Photosynthesis, water-use efficiency and $\delta^{13}C$ of five cowpea genotypes grown in mixed culture and at different densities with sorghum

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Abstract

A field experiment involving two planting densities (83,333 and 166,666 plants per ha), two cropping systems (monoculture and mixed culture) and five cowpea [*Vigna unguiculata* L. (Walp.)] genotypes was conducted at Nietvoorbij (33°54S, 18°14E), Stellenbosch, South Africa, to select cowpea material with superior growth and water-use efficiency (WUE). The results showed significantly higher photosynthetic rates, stomatal conductance and transpiration in leaves of plants at low density and in monoculture due to greater chlorophyll (Chl) levels relative to those at high density and in mixed culture. As a result, C concentration in leaves and the amount of C, P, K, Ca, Mg, Fe, Cu, Zn, Mn, and B accumulated in shoots at low density and under monoculture were also much higher. Even though no marked differences in photosynthetic rates were found between and among the five cowpea genotypes, leaf C concentration and shoot C, P, K, Ca, Mg, Fe, Cu, Zn, Mn, and B contents differed considerably, with Sanzie exhibiting the highest C concentration and C, P, K, Ca, Mg, Fe, Cu, Zn, Mn, and B contents in shoots, followed by Bensogla and Omondaw, while ITH98-46 and TVu1509 had the lowest shoot concentration and contents of C, P, K, Ca, Mg, Fe, Cu, Zn, Mn, and B. WUE (calculated as photosynthate produced per unit water molecule transpired) was significantly greater in plants at low density and monoculture relative to those at high density and in mixed culture. Isotopic analysis revealed significant differences in $\delta^{13}C$ values of sorghum [*Sorghum bicolor* L. (Moench.)] and cowpea, with higher $\delta^{13}C$ values being obtained for plants at low density and in monoculture relative to those at high density or in mixed culture. The five cowpea genotypes also showed significant differences in $\delta^{13}C$ values, with Sanzie exhibiting the most negative value (*i.e.* low WUE) and ITH98-46, the least negative $\delta^{13}C$ value (*i.e.* high WUE). Whether measured isotopically or from gas-exchange studies, sorghum (a C_4 species) exhibited much higher WUE relative to cowpea (a C_3 species). Both correlation and regression analyses revealed a positive relationship between WUE from gas-exchange studies and $\delta^{13}C$ values from isotopic analysis of cowpea and sorghum shoots.

Additional key words: chlorophyll; cultivars; discrimination; gas exchange; intercropping; isotope; monocropping; plant stand; species; varieties.

Introduction

Carbon nutrition in plants *via* photosynthesis is the second most important physiological process after N nutrition (Drake *et al.* 1997), as at least 90% of plant dry matter is derived from photosynthetic CO_2 reduction (Zelitch 1982). Efficient translocation of photosynthate from source to sink organs is the key factor driving plant growth and increasing crop yields (Goldberg *et al*. 1983, Tollenaar and Daynard 1982, Dong *et al*. 1991). One of the major macromolecules important for photosynthetic functioning is chlorophyll; it is responsible for harvesting light energy for conversion to chemical energy *via* photo-assimilation (Tanaka *et al*. 1998). Several factors affect photosynthetic C yield; these include temperature, light, CO_2 concentration, mineral nutrition and water needed for photolysis during photosynthesis (Usada *et al*. 1985, Drake *et al*. 1997, Balakrishnan *et al*. 2000, Cakmak and Engels 1999). Photosynthesis is also reported to be affected by sink strength as increased photosynthate supply is required to meet increasing demand for photo-assimilates during early vegetative growth and seed

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Abbreviations: *A* – photosynthetic rate; Chl – chlorophyll; Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; C_i – internal CO_2 concentration; DMSO – dimethylsulphoxide; *E* – transpiration rate; g_s – stomatal conductance; WUE – water-use efficiency.

development (Richards 2000). Agronomic practices such as intercropping (or mixed culture) and planting density also affect photosynthesis and plant biomass growth (Hooper 1998, Akunda 2001, Andersen *et al*. 2005, Srinivasan *et al*. 1985, Horton 2000, San-oh *et al*. 2006). Photosynthesis is generally decreased with intercropping (mixed culture) due to reduced light penetration from shading. Net photosynthesis, stomatal conductance and transpiration are also reported to be reduced in *Atriplex prostrate* plants growing at high density relative to low density (Wang *et al*. 2005).

Carbon isotope discrimination during photosynthesis, measured as ^{13}C natural abundance ($\delta^{13}C$), is an indicator of WUE in C_3 plants (Farquhar *et al*. 1989). The combined use of data from gas-exchange and isotopic measurements of ^{13}C permits the selection of crop genotypes with enhanced photosynthetic activity and greater WUE for increased yields. An increase in our understanding of the photosynthetic process should also permit the manipulation of source capacity in crop species for increased photoassimilate supply to sink organs in cropping systems for increased crop yields.

In Africa, cowpea is the most important food grain legume adapted to a wide range of soil ecologies. However, its yield is constrained by a number of factors, such as insect pests and diseases. African farmers depend largely on natural rainfall for crop yields; but the rainfall is erratic and often leads to poor crop yields. Increasing cowpea production requires the selection of cowpea genotypes with improved drought tolerance and water relations. Because cowpea is usually cultivated as an intercrop with millet or sorghum with high plant density, the best way to select drought-tolerant genotypes for small-scale farmers in South Africa would therefore be to assess their water relations against a background of intercropping and high plant density (the most common agronomic practices used by rural African farmers).

