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Plant growth and N_2 fixation in *Cyclopia longifolia* (Vogel L.) supplied with mineral nutrients in pot and field experiments

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ABSTRACT

Cyclopia longifolia Vogel L. is indigenous to the Cape fynbos, and widely grown for the production of Honeybush tea, a beverage with health benefits and economic potential in the cosmetic and nutraceutical industries. The aim of this study was to assess plant growth and symbiotic performance of *Cyclopia longifolia* in field and pot experiments. Field plants were supplemented with different levels of P, Mg and Ca at Kanetberg mountains using 0, 5, 25 and 50 mM of K_2HPO_4, $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ in split application, while P, Ca and Mg were applied as Supergrow (20.3% P), $CaMg \cdot (CO_3)_2$, and $MgSO_4 \cdot 7H_2O$) at 0, 10, 20 and 50 $kg \cdot ha^{-1}$ to potted plants. Shoots were harvested at 240 and 300 days after last fertilisation for pot and field experiments respectively, and analyzed for nodulation and N_2 fixation. The data revealed significantly increased nodule number, nodule dry weight, root and shoot biomass in the pot experiment with P, Ca and Mg supply. Shoot dry matter, N content, amount of N-fixed, soil N uptake and tea yield of *C. longifolia* was significantly increased with increasing supply of P, Mg and Ca to field plants, and was highest at 50 mM level of each element. Applying different levels of Mn, Cu, Zn and Mo to field plants of *C. longifolia* also markedly increased shoot biomass, N content, $\delta^{15}N$, amount of N-fixed, soil N uptake and tea yield. The increase in $\delta^{15}N$ values, and hence reduced %Ndfa, of *Cyclopia longifolia* plants supplied with P, Mg, Ca, Mn, Cu, Zn and Mo suggests that the endogenous soil concentration of these nutrients were more limiting for plant growth than for nodule functioning. As a result, supplying P, Mg, Ca, Mn, Cu, Zn and Mo increased plant growth and biomass, but not %N derived from fixation.

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1. Introduction

Cyclopia longifolia Vogel L. is a shrub legume belonging to the family Leguminosae (Fabaceae). It grows in the wild in acidic, nutrient-poor soils (Kies, 1951; Schutte, 1995; Joubert et al., 2007) of the coastal plains and mountainous areas of the Western Cape, as well as the wetter parts of Eastern Cape of South Africa (Joubert et al., 2011). The annual harvesting of shoots as tea constitutes a major pathway of nutrient depletion of soils in the Cape fynbos (Joubert et al., 2010a, 2010b). This species is nodulated by *Bradyrhizobium, Mesorhizobium* and *Burkholderia* strains (Elliott et al., 2007; Beukes et al., 2013). Thus, symbiotic N_2 fixation is a major source of N among *Cyclopia* species. However N_2 fixation in legumes can be hampered by lack of nutrients (O'Hara et al., 1988). For example, P affects the synthesis of molecules which transcribe bacterial nod genes (Dakora and Le Roux, 1995), and is thus needed for nodulation and N_2 fixation (Tang et al., 2001; Valverde and Wall, 2002). Calcium also elicits the release of nod gene inducers (Richardson et al., 1988; Werner and Hohl, 1990), and can reduce the adverse effect of acidic soils on nodule formation (Reeve et al., 1993). Mineral elements such as Mg and Mo and Mn are also important for legume nodulation and N_2 fixation (Heckman et al., 1993; Fageria et al., 2008; Izaguirre-Mayoral and Sinclair, 2009). Copper and Zn can also limit nodulation and plant growth in legumes (Riley and Dilworth, 1985; Zhang et al., 1998; Andrew et al., 2001).

Although some studies have evaluated the symbiotic performance of *Aspalathus linearis* (Muofhe and Dakora, 1999a, 1999b) and *Cyclopia* species (Spriggs et al., 2003; Spriggs and Dakora, 2007, 2008) in the Cape Floristic Region of South Africa, none has assessed the effect of mineral nutrient supplementation on the relative dependency of *C. longifolia* on atmospheric N_2 fixation for its N nutrition. The objective of this study was to assess the symbiotic performance of *C. longifolia* supplied with different levels of P, Mg, Ca, Mn, Cu, Zn and Mo in pot and field experiments.

