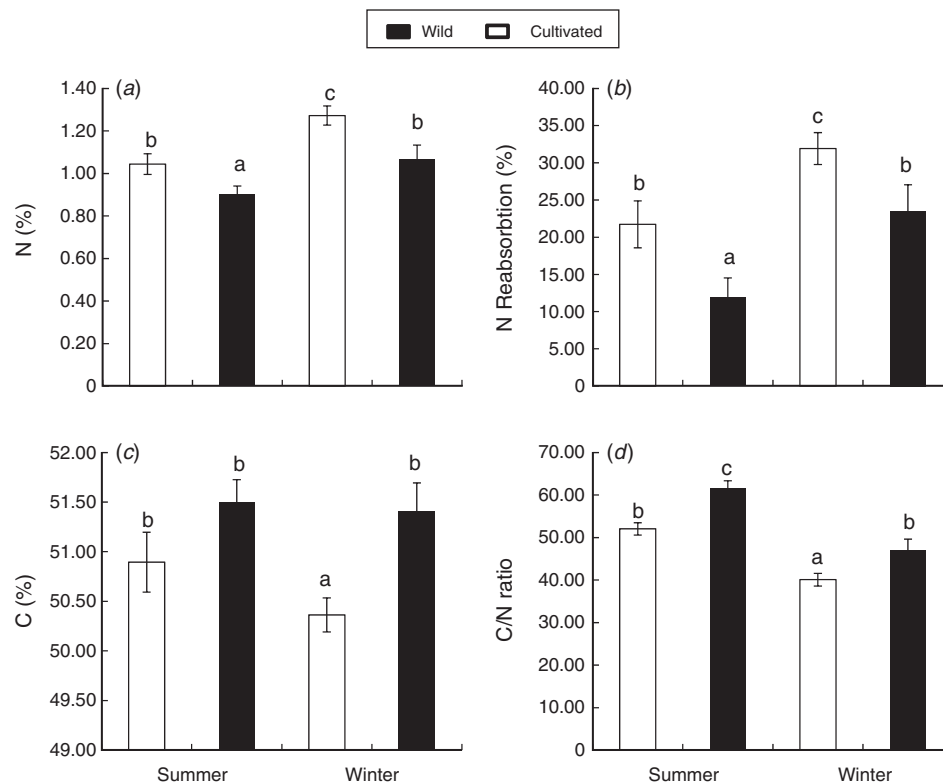
Fig. 3. Differences in (*a*) nitrogen (N) concentrations, (*b*) N reabsorption rates, (*c*) carbon (C) concentrations and (*d*) C : N ratio in leaves and stems of cultivated and wild *Aspalathus linearis* during late summer and winter (mean ± s.e.). The same letter above bars indicates that the treatments are not statistically significantly different from each other ($P \leq 0.05$, *post hoc* Tukey's HSD, multiple-range test, ANOVA; $n = 10$ means).
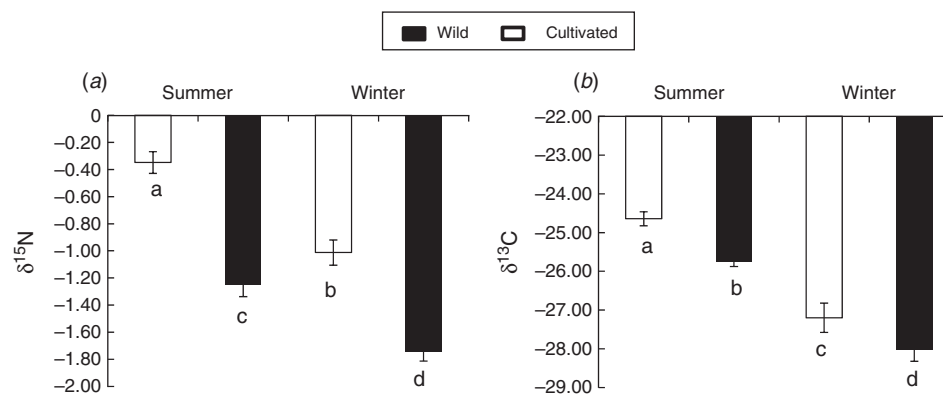