In this study five cowpea genotypes were evaluated for plant growth and WUE using mixed culture and high plant density as background treatments. Plant photosynthesis was measured by leaf gas exchange and as shoot C accumulated at early podding, while WUE was measured as $\delta^{13}C$, and as photosynthate produced per unit water molecule transpired.

Materials and methods

Site description: The study was conducted at the Agricultural Research Council (ARC) Nietvoorbij station in Stellenbosch, South Africa, during the 2005 and 2006 summer seasons. Because the data were similar between the two years, only those of 2006 are reported here. The site is located at 33°54′S and 18º14′E with an elevation of 146 m above mean sea level. The mean annual rainfall was 713.4 mm, and the potential evapotranspiration (ET_O) as measured by Penman Monteith (Monteith 1965) was 1,573 mm. The mean annual day and night temperatures were 22.6ºC and 11.6ºC respectively and the mean monthly radiation 544 MJ m^{-2}. The experimental sites had a history of table grape cultivation with a moderate application of P fertilizer (80 kg ha^{-1} maxfos, 20% P). According to the Soil Classification Working Group, the field soil used for this study is a sandy loam classified as Glenrosa, Hutton form (SCWG 1991) equivalent to skeletic leptosol in the FAO soil classification system (FAO 2001). Prior to planting in each year, four soil samples (0–20 cm soil depth) were collected from each of the 88 experimental plots, pooled, and subsamples were used for chemical analysis. The soil chemical properties before planting are summarized in Table 1.

Experimental design: The experimental treatments included five cowpea genotypes (namely, Bensogla, Sanzie, Omondaw, ITH98-46 and TVu1509), two cowpea densities (83,333 *vs*. 166,666 plants per ha), and two cropping systems (monoculture *vs*. mixed culture). Intercropping and monoculture are the two most commonly used cropping systems in Africa, with intercropping depicting high plant density, and monoculture, low density. A completely randomised block design was used with a 3-factorial arrangement. Four replicate plots were used, each measuring 3.6 m × 3.2 m. Cowpea plants in monoculture were sown with a row-to-row spacing of 60 cm and plant-to-plant distance of 40 cm for the low plant density treatment, while a row-to-row spacing of 60 cm, and plant-to-plant distance of 20 cm were used for high plant density. Sorghum plants in plots with row-to-row spacing of 90 cm and plant-to-plant distance of 40 cm were maintained at a density of 55,555 plants per ha. In mixed culture, cowpea was sown at a row-to-row spacing of 90 cm and plant-to-plant distance of 26.6 cm to obtain the low plant density. Similarly, row-to-row spacing of 90 cm with plant-to-plant distance of 13.3 cm was used to achieve high plant density in mixed culture. Cowpea seeds were inoculated with *Bradyrhizobium* strain CB756 and planted together with sorghum. The seedlings were later thinned to two per stand. Weeding was done manually with a hoe. The plants were irrigated every three days up to flowering stage, and then the frequency reduced to once every seven days until harvest. Soil moisture supply through irrigation was necessary as the study was done during the dry summer season, when temperatures and evaporative demands are both high.

Chl determination: Chl was extracted from each of four trifoliate leaves per plot (n = 16 per treatment) using dimethyl sulphoxide (DMSO), as described by Hiscox and Israelstam (1979). At 67 days after planting (DAP),

Table 1. Soil chemical properties before planting. Each value (mean ± SE, $n = 4$) was obtained from 88 pooled soil samples collected from different points within replicate plots during 2005 and 2006.

Treatment	pH	P	K	Ca	Mg	S	Na [mg kg^{-1}]	Fe	Cu	Zn	Mn	B
Year												
2005	6.2±0.03	18.8±1.8	137.8±4.8	70.5±1.8	16.6±0.5	4.2±0.2	90.5±2.3	253.4±9.6	3.7±0.1	3.4±0.2	8.8±0.3	0.5±0.0
2006	6.2±0.03	18.6±1.8	136.6±4.8	68.7±1.8	15.5±0.5	4.0±0.2	90.4±2.3	255.9±9.7	3.6±0.1	3.2±0.2	8.6±0.3	0.3±0.0
One-way *ANOVA* (F-Statistics)												
	1.30	0.01	0.03	0.51	2.60	0.44	0.00	0.03	4.30	0.54	0.21	120.70

healthy flag leaves were sampled from the field for Chl analysis. The trifoliate leaves were cut into small 1 mm pieces, and 100 mg of the leaf tissue weighed into a 15-ml vial containing 7 ml DMSO, and incubated at 4°C for 72 h. Following incubation, the extract was diluted to 10 ml with DMSO, and 3 ml of extract used to read the absorbance at 645 nm and 663 nm on a spectrophotometer (*UV/Visible Spectrophotometer, Pharmacia LKB, Ultrospec II E*, Lockrom, England) against a DMSO blank.