2. Materials and methods

2.1. Site description and experimental design

2.1.1. Pot experiment

The study was conducted in the glasshouse at Tshwane University of Technology (TUT) in Pretoria from March 2009 to April 2010, using soil

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collected from TUT Research farm near Pretoria. The soil contained 5 mg·kg^{-1} P, 13 mg·kg^{-1} K, 163 mg·kg^{-1} Mg, 725 mg·kg^{-1} Ca, 7.1 mg·kg^{-1} S, 15 mg·kg^{-1} Na, 323.4 mg·kg^{-1} Fe, 18.2 mg·kg^{-1} Zn, 9.0 mg·kg^{-1} Cu, 624.3 mg·kg^{-1} Mn, and 0.67 mg·kg^{-1} B, with pH ($CaCl_2$) 5.92.

Each pot was filled with 28 kg soil. The pots were arranged using a randomised complete block design by random numbers from a GenStat Release 7.22 DE, 2008 programme. Four replicate pots were used per treatment. Plantlets developed from cuttings were transplanted at a rate of one plant per pot. At 150 days after transplanting, different levels of P, Ca and Mg were applied as 0, 10, 20 and 50 kg·ha^{-1} of supergrow (20.3% P), $CaMg.(CO_3)_2$, and $MgSO_4 \cdot 7H_2O$.

2.1.2. Field experiment

The study was conducted on a commercial Honeybush tea-producing farm at Kanetberg about 6 km from Brandrivier located at longitude 33°S and latitude 20°W. The mean annual rainfall is 486 mm. Ten healthy plants were selected per row as one replicate in a completely randomised manner within a one-year-old tea plantation. Four replicates were used, and three macronutrients (i.e., P, Mg and Ca) in the form of K_2HPO_4, $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ were split-applied at four levels (i.e. 0, 5, 25 and 50 mM) at 365 days after transplanting. The nutrients were prepared in de-ionised water as single-nutrient solutions and applied at 100 mL per plant in three split-applications in November 2008, March 2009 and July 2009 over 9 months.

The micronutrients Cu, Mo, Mn and Zn were also split-applied as solutions of $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $MnSO_4 \cdot 2H_2O$ and $ZnSO_4 \cdot 7H_2O$, respectively at four levels (namely, 0, 0.5, 1.0 and 1.5 mM) at100 mL per plant over 9 months in November 2008, March 2009 and July 2009.

2.2. Plant growth and nodulation in pot experiment

In the pot experiment, whole plants were washed free of soil and separated into nodules, roots, stems and leaves to determine biomass yield per pot. These organs were put into brown paper bags and oven-dried at 60 °C for 48 h to constant weight. The mass of the various plant parts were recorded as dry matter yield. The nodules were counted and dry nodule mass also determined.

In the field experiment, the plants were harvested by cutting stems about 2 cm above ground level. Only the shoots (stems and leaves) were harvested and air-dried. Representative sub-samples were oven-dried to determine dry mass of leaves and stems (or shoots).

2.3. Plant harvest and processing

At 240 days after mineral application to the pot experiment, and 300 days to the field experiments, *C. longifolia* plants (leaves and stems) were harvested and oven-dried at 60 °C for 48 h to constant weight, and ground into fine powder (<0.85 mm sieve). Smaller plant samples (e.g. nodules and roots) from the pot experiment were ground using a pestle and mortar. The ground samples from both pot and field experiments were stored in vials prior to ^{15}N analysis.

2.3.1. ^{15}N isotopic analysis

About 3.0 and 2.0 mg, sample of *Cyclopia* and the non-fixing reference plant samples were respectively weighed into tin capsules (Elemental Microanalysis Ltd.,UK) and analyzed for $\delta^{15}N$ and % N using a Carlo-Erba NA1500 elemental analyzer (Fisons Instruments SpA, Strada, Rivoltana, Italy) coupled to a Finan MAT252 mass spectrometer (Finnigan, MAT CombH, Bremen, Germeny) VIA A CONFLO II open-split device.

