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An assessment of symbiotic N nutrition in species of the genus *Aspalathus* endemic to the Cape Floristic Region of South Africa

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ABSTRACT

Nitrogen fixation is important for the growth and yield of nodulated legumes. In this study, the ^{15}N natural abundance technique was used to assess the symbiotic N nutrition of 15 *Aspalathus* species sampled from 13 locations. The results showed that all 15 species met part of their N requirements from N_2 fixation, as shown by the different levels of symbiotic dependency. Seven species, for example, derived 54 to 77% of their N nutrition from symbiosis, nine obtained 36 to 49% from fixation, and five species derived < 20% of their N from the atmosphere. More specifically, *A. acuminata* and *A. clada* both from Worcester Langerug derived 76.5% and 72.1% N, respectively, from symbiosis, *A. acuminata* from Rondebosch obtained 68.4% N, *A. acuminata* from Kalbaskraal 59.5% N, *A. aculeata* from Mamesbury 56.8% N, *A. divericata* from Penhill 56.6% N and *A. cordata* from Dutoitkloof 53.9% N from the atmosphere. In contrast, *A. zeyheri* sampled from Kokrivier derived only 4.3% of its N from symbiosis, followed by *A. capensis* (13.3%) and *A. carnosa* (14.6%) both from Silvermine. The C/N values found for the *Aspalathus* species in this study were unusually high for a legume, and could suggest an ecological adaptation to the low N soils of the Cape fynbos as under such N-poor plants metabolically switch from N-containing storage compounds to N-free storage molecules such as sucrose and glycerol, glucosides, galactoses and disaccharides.

1. Introduction

Aspalathus is a genus of the Leguminoseae family, with 291 species endemic to the fynbos of the Cape Floristic Region of South Africa, where the soils are acidic and low in nutrients, especially N, P and Ca (Maseko and Dakora 2013). The Cape Floristic Region has a mediterranean-type climate, with long warm, dry summers and cool, wet, short winters (Bradshaw et al. 2014; Slingsby et al. 2017). *Aspalathus* species occur over a wide geographic range in the western and south-eastern parts of the Western Cape Province, as well as in limited areas in the south-western part of the Northern Cape Province (Dahlgren 1988).

The species from the summer rainfall parts of South Africa, mostly the eastern region of the Cape Province, are few in numbers and are less studied and collected than the winter rainfall species of the western and southern Cape regions (Dahlgren 1988). Five new species have recently been described, and they include *A. theresae* (Cupido 2007); *A. crewiana* (Boatwright and Cupido 2011); *A. abbottii* (Stirton and Muasya 2011); *A. microlithica* and *A. quartzicola* (Curtis et al. 2013).

Due to the poor nutrient status of fynbos soils, especially plant-available N (0.001–0.002% N, or 1 to 2 mg N g^{-1} soil) and P (0.00004 to 0.00037% P, or 0.4–3.7 μg P g^{-1}) (Cramer 2010), most fynbos legumes have adopted various strategies (Maseko and Dakora 2013; Richardson et al. 2009) for meeting their nutritional requirements which include N_2 fixation, mycorrhizal infection, cluster root formation and change in root architecture. Maseko and Dakora (2015) have reported high symbiotic N dependency as nutritional strategy by wild legumes in the Cape fynbos, especially species of the genus *Cyclopia* and *Aspalathus*. Kanu and Dakora (2012) have similarly found high dependency of *Psoralea* species on N fixation for their N nutrition in the low N soils of the Cape fynbos soil. *Wiborgia, Wiborgiella* and *Polhillia* species, which are endemic to the Cape fynbos, also were reported to derive up to 91% of their N_2 needs from symbiotic nitrogen fixation (Mpai et al. 2020).

Although there is a huge population of the Leguminoseae in the Cape fynbos (Boatwright and Cupido 2011; Gerber and Hoffman 2012), it has been predicted that climate change and global warming are likely to result in a loss of plant species diversity, including members of the Leguminoseae. Therefore, ecophysiological studies are important for conserving these plant species in the Cape fynbos. Notwithstanding that some work has been done on symbiotic N_2 fixation in Fynbos legumes (Maseko and Dakora 2015; Mpai et al. 2020),

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Table 1
Agro-ecological description of the study sites used for plant and soil sampling from the Cape fynbos.

Districts	Province	Nearest town	Soil pH	Agro-ecological zone	Altitudes (m)	Annual rainfall (mm)
West Coast	Western Cape	Malmesbury	5.02	Cape Fynbos	135	490
West Coast	Western Cape	Kalbaskraal	4.23	Cape Fynbos	81	487
West Coast	Western Cape	Yzerfontein	5.65	Cape Fynbos	20	314
Cape Town	Western Cape	Rondebosch	4.09	Cape Fynbos	28	475
Overberg	Western Cape	Elim	4.56	Cape Fynbos	41	492
Overberg	Western Cape	Fernskloof	4.27	Cape Fynbos	110	577
Overberg	Western Cape	Kokrivier	4.95	Cape Fynbos	112	641
Cape Winelands	Western Cape	Worcester Langerug	4.76	Cape Fynbos	220	400
Cape Town	Western Cape	Silvermine	4.82	Cape Fynbos	347	1187
West Coast	Western Cape	Silwerstroomstrand	4.17	Cape Fynbos	48	300
Cape Winelands	Western Cape	Dutoitskloof	4.68	Cape Fynbos	820	770
Cape Winelands	Western Cape	Rawsonville	4.97	Cape Fynbos	223	567
Cape Town	Western Cape	Penhill	4.23	Cape Fynbos	89	470

however little is known of the genus *Aspalathus*, which is the largest in the Fabaceae Family in the Cape fynbos. So far, however, only about 70 species of the Leguminosae family have been evaluated for their N nutrition from symbiotic fixation (Sprent 2009). The aim of this study was to assess the symbiotic dependency and N contribution by *Aspalathus* growing in thirteen different locations of the Cape fynbos using ^{15}N natural abundance technique.