Chl levels were calculated using equations by Arnon (1949) with unit of mg l^{-1}:

Chl $a = 12.7D_{663} - 2.69D_{645}$
Chl $b = 22.9D_{645} - 4.68D_{663}$
Total Chl $= 20.2D_{645} + 8.02D_{663}$

Photosynthetic gas-exchange measurements: At 67 DAP, A, g_s, C_i, and E were measured in four young leaves (flag leaves) per plot for each species using a portable infrared red gas analyser (*LCpro+ 1.0 ADC, Bioscientific Ltd*., Hoddesdon, Hertfordshire, UK). Measurements were made from 8 to 11 h and from 14 to 16 h for each replicate plot per day. Leaves were allowed 4–5 min to acclimate to the light environment in the chamber. Without troubleshooting, each measurement took approximately 2 min, which was the minimum time allowed for the readings to stabilize before they were recorded. Measurements were taken with the following conditions in the leaf chamber: photosynthetic photon flux density (PPFD) = 1100 µmol(quantum) m^{-2} s^{-1}, relative humidity = 44%, leaf vapour pressure deficit = 1.83 kPa, flow rate = 400 µmol s^{-1}, reference CO_2 = 400 ppm, and leaf temperature = 25°C. WUE was calculated as (Hamid *et al*. 1990):

$$\text{WUE} = \frac{A}{E}$$

Plant harvest and processing for isotope analysis: At 67 DAP (*i.e.* during early pod development), the shoots of 16 cowpea plants and 8 sorghum plants were harvested from the middle rows of each plot, pooled, oven-dried at 60°C for 48 h, weighed, and ground to fine powder (0.85 mm sieve) for analysis of ^{13}C.

Measurement of ^{13}C and %C in shoots: Shoot samples were weighed (2 mg for cowpea and 2.5 mg for sorghum), transferred into tin capsules (*Elemental Microanalysis Ltd*., Okehamptom, UK), and injected into a *Thermo Flash Elemental Analyser 1112* (*Fisions Instruments SpA*, Strada Rivoltana, Italy) *via* a *Thermo Conflo III* devise coupled to a *Thermo Finnigan Delta Plus XP Stable Light Isotope Mass Spectrometer* (*Finnigan MAT GmbH*, Bremen, Germany). The ratio of $^{13}C/^{12}C$ in each sample was used to calculate the ^{13}C natural abundance or $\delta^{13}C$ (‰) of shoot, as (Farquhar *et al*. 1989):

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

where $(^{13}C/^{12}C)_{sample}$ is the isotopic ratio of the sample, and $(^{13}C/^{12}C)_{standard}$ is the isotopic ratio of PDB, a universally accepted standard from belemnite Pee Dee limestone formation (Craig 1957).

Shoot C content per plant [g $plant^{-1}$] was calculated as: %C × shoot dry mass per plant.

Determination of plant-available minerals in rhizosphere soils: Extractable P, K, Ca, and Mg were determined by the citric acid method as developed by Dyer (1894) and modified by the Division of Chemical Services (DCS 1956) and Du Plessis and Burger (1964). A 20 g of the 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 1-h 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 a 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. Measurements of P, K, Ca, and Mg were then done directly by aspiration on a calibrated simultaneous inductively coupled plasma-mass spectrophotometer (*IRIS/AP HR DUO Thermo Electron Corporation*, Franklin, MA, USA).

The determination of S and B in the soil was done by adding 20 g of soil in 0.01 M $Ca(H_2PO_4)_2{\cdot}H_2O$ extracting solution (FSSA 1974), followed by filtering. Sulphur was determined by direct aspiration on a calibrated simultaneous inductively coupled plasma-mass spectrophotometer (*IRIS/AP HR DUO Thermo Electron Corporation*, Franklin, MA, USA).

The trace elements Fe, Cu, Zn, and Mn, were extracted from soil using diammonium ethylenediaminetetraacetic (EDTA) acid solution [Trierweiler and Lindsay (1969), modified by Beyers and Coetzer (1971)]. The extractants were analysed for Fe, Cu, Zn, and Mn using ICP-MS spectrometry (*IRIS/AP HR DUO Thermo Electron Corporation*, Franklin, MA, USA).

Measurement of mineral elements in plant shoots: Measurements of mineral elements (P, K, Ca, Mg, Cu, Zn, Mn, Fe, and B) were done by ashing of 1 g of ground sample in a porcelain crucible at 500°C overnight. This was followed by dissolving the ash in 5 ml of 6 M HCl and placing it in an oven at 50°C for 30 min and 35 ml of deionised water was added. The mixture was filtered through *Whatman no. 1* filter paper. Mineral element concentration in plant extracts was determined using the inductively coupled plasma-mass spectrometer (*ICP-MS*) (Giron 1973). Sulphur was determined by wet digestion procedure using 65% nitric acid. In each case, 1 g of milled plant material was digested overnight with 20 ml

of 65% nitric acid in a 250-ml glass beaker. The beaker containing the extract was then placed on a sand bath and gently boiled until approximately 1 ml of the extract was left. After that, 10 ml of 4 M nitric acid was added and boiled for 10 min. The beaker was removed from the sand bath, cooled, and the extract washed completely in a 100-ml volumetric flask and the extract filtered through *Whatman no. 2* filter paper. S in the sample was then determined (FSSA 1974) by direct aspiration on the calibrated simultaneous ICP-MS. Shoot nutrient content was calculated as: Nutrient content per plant [mg plant^{-1}] = % nutrient in shoot × shoot dry mass per plant [g plant^{-1}].