The $\delta^{15}N$ value was expressed as per mil excess of ^{15}N relative to the atmospheric N_2 (= 0.36637 atom % ^{15}N) as described by Junk and Svec (1958) or (Mariotti et al., 1981):

$$\delta^{15}N = \frac{atom\%\,^{15}Nsample - atom\%\,^{15}Nair}{atom\%\,^{15}Nair} * 1000.$$

To correct errors associated with ^{15}N isotope analysis, internal standards of *Nasturtium* were included for every eight runs. The percentage of plant N derived from atmospheric fixation (%Ndfa) was determined as (Shearer and Kohl, 1986; Unkovich et al., 2008):

$$\%Ndfa = \left\{ \frac{\delta^{15}N_{ref} - \delta^{15}N_{leg}}{\delta^{15}N_{ref} - B} \right\} \times 100$$

where $\delta^{15}N_{ref}$ is the natural abundance of reference plants, $\delta^{15}N_{leg}$ is the ^{15}N natural abundance of the test legume, and B is the ^{15}N natural abundance of test legume solely dependent on N_2 fixation for its N nutrition. The B value replaces atmospheric N_2 as it incorporates the isotopic fractionation associated with N_2 fixation (Shearer and Kohl, 1986). In this study, the mean B values used to calculate %Ndfa ranged from −1.71 to 0.35 ‰ (Spriggs, 2004).

Different reference plant species were sampled and their combined mean $\delta^{15}N$ value also used for estimating %Ndfa. In the field experiment, shoots of Protea (*Protea neriifolia*), red bearded Protea (*Protea grandiceps*), bracken fern (*Pteridium aquilinum*), strictum female (*Leucadendron salicifolium*), and strictum male (*Leucadendron sabulosum*) were analyzed as reference plants for their ^{15}N isotopes. The combined mean $\delta^{15}N$ of these reference plants (+2.91‰) was used for estimating %Ndfa of *C. longifolia* shoots. The reference plants used in the pot experiment included red amaranth (*Amaranthus cruentus*), spider flower (*Cleome spinosa*), jute (*Corchorus olitorius*) and nightshade (*Solanum retroflexum*). The combined mean $\delta^{15}N$ of shoots of these reference plants (+8.23‰) was used to estimate %Ndfa of potted *C. longifolia* plants.

The amount of N-fixed was calculated as (Shearer and Kohl, 1986; Maskey et al., 2001):

$$N-fixed = \%Ndfa \times \text{legume biomass N}$$

where legume biomass N is the N content of shoots.

Whole-plant ^{15}N natural abundance of potted plants was calculated as an average of the ^{15}N natural abundance values of all shoots and roots weighted by their respective total N accumulated as (Robinson et al., 2000):

$$\delta^{15}N_{whole\ \ plant} = \frac{\sum\left(\delta^{15}N_{root} \times N_{root} + \delta^{15}N_{shoot} \times N_{shoot}\right)}{\sum(N_{root} + N_{shoot})}.$$

2.3.2. Nitrogen content

The N content of plant samples was calculated as the product of %N and sample mass (Pausch et al., 1996).

2.3.3. Soil N uptake

The soil N uptake was calculated as the difference between plant total N and N-fixed.

2.4. Statistical analysis

The data on nodulation, plant growth and symbiotic performance data from pot and field experiments were subjected to analysis of variance (ANOVA) using a STATISTICA 2007 programme, version 7.1 (Statsoft Inc., Tulsa, OK, USA). A One-Way ANOVA was used to compare treatments in nodulation, plant growth, N content, %Ndfa, N-fixed and

Table 1
Chemical and physical characteristics of Kanetberg soil used in this study. These data were obtained from Matie Taljaard, a Honeybush farmer at Kanetberg mountains in 2009.