2. Materials and methods

2.1. Study site description

The study was conducted in the Cape Floristic Region in the Western Cape Province, which stretches from the West Coast district to the Cape Wineland district, (Table 1). Thirteen locations were selected for plant and soil sampling (namely; Silvermine, Malmesbury, Fernskloof Nature Reserve, Dutoitskloof, Kalbaskraal, Kokrivier, Rondebosch, Silwerstroomstrand junction, Rawsonville, Worcester Langerug, Elim and Penhill, Table 1). The soil features of the study sites varied from sand to sandy-loam soil with low nutrient levels.

The Malmesbury, Kalbaskraal and Yzerfontein sites comprised of farmer's fields located in the Cape West coast district with altitudes of 135 m, 81 m and 20 m, and mean annual rainfall of 490 mm, 487 mm and 314 mm, respectively. The Kokrivier and Elim sites were also farmer's fields located in the Overberg district with altitudes of 112 m and 41 m and mean annual rainfall of 661 mm and 492 mm, respectively, during the winter season from May to September each year (Table 1).

Fernskloof Nature Reserve is situated at an altitude of 110 m in the Overberg district of the Western Cape and has an annual mean rainfall of 577 mm. The Rondebosch, Silvermine and Penhill sites were Nature Reserves in the Cape Town district with altitudes of 28 m, 347 m and 89 m, and mean annual rainfall of 475 mm, 1187 mm and 470 mm in the winter season. The Rawsonville, Worcester and Dutoitskloof sites were farmer's fields located in the Cape Wineland district with altitudes 223 m, 220 m and 820 m, respectively (Table 1) and mean annual rainfall of 567 mm, 220 mm and 770 mm, respectively. Silwerstroomstrand junction is located in the West Coast district with an altitude of 48 m and mean annual rainfall of 300 mm.

2.2. Soil sample collection

Rhizosphere soil samples (0–30 cm) were collected from *Aspalathus* species at the 13 study sites in the Cape fynbos, placed in plastic bags, and taken to the laboratory. They were air-dried, sieved (2 mm-mesh), and a sub-sample used for measuring pH ($CaCl_2$) and mineral analysis. Soil samples collected from farms adjacent to each other or separated by a road, were pooled and processed before analysis.

2.3. Soil Analysis

The citric acid method by Du Plessis and Burger (1965) was used to determine exchangeable phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sodium (Na). A 20 g of the sieved, air-dried soil sample was extracted in 200 mL of 1% (w/v) citric acid, heated to 80°C, shaken for 2 min at 10 min intervals over 1h period, and filtered. A 50 mL aliquot was heated to dryness on a water bath, digested with 5 mL of concentrated HCl and HNO_3, evaporated to dryness on water bath, and 5 mL of concentrated HNO_3 and 20 mL of deionized water added. The mixture was then heated to dissolve the dry residue, and the sample filtered. Measurement of P was done directly by aspiration on a calibrated simultaneous inductively coupled plasma-mass spectrometer (IRIS/AP HR DUO Thermo Electron Corporation, Franklin, Massachusetts, USA).

2.4. Plant sample collection, processing, and analysis

Aspalathus shoots were sampled from the 13 study sites, placed in separate brown paper bags and transported to the Biological Nitrogen Fixation Laboratory, Tshwane University of Technology, Pretoria, where they were subsampled and oven-dried at 60°C for 72 h, weighed, milled (0.85 mm sieve size), and stored in vials prior to ^{15}N analysis. Non-legume plant species were sampled as reference plants and similarly processed as done for the *Aspalathus* species. Eleven plant species were sampled as reference plants from Elim and Dutoitskloof, 9 from Silvermine and 8 from Rondebosch and Fernskloof, 5 from Yzerfontein, N1-Rawsonville and Penhill, 15 from Malmesbury, 12 from Kokrivier, 3 from Silwerstroomstrand junction and 6 from Kalbaskraal and Worcester Langerug.

2.5. Measurement of N_2 fixation

2.5.1. $^{15}N/^{14}N$ isotopic analysis

The ratio of $^{15}N/^{14}N$ and the N concentration (%N) in plant shoots were determined with a Carlo Erba NA1500 Elemental Analyzer coupled to a Finnigan MAT 252 Mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) via Conflo II Open-Split Device at the University of Cape Town. A 2 mg finely ground legume sample was weighed into aluminium capsules, loaded onto the Carlo Erba system, combusted in evacuated quartz tubes in the presence of cupric oxide and metallic copper, and the resultant gases cleaned online before being introduced into the mass spectrometer. The reference plants were similarly analysed using the mass spectrometer.