Statistical analysis: Data were collected in four replicates and analysed statistically using a 3-factorial *ANOVA*. The analysis was performed using *STATISTICA 2007* (*StatSoft Inc*., Tulsa, OK, USA). One-way *ANOVA* was also used to compare Chl, gas exchange parameters, $\delta^{13}C$ and % C in cowpea and sorghum species. Correlation and regression analysis between $\delta^{13}C$ and photosynthetic WUE was done for cowpea and sorghum. Fisher's least significant difference (LSD) was used to compare treatment means at $P \leq 0.05$ (Steel and Torrie 1980).

Results

Effect of plant density and cropping system on Chl levels: Analysis of data using 3-way *ANOVA* revealed significant differences in Chl concentration of cowpea and sorghum leaves (Table 2). The levels of Chl *a*, Chl *b*, and total Chl were markedly higher in plants at low density relative to high density irrespective of the plant species. Similarly, the leaves of plants under monoculture showed much greater levels of Chl *a*, Chl *b*, and total Chl when compared with their counterparts in mixed culture (Table 2). The concentrations of Chl *a*, Chl *b*, and total Chl were similar in all five cowpea genotypes.

Effect of plant density and cropping system on photosynthesis, stomatal conductance and transpiration: *A*, g_s, and *E* were all significantly higher in the leaves of plants at low density relative to those at high density, irrespective of the plant species (Table 3). Similarly, the same gas-exchange parameters were markedly greater under monoculture relative to mixed culture (Table 3). However, no differences were found in leaf *A*, g_s, and *E* of the five cowpea genotypes (Table 3).

Table 2. Effect of plant density and cropping system on chlorophyll (Chl) level in leaves of cowpea [Vigna unguiculata L. (Walp.)] and sorghum [Sorghum bicolor L. (Moench.)] plants. Values (mean ± SE, n = 16) followed by dissimilar letters in a column for each treatment are significantly different at $^{*}(P \leq 0.05)$, $^{**}(P \leq 0.01)$, and $^{***}(P \leq 0.001)$. Genotype and interactive effects are not shown here as they were not significantly different. Chl levels were calculated using equations by Arnon (1949).

,	Chl *a* [mg g^{-1}]	Chl *b* [mg g^{-1}]	Chl (*a*+*b*) [mg g^{-1}]
COWPEA			
Density [plants ha^{-1}]			
83,333	1,090±40^{a}	250±10^{a}	1,340±50^{a}
166,666	790±50^{b}	170±10^{b}	960±60^{b}
Cropping system			
Monoculture	1,090±40^{a}	240±10^{a}	1,330±60^{a}
Mixed culture	790±40^{b}	180±10^{b}	970±50^{b}
3-way *ANOVA* (F-Statistics)			
Density	28.3***	28.1***	28.8***
Cropping system	26.8***	21.0***	26.1***
SORGHUM			
Density [plants ha^{-1}]			
83,333	880±40^{a}	160±10^{a}	1,004±50^{a}
166,666	800±50^{b}	1.4±0.1^{b}	940±60^{b}
Cropping System			
Monoculture	1,090±00^{a}	190±00^{a}	1,280±00^{a}
Mixed culture	590±30^{b}	110±10^{b}	700±40^{b}
3-way *ANOVA* (F-Statistics)			
Density	6.3*	7.2**	6.7*
Cropping system	261.3***	172.9***	251.1***

Table 3. Effect of plant density and cropping system on photosynthesis and gas exchange of cowpea [*Vigna unguiculata* L. (Walp.)] and sorghum [Sorghum bicolor L. (Moench.)] leaves. Values (mean ± SE, n = 16) followed by dissimilar letters in a column for each treatment are significantly different at $^{*}(P\leq0.05)$, $^{**}(P\leq0.01)$, and $^{***}(P\leq0.001)$. Interactive effects are not shown here as they were not significantly different.

Treatment	A [μmol(CO_2) m^{-2} s^{-1}]	E [mmol(H_2O) m^{-2} s^{-1}]	C_i [μmol(CO_2) mol^{-1}]	g_s [mol m^{-2} s^{-1}]	WUE [mmol(CO_2) mol^{-1}(H_2O)]
COWPEA					
Density [plants ha^{-1}]					
83,333	20.4±0.7^{a}	4.0±0.1^{a}	234.8±5.1^{a}	0.4±0.0^{a}	5.1±0.1^{a}
166,666	15.6±0.7^{b}	3.4±0.1^{b}	209.1±4.5^{b}	0.3±0.0^{b}	4.5±0.1^{b}
Cropping system					
Monoculture	20.6±0.6^{a}	4.0±0.1^{a}	238.9±5.2^{a}	0.40±0.0^{a}	5.2±0.1^{a}
Mixed culture	15.5±0.7^{b}	3.4±0.1^{b}	205.0±3.5^{b}	0.31±0.0^{b}	4.5±0.1^{b}
Genotypes					
Bensogla	18.2±1.1^{a}	3.6±0.1^{a}	221.1±6.7^{a}	0.3±0.0^{a}	5.0±0.2^{a}
ITH98-46	17.9±1.4^{a}	3.6±0.2^{a}	207.4±6.8^{a}	0.3±0.0^{a}	4.9±0.2^{a}
Sanzie	17.5±1.3^{a}	3.9±0.1^{a}	232.6±8.4^{a}	0.4±0.0^{a}	4.4±0.2^{a}
TVu1509	17.9±1.3^{a}	3.7±0.1^{a}	228.0±11.5^{a}	0.4±0.0^{a}	4.8±0.3^{a}
Omondaw	18.6±1.1^{a}	3.8±0.1^{a}	220.7±5.8^{a}	0.4±0.0^{a}	4.9±0.2^{a}
3-way *ANOVA* (F-Statistics)					
Density	36.1***	32.5***	21.0***	23.4***	16.4***
Cropping system	40.2***	37.4***	36.5***	16.8***	18.9***
Genotypes	0.2	1.9	2.3	1.4	1.8
SORGHUM					
Density [plants ha^{-1}]					
83,333	19.9±0.4^{a}	2.76±0.1^{a}	142.45±6.8^{a}	0.3±0.0^{a}	8.7±0.2^{a}
166,666	17.7±0.7^{b}	2.4±0.1^{b}	117.7±5.6^{b}	0.2±0.0^{b}	7.7±0.4^{b}
Cropping system					
Monoculture	21.5±0.0^{a}	2.7±0.0^{a}	148.3±0.0^{a}	0.3±0.0^{a}	9.5±0.0^{a}
Mixed culture	16.2±0.5^{b}	2.3±0.1^{b}	111.7±8.2^{b}	0.2±0.0^{b}	6.9±0.3^{b}
3-way *ANOVA* (F-Statistics)					
Density	31.5***	15.0***	11.0**	5.1*	22.2***
Cropping system	183.1***	26.4***	24.3***	21.0***	148.4***