Fig. 4. Seasonal variation in stable carbon (C) and nitrogen (N) isotopic signatures for cultivated and wild *Aspalathus linearis*. (*a*) $\delta^{13}C$ values and (*b*) $\delta^{15}N$ during late summer and winter (mean ± s.e.). The same letter above bars indicates that the treatments are not significantly different from each other ($P \leq 0.05$, *post hoc* Tukey's HSD, multiple-range test, ANOVA; $n = 10$ means).

transpiration rates in winter. In both wild and cultivated rooibos, transpiration rates were higher in winter than in summer. Increases in photosynthetic rates were mostly accompanied by concomitantly higher stomatal conductances.

Both wild and cultivated rooibos exhibited lower WUE_i during the wet season. This coincided with the $\delta^{13}C$-based integrated-WUE variation between winter and summer. There was, however, no difference in instantaneous WUE between wild and cultivated rooibos during winter, although the

integrated WUE did show significantly higher WUE in cultivated plants. During the dry summer period, the wild plants demonstrated a significantly higher instantaneous WUE.

Soil N data revealed a significant difference in soil N concentration between wild and cultivated sites. Although the cultivated site had higher soil N than did the wild site, seasonal differences were not significant (Fig. 6). Soil N displayed a seasonal pattern similar to that of plant N, being highest during late winter. Soil C was significantly higher at the wild
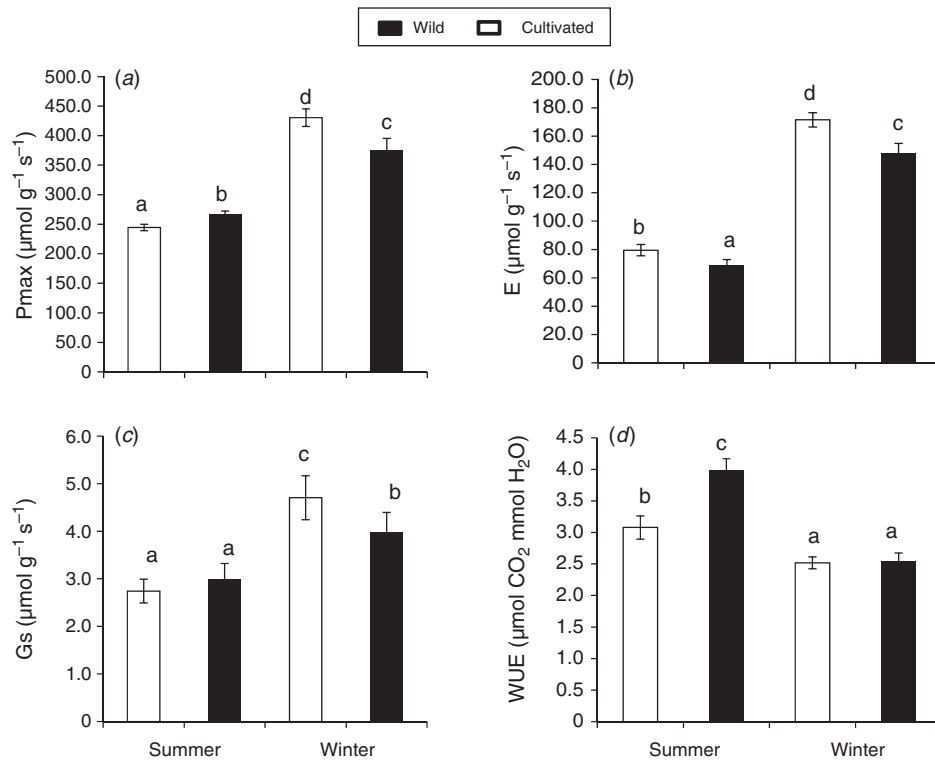
Fig. 5. Differences in (*a*) photosynthetic rate (P_{max}), (*b*) transpiration (*E*), (*c*) stomatal conductance (*G*s) and (*d*) water-use efficiency (WUE) in cultivated and wild *Aspalathus linearis* during late summer and winter (mean ± s.e.). The same letter above bars indicates that the treatments are not significantly different from each other ($P \leq 0.05$, *post hoc* Tukey's HSD, multiple-range test, ANOVA; $n = 6$ means).