Internal standards consisting of *Nasturtium* spp. and *Acacia* spp. were separately included in every five runs to correct for machine errors during isotopic fractionation. The ^{15}N natural abundance expressed as δ (delta) notation, which is the ‰ deviation of the ^{15}N

natural abundance of the sample from atmospheric N_2 (0.36637 atom % ^{15}N) was calculated as (Unkovich and Baldock 2008):

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

where $^{15}N/^{14}N_{sample}$ is the ratio of ^{15}N and ^{14}N abundance in the sample and $^{15}N/^{14}N_{atm}$ is the ratio of ^{15}N and ^{14}N abundance in the atmosphere.

2.5.2. Percent N derived from fixation (%Ndfa)

The proportion of N derived from atmospheric N_2 fixation was determined as (Unkovich et al. 2008):

$$0/0\text{Ndfa} = \frac{\delta^{15}N_{ref} - \delta^{15}N_{leg}}{\delta^{15}N_{ref} - B} \times 100$$

Where $\delta^{15}N_{ref}$ is the mean ^{15}N natural abundance of the reference plant, $^{15}N_{leg}$ is the mean ^{15}N natural abundance of the legume, and the B value is the ^{15}N natural abundance of the test legume grown in the glasshouse and was wholly dependent on N_2 fixation for its N nutrition. The B-value replaces the value of atmospheric N_2 as it incorporates the isotopic fractionation associated with N_2 fixation (Unkovich et al. 2008) and the B value used was a predetermined measurement of -2.0‰ (Muofhe and Dakora 1999).

2.8. Statistical analysis

All the data collected were tested for normal distribution before being analyzed using the STATISTICA program version 7.1. Symbiotic parameters such as $\delta^{15}N$ and %Ndfa were analyzed using a one-way ANOVA to compare species performance within each location. Where treatment means differed significantly, the Duncan's multiple range test was used to separate the means at $p \leq 0.05$.

3. Results

3.1. Analysis of rhizosphere soil

Analysis of rhizosphere soils collected from different *Aspalathus* species at 13 study sites revealed significant differences in macronutrients levels. The rhizosphere soil of *A. aspalathoides* from Fernskloof showed the highest Ca concentration, followed by *A. astroites* from Dutoitskloof and *A. cilliaris* from Kokrivier, with the lowest from *A. acuminata* at Kalbaskraal (Table 2). Potassium concentration range from 19 mg.kg^{-1} in the rhizosphere of *A. carnosa* at Silvermine to 130 mg.kg^{-1} at *A. astroites* at Dutoitskloof, followed by *A. aspalathoides* at Fernskloof (103 mg.kg^{-1}) as the second highest. Magnesium levels also ranged from 38 mg.kg^{-1} for *A. hispida* at Yzerfontein to 408 mg.kg^{-1} for *A. aspalathoides* at Fernskloof, followed by *A. astroites* at Dutoitskloof (256 mg.kg^{-1}) and *A. anus* at Penhill (209 mg.kg^{-1}). The concentration of P was highest in the rhizosphere soil of *A. acuminata* (56 mg.kg^{-1}) from Rondebosch, followed by *A. astroites* (33 mg.kg^{-1}) from Dutoitskloof and lowest for *A. anus* (3 mg.kg^{-1}) from Penhill. Sodium levels were highest in the rhizosphere of *A. aspalathoides* from Fernskloof followed by *A. spinosa* from Kalbaskraal, and lowest for *A. anus* from Penhill (Table 2).

Micronutrient concentration also differed between and among the rhizosphere soils from the different *Aspalathus* species (Table 2). The highest concentration of Cu was found in the rhizosphere soil of *A. astroites*, followed by *A. hispida*, and *A. acuminata* (Table 2). The concentration of Fe was highest in the rhizosphere of *A. acuminata* at Rondebosch, Kalbaskraal and Elim, and lowest in *A. hispida* from Yzerfontein. Similarly, Mn levels were highest in the rhizosphere soil of *A. hispida*, followed by *A. astroites* at Dutoitskloof and lowest in *A. divericata* from Yzerfontein (Table 2). Zinc concentration was also highest in the rhizosphere soil of *A. hispida* and *A. astroites* at