Effect of plant density and cropping system on $\delta^{13}C$ and WUE: WUE (calculated as photosynthate produced per unit water molecule transpired) was significantly higher in photosynthetic leaves of plants at low density relative to those of plants at high density, irrespective of the plant species (Table 3). Plants under monoculture also showed much higher WUE relative to those in mixed culture. There were however no marked differences in WUE between and among the five cowpea genotypes (Table 3).

Data from isotopic analysis revealed significant differences in $\delta^{13}C$ values of sorghum and cowpea plants under different experimental treatments. For example, the $\delta^{13}C$ values of plants were significantly higher (*i.e.* less negative) at the low plant density relative to the high one, irrespective of plant species (Table 4). Similarly, the $\delta^{13}C$ values were much higher in plants under monoculture when compared with those in mixed culture (Table 4). The five cowpea genotypes also showed significant differences in $\delta^{13}C$ with Sanzie exhibiting the most negative value and ITH98-46 the least negative value (Table 4).

As found with photosynthate, the concentration of C in leaves of plants at low density and under monoculture was significantly much higher, irrespective of the plant species (Table 4). The five cowpea genotypes also showed significant differences between and among themselves with regards to leaf C concentration. For example, Sanzie exhibited the highest C concentration in shoots, followed by Bensogla and Omondaw, while ITH98-46 and TVu1509 had the lowest concentration of C in shoots (Table 4). As a result, shoot C content was also significantly much higher in Sanzie, followed Bensogla and Omondaw, and lowest in *cvs*. ITH98-46 and TVu1509 (Table 4).

Table 4. Effect of plant density and cropping system on shoot dry mass, ^{13}C natural abundance, % C, and C content of cowpea [*Vigna unguiculata* L. (Walp.)]and sorghum [*Sorghum bicolor* L. (Moench.)] plants. Values (mean ± SE, $n = 4$) followed by dissimilar letters in a column for each treatment are significantly different at $^{*}(P\leq0.05)$, $^{**}(P\leq0.01)$, and $^{***}(P\leq0.001)$. Interactive effects are not shown here as they were not significantly different.

Treatment	Shoot dry mass [g plant^{-1}]	$\delta^{13}C$ ‰	Shoot C %	Shoot C content [g plant^{-1}]
COWPEA				
Density [plants ha^{-1}]				
83,333	23.0±1.2^{a}	–28.2±0.1^{a}	41.5±0.1^{a}	9.5±0.5^{a}
166,666	18.8±1.0^{b}	–28.4±0.1^{b}	40.8±0.2^{b}	7.8±0.4^{b}
Cropping system				
Monoculture	23.9±1.1^{a}	–28.2±0.1^{a}	41.7±0.1^{a}	9.5±0.5^{a}
Mixed culture	17.8±1.1^{b}	–28.5±0.1^{b}	40.5±0.2^{b}	7.7±0.5^{b}
Genotypes				
Bensogla	20.2±1.9^{ab}	–28.4±0.1^{b}	41.4±0.2^{b}	8.4±0.8^{b}
ITH98–46	18.2±1.6^{b}	–28.0±0.1^{a}	40.6±0.3^{c}	7.4±0.7^{b}
Sanzie	25.4±2.1^{a}	–28.7±0.1^{c}	41.8±0.2^{a}	10.6±0.9^{a}
TVu1509	19.5±2.1^{ab}	–28.1±0.1^{a}	40.6±0.3^{c}	8.0±0.9^{b}
Omondaw	21.1±1.1^{ab}	–28.5±0.1^{b}	41.3±0.2^{b}	8.7±0.5^{b}
3-way *ANOVA* (F-Statistics)				
Density	9.5**	11.6**	22.5***	10.3**
Cropping system	20.5***	26.1***	68.7***	9.6**
Genotypes	3.3*	16.3***	11.5***	3.6*
SORGHUM				
Density [plants ha^{-1}]				
83,333	33.5±1.2^{a}	–12.1±0.0^{a}	43.0±0.4^{a}	14.5±0.6^{a}
166,666	24.1±0.7^{b}	–12.3±0.1^{b}	41.84±0.5^{b}	10.2±0.4^{b}
Cropping system				
Monoculture	33.4±1.1^{a}	–11.91±0.0^{a}	44.6±0.0^{a}	14.9±0.5^{a}
Mixed culture	24.3±0.9^{b}	–12.54±0.1^{b}	40.3±0.4^{b}	9.8±0.4^{b}
3-way *ANOVA* (F-Statistics)				
Density	95.9***	26.0***	12.2***	109.1***
Cropping system	89.2***	239.0***	168.0***	149.1***