Chemical properties	
pH(KCl)	5.0
Calcium	2009 ($mg \cdot kg^{-1}$)
Magnesium	463 ($mg \cdot kg^{-1}$)
Potassium	64.0 ($mg \cdot kg^{-1}$)
Sodium	74.9 ($mg \cdot kg^{-1}$)
Phosphorus	53.8 ($mg \cdot kg^{-1}$)
Copper	0.3 ($mg \cdot kg^{-1}$)
Zinc	1.1 ($mg \cdot kg^{-1}$)
Manganese	6.1 ($mg \cdot kg^{-1}$)
Boron	0.3 ($mg \cdot kg^{-1}$)
NH_4^+	4.18 ($mg \cdot kg^{-1}$)
NO_3^-	<1.00 ($mg \cdot kg^{-1}$)
Physical properties	
Texture	Sand
Water-holding capacity	35.5 ($g \cdot 100^{-1}$)
Sand	10%
Medium	44%
Fine	30%
Clay	4%
Silt	12%

Values are averages of duplicate runs.

soil N uptake. Where significant differences were found, the Tukey HSD test was used to separate treatment means at $p \leq 0.05$.

3. Results

3.1. Soil analysis

Texturally, the Kanetberg soil was sandy with pH 5.0. Except for Ca and Mg which recorded 0.2 and 0.05% respectively, the concentrations of the other mineral nutrients were very low (Table 1).

3.2. Nodulation in potted C. longifolia plants

There were significant differences ($p \leq 0.05$) in nodulation (nodule number and nodule mass) of *C. longifolia* supplied with P, Mg and Ca in potted soil (Table 2). Compared to control, the *C. longifolia* plants exhibited much higher nodulation at 50 $kg \cdot ha^{-1}$ levels of P, Mg and Ca (Table 2). Due to the deep rooting system, nodulation of *C. longifolia* plants was not assessed in the field experiment.

3.3. Plant growth and N content

Plants of *C. longifolia* supplied with P, Mg and Ca showed a marked increase in the dry matter yield of shoots and whole plants in the pot experiment (Table 2). In the field, shoot dry matter also increased significantly with the application of P, Mg, Ca, Mn, Cu, Zn and Mo to *C. longifolia* plants, and was highest at the 1.5 mM and 50 mM levels for trace and major elements, respectively (Table 3). Similarly, N content also increased markedly at 1.5 mM and 50 mM respectively for trace elements and major nutrients (Table 3).

3.4. $\delta^{15}N$ and %Ndfa values

One-Way ANOVA analysis of data from *C. longifolia* plants supplied with P, Mg and Ca in potted soil revealed marked ($p \leq 0.05$) increases in $\delta^{15}N$ of organs, which resulted in much lower %Ndfa values with increasing nutrient application (Table 2). Although whole-plant $\delta^{15}N$ increased with increasing supply of Mg and Ca, no significant differences were observed with P application (Table 2). The application of P, Mg, Ca, Mn, Cu, Zn and Mo to *C. longifolia* plants in the field also resulted in higher $\delta^{15}N$ values, and hence lower %Ndfa values, with increasing nutrient supply (Tables 3 and 4).

3.5. Amount of N-fixed

Because plant biomass increased with increasing application of each nutrient element, the amount of N-fixed also increased in both pot and field experiments (Tables 2 and 3). Although P and Ca applications increased the amounts of N-fixed, Mg showed a decrease (Table 3).

3.6. Soil N uptake

Despite the increased amounts of N-fixed with rising levels of each mineral nutrient (Tables 3 and 4), soil N uptake was also increased in both field and pot experiments with higher mineral application (Tables 2, 3 and 4). In both field and pot experiments, increased fertilisation with P, Mg, Ca, Mn, Cu, Zn or Mo resulted in greater soil N uptake (Tables 2, 3 and 4).

3.7. Tea yield

Applying different concentrations of P, Mg, Ca, Mn, Cu, Zn and Mo to *C. longifolia* plants in the field, caused a gradual but significant accumulation of biomass, which resulted in increased tea yield when compared to the zero-control treatment (Tables 2, 3 and 4).

Table 2
Nodule number, dry matter yield, N content, symbiotic performance and tea yield of *Cyclopia longifolia* treated with phosphorus, magnesium and calcium in the pot experiment, Pretoria, South Africa from 2009 to 2010. Values (Means ± SE) with dissimilar letters are significantly different at *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.001$ and ns = not significant.