Table 2
Chemical properties of soils from the 13 study sites in the Cape fynbos

		Macronutrients					Micronutrients			
Location	species	Calcium	Potassium	Magnesium	Sodium	Phosphorus mg.kg^{-1}	Copper	Iron	Manganese	Zink
Silvermine	*A. cilliaris*	287 ± 42e-g	29 ± 3.75de	73 ± 4.50de	37 ± 14.43c-f	2.5 ± 0.29e	0.08 ± 0.00b	36.70 ± 7.48de	3.19 ± 0.40bc	0.30 ± 0.01c
Kokrivier	*A. cilliaris*	732 ± 12bc	44 ± 3.75de	126 ± 1.04cde	36 ± 0.87c-f	6 ± 0.00de	0.05 ± 0.00b	67.86 ± 0.81b-e	0.37 ± 0.05c	0.16 ± 0.01c
Rondebosch	*A. acuminata*	424 ± 17def	106 ± 49.07ab	7.0 ± 30.83de	49 ± 17.32b-e	56 ± 9.24a	1.34 ± 0.23a	126.90 ± 0.52a	3.42 ± 1.76bc	5.49 ± 1.95bc
Kalbaskraal	*A. acuminata*	128 ± 13g	41 ± 2.08de	61 ± 4.92e	25 ± 2.33c-f	13 ± 1.20cde	0.12 ± 0.02b	106.77 ± 1.90ab	0.90 ± 0.12c	0.50 ± 0.17c
Dutoitskloof	*A. hispida*	448 ± 86de	28 ± 1.44de	68 ± 6.24e	15 ± 3.75ef	18 ± 1.72cd	1.58 ± 0.26a	67.56 ± 23.06b-e	74.27 ± 67.82a	13.26 ± 7.63a
Elim	*A. hispida*	585 ± 83cd	95 ± 15.88abc	187 ± 18.01bcd	52 ± 3.75bcd	15 ± 3.46cde	0.31 ± 0.05b	106.78 ± 25.14ab	7.74 ± 3.42bc	0.58 ± 0.19c
Yzerfontein	*A. hispida*	192 ± 54e-g	28 ± 11.29e	38 ± 12.20e	8 ± 3.18f	13 ± 5.57cde	0.06 ± 0.02b	25.04 ± 19.38e	0.28 ± 0.05c	0.18 ± 0.03c
Silvermine	*A. juniperina*	181 ± 67e-g	50 ± 18.48cde	53 ± 20.44e	29 ± 11.84c-f	10 ± 0.58cde	0.07 ± 0.01b	64.41 ± 6.24b-e	1.18 ± 0.57c	0.28 ± 0.08c
Silvermine	*A. cordata*	204 ± 35e-g	26 ± 4.41e	84 ± 25.06de	17 ± 5.49d-f	7 ± 4.33de	0.11 ± 0.03b	30.96 ± 10.87de	8.34 ± 3.32bc	0.35 ± 0.14c
Silvermine	*A. carnosa*	222 ± 56e-g	19 ± 3.46e	45 ± 2.77e	13 ± 0.29ef	21 ± 6.35c	0.09 ± 0.00b	35.98 ± 2.44de	2.23 ± 0.24bc	1.09 ± 0.30c
Kokrivier	*A. zeyheri*	435 ± 76de	32 ± 6.93de	85 ± 16.97de	24 ± 3.18c-f	4 ± 0.58e	0.05 ± 0.00b	33.18 ± 6.11de	0.52 ± 0.25c	0.12 ± 0.01c
Malmesbury	*A. muraltoides*	176 ± 34e-g	79 ± 5.77bcd	55 ± 2.08e	12 ± 0.87f	4 ± 1.15e	0.18 ± 0.05b	85.21 ± 6.83abc	11.29 ± 6.46bc	1.13 ± 0.78c
Malmesbury	*A. aculeata*	365 ± 31d-g	100 ± 12.35abc	69 ± 10.46e	13 ± 2.65ef	5 ± 1.20de	0.25 ± 0.06b	73.16 ± 8.12bcd	40.40 ± 11.67abc	0.95 ± 0.24c
Kalbaskraal	*A. spinosa*	222 ± 0.00e-g	40 ± 2.60de	131 ± 7.97cde	78 ± 7.51ab	9 ± 0.29cde	0.14 ± 0.02b	103.88 ± 2.61ab	1.13 ± 0.19c	0.72 ± 0.24c
Yzerfontein	*A. divericata*	213 ± 59.60e-g	33 ± 12.35de	48 ± 17.72e	11 ± 3.52f	12 ± 5.36cde	0.09 ± 0.03b	37.14 ± 20.71de	0.38 ± 0.05c	0.23 ± 0.03c
Fernskloof	*A. aspalathoides*	1424 ± 21.62a	103 ± 14.15ab	408 ± 75.52a	99 ± 24.54a	13 ± 3.75cde	0.09 ± 0.01b	71.03 ± 22.64b-e	1.60 ± 0.52bc	0.33 ± 0.09c
Penhill	*A. anus*	162 ± 32.33fg	24 ± 3.18e	209 ± 107.73bc	5 ± 0.00f	3 ± 0.58e	0.43 ± 0.29b	26.26 ± 1.53de	2.54 ± 0.41bc	0.65 ± 0.47c
Dutoitskloof	*A. astroites*	834 ± 19.63b	130 ± 45.90a	256 ± 49.19b	57 ± 27.71bc	33 ± 4.62b	1.76 ± 0.62a	88.78 ± 30.28abc	51.34 ± 31.71ab	8.69 ± 4.75ab

Table 3
Soot δ^{15}N values of reference plants from the study sites

Sites of study	No. of reference plants	Shoot δ^{15}N (‰)				
		Minimum	maximum	Mean	Standard deviation	Standard error
Elim	11	1.18	3.44	1.42	1.43	0.43
Yzerfontein	5	2.28	8.09	4.86	5.48	2.45
Malmesbury	15	-0.65	6.54	3.10	8.08	2.09
N1-Rawsonville	5	1.87	3.23	2.62	1.03	0.46
Penhill	5	1.53	3.52	1.43	1.53	0.68
Rondebosch	8	2.63	5.88	4.33	3.03	1.07
Silvermine	9	-1.20	2.64	0.74	1.16	0.39
Silwerstroomstrand	3	1.99	4.32	3.15	1.65	0.95
Fernskloof	8	-0.25	3.66	0.99	3.56	1.26
Worcester Langerug	6	0.50	6.99	3.63	6.12	2.50
Dutoitskloof	11	-1.35	3.09	0.43	3.99	1.20
Kokrivier	12	-0.75	2.10	0.51	3.59	1.04
Kalbaskraal	6	1.01	2.74	2.06	1.46	0.6

Dutoitskloof and *A. acuminata* at Rondebosch, and lowest in *A. cilliaris* at Kokrivier (Table 2).