Effect of plant species on Chl, photosynthesis, WUE and $\delta^{13}C$: The levels of Chl *a* and Chl *b*, as well as total Chl were significantly higher in cowpea relative to sorghum. Although photosynthesis rates were similar, E, g_s and C_i were all significantly higher in cowpea when compared with sorghum (Table 5). However, sorghum showed much higher WUE, $\delta^{13}C$ and % C relative to cowpea (Table 5).

Effect of plant density and cropping system on the mineral concentration in the rhizosphere of cowpea: Increasing cowpea plant density from 83,333 to 166,666 plants per hectare significantly decreased ($P\leq0.05$) the concentrations of P, K, Ca, Mg, S, Fe, Cu, Zn, Mn, and B in the rhizosphere of cowpea plants (Table 6), leading to marked decreases in corresponding contents in plant tissues (Table 7). Growing cowpea in mixed culture with sorghum significantly ($P\leq0.05$) decreased the concentrations of P, K, Ca, Mg, S, Fe, Cu, Zn, Mn, and B in the rhizosphere of cowpea plants (Table 6), leading to marked decreases in corresponding contents in plant tissues (Table 7).

Effect of plant density, cropping system and genotypes on the amount of mineral elements in shoots of cowpea: Increasing plant density significantly ($P\leq0.05$) changed the contents of mineral elements in shoots of cowpea. Similar trend was also observed when cropping system was changed from monoculture to mixed culture. For example, raising cowpea plant density from 83,333 to 166,666 plants per hectare and changing cropping system from monoculture to mixed culture significantly ($P\leq0.05$) decreased the amounts of P, K, Ca, Mg, Fe, Cu, Zn, Mn, and B in shoots (Table 7). The results also showed significant ($P\leq0.05$) differences in the amount of mineral elements in cowpea shoots. Sanzie had greater amounts of mineral elements in its shoot, followed by Bensogla and Omondaw (Table 7).

Table 5. Comparison of chlorophyll (Chl) levels, photosynthetic rates and gas-exchange parameters in cowpea [*Vigna unguiculata* L. (Walp.)] and sorghum [*Sorghum bicolor* L. (Moench.)]. Values (mean ± SE, $n = 16$) followed by dissimilar letters in a column are significantly different at $^{*}(P \leq 0.05)$, $^{**}(P \leq 0.01)$, and $^{***}(P \leq 0.001)$. Chl levels were calculated using equations by Arnon (1949) and expressed per dry mass.

Treatment	A	E	C_i	g_s	WUE	Chl *a*	Chl *b*	Total Chl	$\delta^{13}C$	Shoot C
	[$\mu mol(CO_2)\ m^{-2}\ s^{-1}$]	[$mmol\ m^{-2}\ s^{-1}$]	[$\mu mol(CO_2)\ mol^{-1}$]	[$mol\ m^{-2}\ s^{-1}$]	[$mmol(CO_2)\ mol^{-1(}H_2O)$]	[$mg\ g^{-1}$]	[$mg\ g^{-1}$]	[$mg\ g^{-1}$]	[‰]	[%]
Cowpea	18.0 ± 0.5^{a}	3.7 ± 0.1^{a}	221.9 ± 3.7^{a}	0.4 ± 0.0^{a}	4.8 ± 0.1^{b}	940 ± 40^{a}	210 ± 10^{a}	1150 ± 40^{a}	-28.3 ± 0.1^{b}	41.1 ± 0.1^{b}
Sorghum	18.8 ± 0.4^{a}	2.5 ± 0.1^{b}	130.0 ± 4.6^{b}	0.2 ± 0.0^{b}	8.2 ± 0.2^{a}	840 ± 30^{b}	150 ± 10^{b}	990 ± 40^{b}	-12.2 ± 0.1^{a}	42.4 ± 0.3^{a}
One-way *ANOVA* (F-Statistics)										
	1.5	225.4^{***}	246.8^{***}	45.0^{***}	238.8^{**}	4.4^{*}	32.0^{***}	7.6^{**}	$63{,}961.1^{**}$	15.9^{***}

Table 6. Concentration of mineral elements in the rhizosphere soil of cowpea [*Vigna unguiculata* L. (Walp.)] at different plant densies and cropping systems. Values (mean ± SE, $n = 4$) followed by dissimilar letters in a column for each treatment are significantly different at $^{*}(P \leq 0.05)$, $^{**}(P \leq 0.01)$, and $^{***}(P \leq 0.001)$. Genotype effects are not shown here as they were not significantly different.