Fig. 6. Differences in soil (*a*) nitrogen (N), (*b*) phosphorus (P) and (*c*) carbon (C) concentrations between the cultivated and wild sites during late summer and winter (mean ± s.e.). The same letter above bars indicates that the treatments are not significantly different from each other ($P \leq 0.05$, *post hoc* Tukey's HSD, multiple-range test, ANOVA; $n = 10$ means).
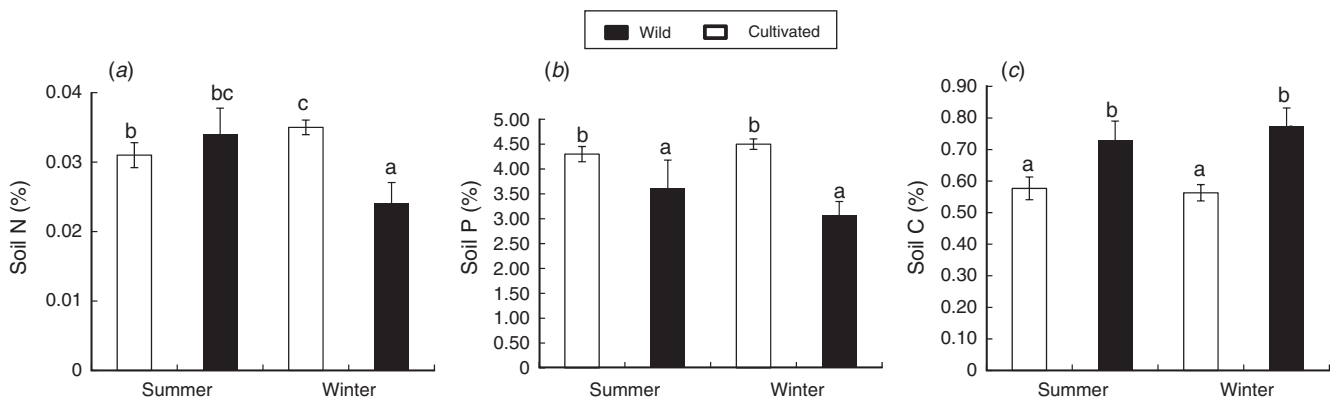
site than at the cultivated site during both seasons. Soil P, however, was significantly higher at the cultivated site. There were no significant differences between seasons for either soil C or P for the respective sites.

Discussion

Dry and wet seasonal variations in N cycling and gas exchange of wild and cultivated *A. linearis* legumes were investigated. It was found that the two types of rooibos tea had different

methods of adapting to N nutrition and C assimilation during wet and dry seasons.

The $\delta^{13}C$ comparison between the wet and dry season indicated that both wild and cultivated rooibos experienced water stress during the dry period of the year. Increasing aridity may cause stomatal closure, which can result in less discrimination against ^{13}C during photosynthesis, with associated elevated $\delta^{13}C$ values (Ehleringer 1994; Swap *et al.* 2004). However, on the basis of the significantly lower $\delta^{13}C$ values of the wild tea type than the cultivated type during

the dry period, it appears that the wild type was less water stressed.

This improved resistance of the wild type to drought may be related to the increased C : N ratio, as an indication of developing schlerophylly. Schlerophylly is often associated with dry climates as an adaptation to low water levels, but also to low nutrient availability (Groom and Lamont 1999; Gutschick 1999; Sardans *et al*. 2008). Bussotti *et al*. (1998, 2000) found that the level of sclerophylly increases during the summer months in response to adverse environmental field conditions. In this regard, both types had lower C : N during winter. Seasonal changes may also cause differences in nutrient availability, and this may be enhanced in nutrient-poor ecosystems such as the fynbos.