3.2. Shoot δ^{15}N of the reference plants

The branches of nine non-legume species were sampled at the Silvermine site as reference plants for estimating %Ndfa. The δ^{15}N values of these species ranged from -1.2‰ to +2.64‰, with a combined δ^{15}N mean of +0.74‰ (Tables 3 and S1). At Malmesbury, 15 reference plants collected for estimating %Ndfa recorded a mean δ^{15}N value of +3.10, with a range of -0.65‰ to +6.54‰ (Tables 3 and S1).

Eight non-legume plant species were sampled from the Rondebosch site as reference plants, and their δ^{15}N values ranged from +2.63 to +5.88 ‰ (Tables 3 and S1), with a combined δ^{15}N value of +4.33 ‰. At Elim, 11 non-legume species were collected as reference plants and their δ^{15}N values ranged from +1.18‰ to +3.44‰, with a combined mean δ^{15}N value of +1.85 (Tables 3 and S1).

At Dutoitskloof, 11 non-legume species were collected as reference plants and their δ^{15}N values ranged from -1.35 to +3.09‰, with a combined mean δ^{15}N value of +0.43‰. At Kokrivier, 12 non-legume species were sampled as reference plants with a range of -0.75 to +2.1‰, and a combined mean δ^{15}N value of +0.51‰ (Tables 3 and S1). At Kalbaskraal, non-legume plant species were sampled as reference plants. Their δ^{15}N values ranged from +1.01 to +2.74‰ with a combined mean δ^{15}N value of +2.06‰. Five non-legume species were collected as reference plants from each of Penhill, N1 Rawsonville and Yzerfontein sites. The δ^{15}N values obtained at Penhill was +1.53 to +3.52‰, with a combined mean δ^{15}N value of +2.43‰. At Rawsonville, the δ^{15}N values ranged from +1.87 to +3.23‰, with a combined mean δ^{15}N value of 2.62‰. At Yzerfontein, the δ^{15}N of reference plants ranged from +2.28 to +8.09‰, with a combined mean δ^{15}N value of +4.86‰ which was used to estimate %Ndfa (Tables 3 and S1).

Eight non-legume species were sampled from Fernskloof Nature Reserve as reference plants and their δ^{15}N values ranged from -0.25 to +3.66‰ with a combined mean δ^{15}N value of +0.99‰ (Tables 3 and S1). At Silwerstroomstrand junction, three non-legume plant species were collected as reference plants, their δ^{15}N values ranged from +1.99 to +4.32‰ with a combined mean δ^{15}N value of +3.15‰. At Worcester Langerug, six non-legume species were sampled as reference plants, and their δ^{15}N values ranged from +0.5 to +6.99‰ with a combined mean δ^{15}N value of +3.63‰. (Tables 3 and S1). The B-value that was used in this study was -2.0‰ (Muofhe and Dakora, 1999).

3.4. Shoot δ^{15}N of Aspalathus species

The ^{15}N isotopic analysis of *Aspalathus* shoots showed significant differences in δ^{15}N across locations, with values ranging from -0.88‰ to +3.66‰ (Table 4). *Aspalathus* species with the highest δ^{15}N values included, *A. hispida* and *A. divericata* from Yzerfontein, followed by *A. muraltoides* from Malmesbury and *A. spinosa* from Kalbaskraal (Table 4). The lowest δ^{15}N values ranged from -0.88‰ to -0.43‰ and were recorded by *A. cordata* from Dutoitskloof, and *A. acuminata* from Worcester Langerug, *A. acuminata* from Kalbaskraal and *A. clada* from Worcester Langerug (Table 4).

3.5. Symbiotic performance

The *Aspalathus* species with the lowest δ^{15}N values (namely, *A. acuminata, A. clada, A. acuminata* and *A. cordata*) derived the most N from atmospheric N_2 fixation. In contrast, those with much higher δ^{15}N values (namely, *A. hispida, A. divericata, A. muraltoides* and *A. spinosa*) showed the least dependency on symbiotic N_2 fixation for their N nutrition. Percent N derived from N_2 fixation differed markedly among the *Aspalathus* species, with a range of 4.3 to 76.5% across the sites sampled (Table 4). The highest N derived from fixation was by *A. acuminata,* and *A. clada* at Worcester Langerug, while the lowest N obtained from symbiosis was by *A. zeyheri* (4.3%) at Kokrivier and *A. capensis* (13.3%) at Silvermine. Of the 27 *Aspalathus* species studied, only six could derive over 50% (56.6 to 76.5%) of their N nutrition from symbiotic fixation. The remaining *Aspalathus* species obtained less than 50% of their N nutrition from symbiosis, and this ranged from 17.1% to 48.8% (Table 4).