Treatment	P	K	Ca	Mg	S	Fe	Cu	Zn	Mn	B
					[mg kg^{-1}]					
COWPEA										
Density [plants ha^{-1}]										
83,333	17.7±1.5^{a}	122.3±3.7^{a}	817.2±29.8^{a}	207.9±6.4^{a}	4.9±0.4^{a}	280.0±10.6^{a}	4.4±0.1^{a}	3.6±0.2^{a}	9.3±0.4^{a}	0.53±0.02^{a}
166,666	11.2±0.7^{b}	102.7±2.7^{b}	641.2±26.6^{b}	165.8±5.0^{b}	2.2±0.2^{b}	218.3±8.9^{b}	3.5±0.1^{b}	2.5±0.1^{b}	7.4±0.3^{b}	0.45±0.01^{b}
Cropping system										
Monoculture	17.6±1.5^{a}	120.2±3.4^{a}	798.6±32.5^{a}	205.8±6.7^{a}	4.4±0.4^{a}	281.9±10.7^{a}	4.2±0.1^{a}	3.4±0.2^{a}	9.4±0.4^{a}	0.54±0.02^{a}
Mixed culture	11.2±0.7^{b}	104.8±3.3^{b}	659.8±26.3^{b}	167.9±5.0^{b}	2.7±0.2^{b}	216.4±8.4^{b}	3.7±0.1^{b}	2.7±0.1^{b}	7.4±0.3^{b}	0.44±0.01^{b}
3-way *ANOVA* (F-Statistics)										
Density	21.8***	19.5**	23.6***	39.6***	60.5***	31.5***	61.7***	23.1***	19.2***	15.8***
Cropping system	20.9***	12.1**	14.7***	32.1***	21.4***	35.5***	16.2***	13.0***	20.6***	23.4***

Table 7. Effect of plant density and cropping system on mineral elements in shoots of cowpea [Vigna unguiculata L. (Walp.)] and sorghum [*Sorghum bicolor* L. (Moench.)] plants. Values (mean ± SE, $n = 4$) followed by dissimilar letters in a column for each treatment are significantly different at $^{*}(P \leq 0.05)$, $^{**}(P \leq 0.01)$, and $^{***}(P \leq 0.001)$.

Treatment	P	K	Ca	Mg	Fe [µg plant^{-1}]	Cu	Zn	Mn	B
COWPEA									
Density [plants ha^{-1}]									
83,333	64.8±4.3^{a}	625.0±71.1^{a}	463.0±37.6^{a}	131.8±8.3^{a}	18,552.7±1489.9^{a}	309.4±20.9^{a}	2,016.0±154.5^{a}	791.5±70.4^{a}	1,086.5±67.0^{a}
166,666	41.6±2.9^{b}	375.5±22.3^{b}	268.4±18.1^{b}	85.4±6.3^{b}	9,016.9±836.5^{b}	207.9±14.9^{b}	1,206.8±94.6^{b}	438.3±37.9^{b}	749.1±53.4^{b}
Cropping system									
Monoculture	65.7±4.3^{a}	641.1±70.2^{a}	465.8±36.8^{a}	134.5±8.5^{a}	17,905.6±1549.2^{a}	318.6±20.7^{a}	2,016.1±153.2^{a}	812.2±70.2^{a}	1,126.7±69.0^{a}
Mixed culture	40.6±2.7^{b}	359.4±20.1^{b}	265.6±19.1^{b}	82.6±5.4^{b}	9,664.0±902.4^{b}	198.7±13.4^{b}	1,206.7±96.7^{b}	417.6±32.8^{b}	708.9±42.5^{b}
Genotypes									
Bensogla	55.8±7.2^{ab}	581.6±142.0^{a}	386.9±57.4^{ab}	111.0±13.6^{ab}	13,967.1±2239.8^{ab}	271.7±35.8^{ab}	1,643.4±235.3^{ab}	620.6±92.6^{ab}	963.4±121.5^{ab}
ITH98-46	42.7±4.7^{b}	373.1±36.3^{a}	277.0±32.1^{b}	89.3±11.5^{b}	10,359.3±1562.0^{b}	198.6±20.3^{b}	1,232.8±138.4^{b}	492.6±73.1^{b}	751.4±77.1^{b}
Sanzie	65.7±8.0^{a}	628.7±104.9^{a}	473.8±69.0^{a}	135.7±14.1^{a}	18,226.2±2777.2^{a}	324.7±35.6^{a}	2,101.5±276.6^{a}	809.3±130.0^{a}	1,121.0±114.2^{a}
TVu1509	50.5±7.2^{ab}	469.5±72.2^{a}	339.9±56.2^{b}	99.3±14.2^{ab}	13,169.0±2750.1^{ab}	241.5±36.0^{ab}	1,537.3±269.0^{ab}	595.1±123.1^{ab}	867.6±123.0^{ab}
Omondaw	51.1±3.2^{ab}	448.5±27.4^{a}	350.8±29.1^{ab}	107.6±9.3^{ab}	13,202.4±1294.0^{ab}	256.6±18.0^{ab}	1,542.1±134.6^{ab}	556.9±46.9^{b}	885.6±58.0^{ab}
3-way *ANOVA* (F-Statistics)									
Density	38.6***	14.7***	43.2***	36.3***	65.5***	28.1***	33.7***	40.1***	29.0***
Cropping system	45.1***	18.8***	45.7***	45.5***	48.9***	39.3***	33.7***	50.1***	44.5***
Genotypes	4.1**	2.0	4.8**	4.0**	4.6**	4.6**	4.1**	3.6*	3.8**
SORGHUM									
Density [plants ha^{-1}]									
83,333	137.8±6.1^{a}	927.1±40.7^{a}	206.1±11.5^{a}	142.0±6.9^{a}	142,619.7±17442.1^{a}	555.0±23.3^{a}	2,684.1±157.2^{a}	1,725.4±92.5^{a}	181.5±6.4^{a}
166,666	115.6±9.3^{b}	801.5±57.4^{b}	176.3±15.4^{b}	123.3±9.6^{b}	129,978.2±19183.1^{b}	469.1±32.7^{b}	2,319.5±209.9^{b}	1,399.9±130.9^{a}	149.6±10.2^{b}
Cropping system									
Monoculture	170.4±0.0^{a}	1,149.1±0.0^{a}	270.6±0.0^{a}	180.4±0.0^{a}	249,593.5±0.0^{a}	659.4±0.0^{a}	3,597.6±0.0^{a}	2,203.3±0.0^{a}	209.4±0.0^{a}
Mixed culture	83.1±5.7^{b}	579.5±31.5^{b}	111.8±7.6^{b}	84.9±5.2^{b}	23,004.4±4003.5^{b}	364.7±24.4^{b}	1,405.9±94.2^{b}	922.0±77.5^{b}	121.7±7.6^{b}
3-way *ANOVA* (F-Statistics)									
Density	29.6***	32.7***	30.6***	23.8***	16.3***	21.7***	25.9***	44.2***	38.0***
Cropping system	457.8***	672.2***	868.5***	617.3***	5227.8***	255.8***	934.8***	685.4***	287.0***