This effect is evident in the leaf N nutrition of both plants during the dry and wet seasons. The highest N concentrations were found in cultivated tea during winter (1.27%), whereas the wild tea during the arid summer had the lowest N concentrations (0.85%). These values are well within the range of other evergreen sclerophyllous mountain fynbos plants (Herppich *et al*. 2002). It has been shown that plants in Mediterranean regions take up N during the winter rainy season, before any above-ground growth, and that these minerals are stored in the old evergreen leaf tissue before being translocated to new growth in spring (Mooney and Rundel 1979). Similar seasonal patterns have been reported for other endemic fynbos species (Stock *et al*. 1987; Witkowski *et al*. 1990; Herppich *et al*. 2002). Maier *et al.* (1995) suggested that the mature leaves may function as sink organs during times of high nutrient availability and eventually reallocate this shared N to the newly formed sinks during spring growth.

Because cultivated tea plants were less reliant on BNF, they may have been able to conserve more N from their leaves through reabsorption, before abscission. In contrast, the wild plants relied more heavily on BNF and resorbed less N from leaves. This reliance of wild plants on BNF was particularly important in winter, when the soil N was poorer than in cultivated plant plots. Reabsorption from senescing material is primarily a N-conservation strategy (Eckstein *et al*. 1999; Aerts and Chapin 1999) and essential in assisting in alleviating nutrient scarcity. Although the values reported for N reabsorption in other fynbos species (35–70%) (Stock *et al*. 1987; Witkowski 1991) are somewhat higher, the reabsorption of N, coupled with the ability to fix N, is an important mechanism to overcome nutrient deficiency in this nutrient-constrained environment.

The effect of the dry season on symbiotic N$_2$ fixation was more evident in the cultivated than the wild plants, evidenced by the greater percentage decline in BNF during summer relative to winter, in the cultivated plants. Because these plants did not show a proportional increase in the reabsorption of leaf N, the remaining N had to be acquired from soil uptake via the roots. This increased reliance on soil N in the cultivated plants during summer may be the result of the lower energy costs associated with soil mineral uptake of N. N$_2$-fixing plants incur additional C costs for the synthesis of roots and nodules and maintenance of mycorrhizal fungi (Vance and Heichel 1991). Nodule initiation, growth, and activity are also more sensitive to moisture stress than are general root and shoot

metabolism (Albrecht *et al*. 1984). During the summer stress, this ability to switch N sources may be a distinct advantage.

The seasonal pattern in net assimilation rates, observed in *A. linearis*, coincided with the results obtained in other studies investigating schlerophyllous shrubs from Mediterranean climate regions (van der Heyden and Lewis 1989; Flexas *et al*. 2001). These studies showed lowest rates of photosynthesis during summer when photosynthesis is reduced by stomatal limitation, triggered by low water availability. According to Louw (2006), drought limits biomass production in *A. linearis*, as was confirmed in our study, by the lower photosynthetic rates of both wild and cultivated rooibos during the late summer months when aridity is most pronounced. However, during late winter, under favourable soil moisture conditions, cultivated tea had elevated gas-exchange rates compared with the wild tea. Conversely, during the dry summer months, the wild tea exhibited an increased ability to adapt to the limited soil-moisture conditions by sustaining higher photosynthetic rates. This was also accompanied by their ability to maintain higher WUEs, facilitated by effective control of transpirational water loss. This enhanced ability to adapt to dry conditions may be related to their higher sclerophylly index, which Turner (1994) argues to be an important protective mechanism to resist extreme climatic events. Bussotti *et al*. (2002) demonstrated that leaves with a higher level of sclerophylly are able to maintain their internal moisture status, even in extreme drought conditions.

The results of our study suggest that, under the present climate conditions, the wild plants are better able to tolerate summer drought, with greater water economy, and more reliance on BNF for N nutrition. These wild plants may therefore be better adapted to tolerate the warmer and drier conditions predicted for the next decades, which will underpin the survival of this legume species under current models of climate change.

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