Treatment	Nodule number $plant^{-1}$	Nod Dm ($g.plant^{-1}$)	Root Dm ($g.plant^{-1}$)	Shoot Dm ($g.plant^{-1}$)	N content ($mg\ plant^{-1}$)	Shoot $\delta^{15}N$ (‰)	whole-plant $\delta^{15}N$ (‰)	Ndfa (%)	N-fixed ($mg.plant^{-1}$)	Soil N uptake ($mg.plant^{-1}$)
Phosphorus										
P_0	268 ± 1d	1.43 ± 0.02d	15.3 ± 1.1c	25.7 ± 1.5d	212.8 ± 28.1c	1.03 ± 0.03b	2.2 ± 0.3a	77.8 ± 0.3a	165.7 ± 22.2c	47.2 ± 6.0b
P_{10}	309 ± 3c	2.67 ± 0.06c	19.5 ± 2.1bc	36.9 ± 0.2c	303.6 ± 16.9c	0.87 ± 0.05b	1.6 ± 0.1a	79.5 ± 0.5a	241.4 ± 14.9bc	62.2 ± 2.0b
P_{20}	454 ± 9b	2.95 ± 0.12b	25.8 ± 1.7b	44.3 ± 1.2b	449.2 ± 37.8b	1.78 ± 0.03a	2.5 ± 0.1a	69.7 ± 0.4b	298.9 ± 38.0b	129.8 ± 15.7b
P_{50}	816 ± 6a	3.65 ± 0.05a	47.5 ± 2.8a	68.6 ± 0.7a	1248.7 ± 46.3a	2.08 ± 0.39a	2.6 ± 0.4a	66.5 ± 4.2b	514.1 ± 31.6a	269.2 ± 63.9a
F-statistics	2066***	164.04***	50.7***	317.6***	193***	8.59***	2.6 ns	8.6***	28.4***	9.4***
Magnesium										
Mg_0	268 ± 1c	1.43 ± 0.02c	15.3 ± 1.1b	26.72 ± 0.96b	212.8 ± 28.1b	1.03 ± 0.03c	2.11 ± 0.34b	78 ± 1a	165.70 ± 22.20a	47.15 ± 5.97b
Mg_{20}	642 ± 8b	1.82 ± 0.08b	21.4 ± 0.7b	36.14 ± 0.68b	248.6 ± 12.7b	2.40 ± 0.22b	3.01 ± 0.10b	63 ± 2a	156.19 ± 7.21a	92.42 ± 8.93b
Mg_{50}	725 ± 14a	2.93 ± 0.17a	47.6 ± 4.6a	73.61 ± 0.16a	328.1 ± 2.4a	5.33 ± 0.69a	5.42 ± 0.63a	31 ± 7b	88.83 ± 14.14b	207.12 ± 40.48a
F-statistics	657***	50.18***	38.3***	1303.7fac6***	11**	27.24***	16.85***	27**	7.07***	11.63***
Calcium										
Ca_0	268 ± 1c	1.43 ± 0.02c	15.3 ± 1.1b	25.72 ± 1.52c	212.8 ± 28.1b	1.03 ± 0.03b	2.01 ± 0.26b	78 ± 0.3a	165.70 ± 22.20a	47.15 ± 5.97c
Ca_{10}	702 ± 5b	2.97 ± 0.53b	18.6 ± 1.4b	35.94 ± 0.39b	348.1 ± 5.1a	2.08 ± 0.37ab	2.24 ± 0.32b	66 ± 4ab	230.79 ± 10.67a	117.35 ± 15.80b
Ca_{50}	811 ± 6a	4.75 ± 0.30a	26.7 ± 2.4a	42.51 ± 0.08a	402.1 ± 43.6a	2.74 ± 0.39a	3.49 ± 0.47a	59 ± 4b	242.24 ± 42.37a	159.86 ± 3.67a
F-statistics	4211***	22.31***	11.7***	86.68***	10.5**	7.69**	4.89*	7.69**	2.13 ns	32.53***

Table 3
Dry matter yield, N content, symbiotic performance and tea yield of *Cyclopia longifolia* treated with phosphorus, magnesium and calcium in field experiment at Kanetberg, South Africa. Values (means ± SE) with dissimilar letters are significantly different at ****p ≤ 0.001.