3.6. Shoot C:N ratio of Aspalathus species

The C:N ratios differed among the *Aspalathus* species studied, and ranged from 16.0 to 53.6 g.g^{-1} across the locations (Table 4). The highest C:N ratios were found in *A. aspalathoides* from Fernskloof, *A. cilliaris* and *A. incurvifolia* from Kokrivier (Table 4). In contrast, the lowest C:N ratios were recorded in *A. wildenowiana* and *A. hispida* from Dutoitskloof, as well as in *A. bracteata* from Rawsonville. The remaining *Aspalathus* species exhibited intermediate C:N ratios ranging from 24.7 to 39.3 g.g^{-1} (Table 4).

3.7. %N in shoots of Aspalathus species

There were significant differences in shoot %N of *Aspalathus* species sampled across locations. The highest shoot %N was found in *A. wildenowiana* (2.8%) from Dutoitskloof, followed by *A. bracteata* (2.4%) from Rawsonville, and *A. hispida* (2.1%) from Dutoitskloof (Table 4). The lowest shoot %N values (0.89% to 1.08%) were found in *A. aspalathoides* from Fernskloof, *A. incurvifolia* and *A. cilliaris* from Kokrivier (Table 4). The remaining *Aspalathus* species exhibited

Table 4
Comparison of symbiotic performance of Aspalathus species from the Western Cape Province of South Africa. Mean values with dissimilar letters are significantly different at *** $p \leq 0.001$.

Location	Species	Shoot N %	Shoot δ^{15}N ‰	Ndfa %	C:N ratio g.g^{-1}
Silvermine	*A. juniperina*	1.23 ± 0.02g-k	0.05 ± 0.02d-g	25.08 ± 0.69e-j	39.26 ± 0.95bcd
Silvermine	*A. capensis*	1.55 ± 0.35d-h	0.38 ± 0.01c-f	13.30 ± 0.30i-k	36.73 ± 8.88b-e
Silvermine	*A. carnosa*	1.47 ± 0.06d-i	0.78 ± 0.26cde	14.6 ± 9.54jk	33.34 ± 1.06c-f
Kokrivier	*A. zeyheri*	1.19 ± 0.04h-k	0.40 ± 0.06c-f	4.32 ± 2.56k	39.94 ± 1.96bc
Kokrivier	*A. incurvifolia*	1.01 ± 0.05jk	-0.21 ± 0.13efg	28.53 ± 5.00e-j	47.19 ± 2.11ab
Kokrivier	*A. cilliaris*	1.08 ± 0.24ijk	0.10 ± 0.01c-g	23.08 ± 3.50f-j	52.89 ± 12.23a
Malmesbury	*A. muraltoides*	1.90 ± 0.08cd	1.26 ± 0.40bc	36.05 ± 7.88d-i	24.74 ± 0.48e-h
Malmesbury	*A. aculeata*	1.80 ± 0.14c-f	0.20 ± 0.52c-g	56.81 ± 10.14a-d	24.83 ± 1.15e-h
Kalbaskraal	*A. spinosa*	1.49 ± 0.14d-i	1.06 ± 0.47bcd	24.57 ± 11.46e-j	33.91 ± 3.40c-f
Yzerfontein	*A. divericata*	1.47 ± 0.18d-i	1.94 ± 1.46b	42.48 ± 21.23d-g	33.67 ± 3.96c-f
Fernskloof Nature Reserve	*A. aspalathoides*	0.89 ± 0.03k	-0.38 ± 0.11efg	45.96 ± 3.74c-f	53.58 ± 1.97a
Worcester Langerug	*A. clada*	1.44 ± 0.04e-j	-0.43 ± 0.21efg	72.10 ± 3.77ab	30.66 ± 1.10c-g
Dutoitskloof	*A. astroites*	1.47 ± 0.04d-i	-0.40 ± 0.01efg	21.87 ± 0.39f-k	29.01 ± 1.00c-g
Dutoitskloof	*A. wildenowiana*	2.77 ± 0.05a	-0.01 ± 0.21d-g	18.04 ± 8.68g-k	15.99 ± 0.46h
Elim	*A. pinguin*	1.53 ± 0.02d-h	0.33 ± 0.07c-g	39.49 ± 1.73d-h	30.70 ± 1.22c-g
N1 Rawsonville	*A. bracteata*	2.43 ± 0.17ab	0.54 ± 0.14c-f	45.07 ± 3.03c-f	19.73 ± 1.44gh
Silvermine	*A. cordata*	1.65 ± 0.14d-g	-0.30 ± 0.42efg	37.98 ± 15.28d-i	27.56 ± 2.53d-g
Silwerstroomstrand junction	*A. hispida*	1.77 ± 0.17c-f	0.74 ± 0.27cde	46.84 ± 5.24c-f	26.73 ± 2.65e-h
Rondebosch	*A. acuminata*	1.88 ± 0.21cde	0.01 ± 0.07d-g	68.37 ± 1.15abc	25.56 ± 2.67e-h
Kalbaskraal	*A. acuminata*	1.53 ± 0.06d-h	-0.35 ± 0.12efg	59.48 ± 2.90a-d	30.89 ± 1.41c-g
Yzerfontein	*A. hispida*	1.42 ± 0.07f-j	3.68 ± 0.03a	17.12 ± 0.48h-k	33.66 ± 1.76c-f
Worcester Langerug	*A. acuminata*	1.47 ± 0.07d-i	-0.68 ± 0.09fg	76.53 ± 1.66a	28.49 ± 1.55c-g
Dutoitskloof	*A. cordata*	1.31 ± 0.03g-k	-0.88 ± 0.25g	53.90 ± 10.21a-d	35.39 ± 0.93cde
Dutoitskloof	*A. hispida*	2.12 ± 0.13bc	-0.163 ± .13d-g	24.43 ± 5.49e-j	22.24 ± 1.37fgh
Elim	*A. hispida*	1.65 ± 0.10d-g	-0.03 ± 0.30d-g	48.77 ± 7.85b-e	28.78 ± 1.60c-g
Elim	*A. acuminata*	1.47 ± 0.13d-i	0.25 ± 0.12c-g	41.52 ± 2.99d-h	31.17 ± 3.44c-g
Penhill	*A. divericata*	1.57 ± 0.08d-h	-0.08 ± 0.07d-g	56.57 ± 1.52a-d	31.80 ± 1.38c-f
F-statistic		9.41***	6.55***	7.41***	6.48***