Treatment (mM)	Shoot dry matter	N-content	$\delta^{15}N$	Ndfa	N-fixed	Soil N uptake		Tea yield
	g·plant^{-1}	mg·plant^{-1}	‰	%	kg·ha^{-1}	mg·plant^{-1}	kg·ha^{-1}	kg·ha^{-1}
Phosphorus								
P_0	351 ± 26b	4087 ± 68b	-0.27 ± 0.04b	81 ± 1a	45.8 ± 1.2b	786.3 ± 33.3b	10.9 ± 0.5b	3505 ± 257b
P_5	545 ± 69ab	4427 ± 160b	-0.24 ± 0.02b	80 ± 1a	51.8 ± 2.3b	935.3 ± 64.0b	13.0 ± 0.9b	5450 ± 686ab
P_{50}	651 ± 58a	10,229 ± 156a	0.08 ± 0.02a	72 ± 1b	74.6 ± 4.2a	2104.5 ± 98.3a	29.2 ± 1.4a	6505 ± 582a
F-statistics	8***	654***	52.72***	53***	28.7***	105.2***	105.2***	8***
Magnesium								
Mg_0	351 ± 26b	4087 ± 68d	-0.27 ± 0.04b	81 ± 1a	45.85 ± 1.23bc	786.34 ± 33.32d	10.92 ± 0.46d	3505 ± 257b
Mg_5	513 ± 26ab	4761 ± 143c	0.16 ± 0.06a	70 ± 1b	40.17 ± 1.34c	1252.48 ± 69.30c	17.40 ± 0.96c	5132 ± 265ab
Mg_{25}	559 ± 35ab	8013 ± 125b	0.06 ± 0.00a	72 ± 0b	52.34 ± 1.93b	1438.95 ± 46.02b	19.99 ± 0.64b	5590 ± 350ab
Mg_{50}	626 ± 67a	13,379 ± 324a	0.18 ± 0.05a	69 ± 1b	69.65 ± 3.96a	2191.86 ± 50.38a	30.44 ± 0.70a	6255 ± 670a
F-statistics	3*	495***	25.96***	26***	28.77***	129.41***	129.41***	3*
Calcium								
Ca_0	351 ± 26b	4087 ± 68b	-0.27 ± 0.04b	81 ± 1b	45.85 ± 1.23c	786.34 ± 33.32bc	10.92 ± 0.46bc	3505 ± 257b
Ca_5	639 ± 77ab	4020 ± 254b	-0.37 ± 0.04bc	83 ± 1ab	41.62 ± 0.73c	604.18 ± 43.68c	8.39 ± 0.61c	6394 ± 773ab
Ca_{25}	866 ± 69a	5387 ± 135a	-0.39 ± 0.03c	84 ± 1a	58.61 ± 3.50b	822.28 ± 83.89b	11.42 ± 1.17b	8665 ± 690a
Ca_{50}	1005 ± 193a	5985 ± 281a	-0.04 ± 0.01a	75 ± 0c	74.29 ± 3.23a	1785.37 ± 73.53a	24.80 ± 1.02a	10,049 ± 1927a
F-statistics	7***	47***	22.91***	23***	34.97***	73.35***	73.35***	7***

4. Discussion

Cyclopia longifolia showed significantly increased root and shoot growth, higher nodulation, and greater amounts of N-fixed in plants supplied with P, Mg and Ca in a pot experiment. The application of P, Mg, Ca, Mn, Cu, Zn and Mo to *C. longifolia* plants in the field similarly resulted in increased plant biomass, amount of N-fixed and soil N uptake, but significantly lower percent N derived from fixation (Tables 3 and 4). Many of these mineral nutrients are reported to have specific effects on legume plant growth, symbiotic establishment, nodule formation and N_2 fixation (Gates and Muller, 1979; Cassman et al., 1980; Bethlenfalvay and Yoder, 1981). As found in other studies (Kliewer and Kennedy, 1978; Togay et al., 2008), supplying Mo to *Cyclopia longifolia* plants increased plant growth and symbiotic performance. The increased nodulation of *Cyclopia longifolia* with Ca supply in this study is consistent with the reported Ca^{2+} requirement for root hair infection and nodule formation in symbiotic legumes (Lowther and Loneragan, 1968; Munns, 1970). As found in this study, Mn promotes plant growth, nodulation, and symbiotic performance in legumes (Fageria et al., 2008).