intermediate shoot N concentrations ranging from 1.90 to 1.19% in *A. muraltoides* from Malmesbury and *A. zeyheri* from Kokrivier (Table 4).

4. Discussion

Mineral nutrition is a major challenge to plants in the Cape fynbos, especially N_2-fixing legumes which require more mineral nutrients for plant growth and N_2 fixation than non-legumes (Belane, et al. 2011). Soils of the Cape fynbos are very low in nutrients, especially N and P (Cramer 2010; Maseko and Dakora 2017). However, legumes in this nutrients-poor environment have developed adaptations for survival which include biological N_2 fixation. The genus *Aspalathus* has 281 species and is one of the largest group of plants in the Cape fynbos. Although some studies have been done on the nodulation biology of this genus (Dakora 1998; Sprent 2009), the number of species involved are rather few. The aim of this study was to assess the relative dependency of *Aspalathus* species on N_2 fixation for their N nutrition.

The ^{15}N natural abundance method was used, with a wide range of reference plants analysed, for estimating percent N derived from fixation (Tables 3 and S1). Although as part of the adaptation to low nutrients, fynbos plants can be mycorrhized, leading to difficulties in estimating N_2 fixation using the^{15}N natural abundance technique (Spriggs et al. 2003), the δ^{15}N values of reference plants (Table 3 and S1) and *Aspalathus* species was sufficiently high enough to permit estimation of N_2-fixation (Unkovich et al. 1993; Sanford et al, 1994). Shoot N concentration, which is a measure of nutrition, varied strongly among the *Aspalathus* species, with a range of 0.89% for *A. aspalathoides* from Fernskloof Nature Reserve to 2.77% for *A. wildenowiana* from Dutoitskloof (Table 4).

There appeared to be no direct relationship between shoot %N and δ^{15}N and or percentage N derived from fixation. For example, although *A. wildenowiana* recorded 2.77% N in shoot which was the highest, the percentage N obtained from fixation was only 18.04%. Similarly, *A. hispida* from Dutoitskloof had a high (2.12%) shoot N but derived only 24.43% N from fixation. In contrast, *A. acuminata* from Worcester Langerug revealed leaf N concentration of 1.47% and yet obtained 76.5% N from atmospheric fixation (Table 4). Although shoot δ^{15}N values were generally very low, the %Ndfa values were not high (Table 4), especially when compared to similar δ^{15}N values obtained with grain legumes. This could be attributed to the fact that mycorrhizal infection of the *Aspalathus* species discriminated against the δ^{15}N isotope in favour of ^{14}N isotope, and made the shoot δ^{15}N even more negative (Spriggs et al. 2003). Similar results have been reported in Australia (Sanford et al. 1994). Although all the *Aspalathus* species studied obtained N from symbiosis, the levels of dependency on N_2 fixation for their N nutrition differed markedly (Table 4). For example, %Ndfa ranged from 4.3% for *A. zeyheri* at Kokrivier to 76.5% for *A. acuminata* at Worcester Langerug. Of the 27 locations where *Aspalathus* species were sampled, only seven recorded %Ndfa values greater than 50% (Table 4). In that order, *A. cordata* from Dutoitskloof obtained 53.9% N from fixation, *A. divericata* from Penhill 56.6%, *A. aculeata* from Malmesbury 56.8%, *A. acuminata* from Kalbaskraal 59.4%, *A. acuminata* from Rondebosch 68.3%, *A. clada* from Worcester Langerug 72.1% and *A. acuminata* from Worcester Langerug 76.5% N (Table 4). Whether sampled from Kalbaskraal, Rondebosch or Worcester Langerug, *A. acuminata* was one of the *Aspalathus* species that derived over 50% of its N nutrition from symbiosis, a clear indication of its better adaptation to different zones within the Cape Fynbos Floristic Region.

It was interesting to note that different *Aspalathus* species occurring in the same location could differ in symbiotic performance. For example, *A. capensis, A. carnosa* and *A. juniperina*, which collected from Silvermine, showed significantly different δ^{15}N values, and hence percent N derived from fixation. Similarly, *A. muraltoides* and *A. aculeata* from Malmesbury also differed markedly in %Ndfa values (Table 4). These variations in symbiotic performance can be attributed to the type and symbiotic effectiveness of the rhizobia nodulating each *Aspalathus* species in the same location. While inter-site differences in symbiotic performance can also be attributed to

differences in N_2-fixing efficacy of the native rhizobia nodulating *Aspalathus*, an additional factor could also be the levels of mineral nutrients in the soil. For example, the rhizosphere soil of *A. hispida* collected from Yzerfontein was low in Ca, K, Mg, Na, Fe, and, to some extent, Mn when compared to Elim. As a result, the δ^{15}N of *A. hispida* from Yzerfontein was much higher and its %Ndfa significantly lower relative to Elim (Table 4).

The C/N ratio in plants is a direct measure of the relationship between C accumulation from photosynthesis and N nutrition from symbiosis and/or soil N uptake. Previous studies have shown that N_2-fixing species tend to have C/N values less than 24 $g.g^{-1}$ and non-fixing plants more than 24 $g.g^{-1}$ (Mohale et al. 2014). In this study, the C/N ratios ranged from 16 $g.g^{-1}$ for *A. wildenowiana* collected from Dutoitskloof to 53.6 $g.g^{-1}$ for *A. aspalathoides* sampled from Fernskloof Nature Reserve. Furthermore, 24 out of the 27 *Aspalathus* plants sampled had C/N ratios greater than 24 $g.g^{-1}$, indicating that the C/N values found for the *Aspalathus* species were unusually high for a legume. In fact, 16 out of the 27 *Aspalathus* plants studied, had C/N ratios greater than 30 $g.g^{-1}$, the highest being 53.6 $g.g^{-1}$ (Table 4).

A close scrutiny of the relationship between %Ndfa and C/N ratios showed that *Aspalathus* species such as *A. zeyheri* from Kokrivier, *A. capensis* from Silvermine, *A. carnosa* from Silvermine and *A. hispida* from Yzerfontein had low %Ndfa values (4.3, 13.3, 14.6 and 17.1% respectively) and high C/N ratios (39.9, 36.7, 33.3 and 33.7 $g.g^{-1}$) greater than 24 $g.g^{-1}$. But *A. clada* from Worcester Langerug, *A. acuminata* from Kalbaskraal, *A. divericata* from Penhill and *A. cordata* from Dutoitskloof, which showed high %Ndfa values (72.1, 59.5, 56.6 and 53.9%, respectively), also recorded high C/N ratios (30.7, 30.9, 31.8 and 35.4 $g.g^{-1}$, respectively) greater than 24 $g.g^{-1}$ (Table 4). These results can be interpreted to mean that regardless of their levels of N nutrition from symbiotic fixation, many members of the genus *Aspalathus* have high C/N ratios, which is not typical of N_2-fixing legumes (Mohale et al. 2014).

A few studies have however shown that, as an adaptation to low soil N conditions, some plant species can increase their C/N ratios without decreasing their growth rate by metabolically switching from N-containing storage compounds to N-free storage molecules such as sucrose and polyols, glycerol, glucosides and galactoses, disaccharides and dimethylphonio-propionate, 4V-B screening compounds like phlorotamins and flavonoids, as well as free radical scavangers such as ascorbate, linear polyols and tannins (Adams et al. 2010). *Aspalathus linearis* is the most studied member of the genus *Aspalathus* because it is the source of Rooibos tea, herbal beverage. The shoots of *A. linearis* and the Rooibos tea beverage contain high levels of phenolics such as aspalathin (dihydrochalcone C-glucoside), aspalanin (a cyclic dihydrochalcone), nothofagin (a rare dihydrochalcone C-glucoside), flavones (e.g. dihydro-orientin, dihydro-isoorientin and hemiphlorin) and flavonols such as quercetin, hyperoside, isoquercetin, ratinand quercetin-3-O-robinobioside (Van Heerden et al. 2003; McKay and Blumberg 2007). Therefore, as an adaptation to the low-N fynbos soils, members of the genus *Aspalathus* store photosynthetic C in the form of phenolics, which are N-free. However, the high C/N ratios found in shoots of the different *Aspalathus* species in this study could also be viewed as a response to the rising atmospheric CO_2 with climate change (Rosenzweig et al. 2008).

In conclusion, there was marked variation in shoot δ^{15}N values between and among the *Aspalathus* species studied, which led to differences in percent N derived from fixation. There was also a location and species effect on in the symbiotic performance of plants, with same species showing variation in symbiotic functioning with location, as well as with different species exhibiting a range of symbiotic performance in the same location. The C/N ratios of *Aspalathus* species differed markedly and were generally greater than 24 $g.g^{-1}$, which suggested that *Aspalathus* stores photosynthetic C as N-free phenotics.

Declaration of Competing Interest

The authors declare that they have no competing interests

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2023.05.023.

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