Fertilising field plants of *Cyclopia longifolia* with P, Mg, Ca, Mn, Cu, Zn and Mo significantly ($p \le 0.05$) increased the $\delta^{15}N$ values of whole-plants (Table 3), an effect which was also observed when potted plants were supplemented with P, Mg and Ca (Table 2). This could be interpreted to mean that the soil concentrations of these nutrients were more limiting for plant growth than for nodule functioning. As a result, supplying P, Mg, Ca, Mn, Cu, Zn and Mo increased plant growth and biomass, but not percent N derived from fixation. As found with other symbiotic legumes (Gurley and Giddens, 1969; Franco and Munns, 1981; Ishizuka, 1982; Brodrick and Giller, 1991), Mo application

Table 4
Dry matter yield, symbiotic performance and tea yield of *Cyclopia longifolia* treated with manganese, copper, zinc and molybdenum in a farmer's field at Kanetberg, South Africa. Values (Mean ± SE) with dissimilar letters are significantly different at ***p ≤ 0.01 and ns = non-significant.

Treatment (mM)	Shoot	N-content	$\delta^{15}N$	Ndfa	N-fixed	Soil N uptake		Tea yield
	g·plant^{-1}	g·plant^{-1}	‰	%	kg·ha^{-1}	mg·plant^{-1}	kg·ha^{-1}	kg·ha^{-1}
Manganese								
$Mn_{0.0}$	351 ± 26a	4087 ± 68b	-0.27 ± 0.04c	81 ± 1a	45.85 ± 1.23c	786.34 ± 33.32c	10.92 ± 0.46c	3505 ± 257a
$Mn_{0.5}$	367 ± 17a	4900 ± 138b	0.03 ± 0.02b	73 ± 1b	51.81 ± 1.51b	1379.09 ± 42.15b	19.15 ± 0.59b	3674 ± 174a
$Mn_{1.0}$	373 ± 22a	6277 ± 382a	0.16 ± 0.02a	70 ± 1c	55.79 ± 1.47b	1736.06 ± 62.72a	24.11 ± 0.87a	3728 ± 491a
$Mn_{1.5}$	437 ± 31a	6723 ± 376a	0.09 ± 0.03ab	72 ± 1c	65.87 ± 3.00a	1895.09 ± 127.80a	26.32 ± 1.77a	4373 ± 683a
F-statistics	2 ns	19***	47.82***	48***	19.02***	41.78***	41.78***	2 ns
Copper								
Cu_0	351 ± 26b	4087 ± 68c	-0.27 ± 0.04b	81 ± 1a	45.8 ± 1.2c	786.3 ± 33.3c	10.9 ± 0.5c	3505 ± 257b
$Cu_{1.0}$	465 ± 52ab	5781 ± 123b	-0.13 ± 0.08ab	77 ± 2ab	64.1 ± 0.9b	1385.2 ± 147.1b	19.2 ± 2.0b	4654 ± 518ab
$Cu_{1.5}$	564 ± 59a	6522 ± 163a	-0.05 ± 0.03a	75 ± 1b	77.8 ± 2.8a	1863.5 ± 93.8a	25.9 ± 1.3a	5637 ± 588a
F-statistics	5**	101***	4.68***	5***	75.6***	27.7***	27.7***	5**
Zinc								
Zn_0	351 ± 26a	4087 ± 68c	-0.27 ± 0.04b	81 ± 1a	45.8 ± 1.2b	786.3 ± 33.3c	10.9 ± 0.5c	3505 ± 257a
$Zn_{0.5}$	412 ± 27a	4988 ± 82b	0.28 ± 0.01a	67 ± 0b	44.4 ± 0.9b	1584.9 ± 24.1b	22.0 ± 0.3b	4119 ± 267a
$Zn_{1.5}$	439 ± 45a	8585 ± 187a	0.29 ± 0.03a	67 ± 1b	70.3 ± 2.2a	2554.8 ± 132.3a	35.5 ± 1.8a	6908.3 ± 447a
F-statistics	2 ns	367***	118.1348***	118***	88.8***	122.6***	122.6***	2 ns
Molybdenum								
Mo_0	351 ± 26a	4087 ± 68c	-0.270 ± 0.038b	81 ± 1a	45.85 ± 1.23c	786.34 ± 33.32d	10.92 ± 0.46d	3505 ± 257a
$Mo_{0.5}$	496 ± 30b	4832 ± 209c	0.006 ± 0.019a	741 ± 0b	43.79 ± 0.52c	1125.04 ± 28.17c	15.63 ± 0.39c	4960 ± 300b
$Mo_{1.5}$	560 ± 29b	5869 ± 299b	0.047 ± 0.038a	73 ± 1b	56.98 ± 1.49b	1549.03 ± 96.69b	21.51 ± 1.34b	5604 ± 295b
$Mo_{1.5}$	569 ± 27b	9925 ± 532a	0.035 ± 0.053a	73 ± 1b	81.93 ± 2.30a	2201.38 ± 180.09a	30.57 ± 2.50a	5688 ± 275b
F-statistics	13***	64***	14.903***	15***	132.04***	34.05***	34.05***	93.12***

in this study also promoted plant growth and nodulation in *Cyclopia longifolia*. This Mo requirement in nodule formation is consistent with the finding that Mo and Fe are both required for nitrogenase biosynthesis in N_2-fixing nodules (Rubio and Ludden, 2008).

The growth and symbiotic response of *C. longifolia* plants to supplementation with P, Mg, Ca, Mn, Cu, Zn and Mo clearly indicate that, in the Cape fynbos, growth of many legumes is limited by low mineral concentrations. This was evidenced by the greater uptake of applied elements by the test plants in both field and potted experiments (data not shown). The low mineral availability in fynbos soils has implications for the Honeybush tea industry, as continued harvesting of foliage for making tea would lead to reduced plant growth in subsequent years, and therefore lower tea yield for local consumption and export. Furthermore, fertilisation of *C. longifolia* induced an increase in the uptake of other minerals in addition to the applied nutrients (data not shown), and this can accelerate the depletion of soil nutrients in the fynbos.

In this study, the $\delta^{15}N$ values of *C. longifolia* shoots increased with increasing mineral concentrations. As a result, the %Ndfa values also steadily decreased with increasing mineral concentrations. Whether this was due to inhibition of symbiotic functioning by the higher levels of applied nutrients, remains to be determined. However, the symbiotic response of *C. longifolia* to supplementation with P, Mg, Ca, Mn, Cu, Zn and Mo is consistent with the results obtained by Muofhe and Dakora (1999a, 1999b) for *Aspalathus linearis subsp. linearis* supplied with different levels of N, P and Ca under field and glasshouse conditions. As found in this study, applying 5, 25 and 50 mM Ca to *A. linearis* (another fynbos legume) promoted plant growth and increased $\delta^{15}N$ in shoots, leading to lower %Ndfa and smaller amounts of N-fixed (Muofhe and Dakora, 1999a, 1999b). The observed increase in dry matter yield of fynbos legumes with fertilisation contradicts the generalised notion that growth of plants adapted to nutrient-poor soils is genetically pre-determined by the low-nutrient conditions of their environment.

New experiments involving the use of mixed nutrient fertilisers are however needed to determine the fertiliser requirements of *C. longifolia* plants and other Honeybush tea species. In conclusion, the supply of P, Mg and Ca promoted plant growth, and increased shoot N content, $\delta^{15}N$, amount of N-fixed and soil N uptake, but decreased percent N derived from fixation. These data suggest that moderate supply of P, Mg, Ca, Mn, Cu, Zn and Mo to *C. longifolia* can stimulate plant growth and increase tea yields in farmers' fields.

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