Effect of plant density and cropping system on mineral elements in sorghum shoots: Increased plant density significantly ($P \leq 0.05$) reduced the amount of P, K, Ca, Mg, Fe, Cu, Zn, Mn, and B in shoots of sorghum (Table 7). Similarly, mixed culture caused a decrease in the amount of P, K, Ca, Mg, Fe, Cu, Zn, Mn, and B in sorghum shoots (Table 7).

Correlation and regression analysis of WUE and $\delta^{13}C$ in cowpea and sorghum: Performing regression as well as correlation analysis of $\delta^{13}C$ *vs*. photosynthetic WUE showed a statistically significant relationship between the two parameters for both cowpea (r = 0.66***) and sorghum (r = 0.96***).

Discussion

In this study, the concentrations of Chl *a* and *b*, as well as total Chl were increased in plants at low density and under monoculture possibly due to the greater N levels in photosynthetic shoots (Makoi *et al*. 2009) when compared with plants at high density or in mixed culture (Table 2). However, the higher Chl concentrations in plants at low density and in monoculture could also be attributed to the observed greater shoot content of Mg (Table 7), a mineral element important for Chl biosynthesis and a co-factor needed for the formation of enzymes involved in CO_2 fixation and energy transfer *via* ATP (Beale 1999, Kaftan *et al*. 2002, Igamberdiev and Kleczkowski 2003). Because of the role Chl plays in CO_2 reduction, photosynthetic rates closely mirrored leaf Chl levels in this study, with plants at low density and in monoculture exhibiting higher photosynthetic rates relative to those at high density or in mixed culture (Table 3). At high plant density (or mixed culture), mineral nutrients probably became limiting from plant-to-plant competition as the concentrations of P, K, Ca, Mg, S, Fe, Cu, Zn, Mn, and B were lower in the rhizosphere of plants at high density and in mixed culture relative to those at low density and in monoculture (Table 6). Such reductions in nutrient supply to plants were likely to lead to reduced photosynthetic functioning as found for common bean (Cornic *et al*. 1992, Lal *et al*. 1996, Wang *et al*. 2005). Analysis of cowpea shoots also revealed low concentrations of P, K, Ca, Mg, Fe, Cu, Zn, Mn, and B in plants from high density and mixed culture relative to low density and monoculture (Table 7). These changes in mineral nutrition could have had subtle effects on plant processes in both cowpea and sorghum, as low levels of Fe, Cu, and Zn were found to negatively affect the photosynthetic electron transport chain in cauliflower, which led to reduced photosynthesis and Chl biosynthesis (Cakmak and Engels 1999, Alloway 2001, Hacisasiloglu *et al*. 2003). In addition, Fe and Zn limitation (observed in this study, Table 7) was also found to decrease the levels of Chl *a*, just as low Mg concentration (as found for plants at high density and in mixed culture in this study) also significantly reduced Chl *b* levels (Balakrishnan *et al*. 2000, Tang *et al*. 2006).

Tissue Chl concentrations were not different in the five cowpea genotypes. Consistently, leaf photosynthetic rates were not significantly different between and among the five cowpea genotypes tested (Table 3). That not withstanding, C accumulation from photosynthesis differed markedly between and among genotypes (Table 4). Shoot C concentration was much higher in Sanzie, followed by Omondaw and Bensogla, relative to ITH98-46 and TVu1509 (Table 4). As a result, the amount of C in cowpea shoots was also significantly much greater in Sanzie compared with the other four cowpea genotypes (Table 4). This inconsistency between photosynthetic rates and shoot C accumulated can be explained by the fact that the former is an instantaneous measure of Rubisco enzyme activity (which is affected daily by environmental factors), while the latter is a measure of the cumulative product of the enzyme's daily activity. Clearly, there are dangers in using only instantaneous enzyme activities to measure long-term growth. Whatever the case, in this study, Sanzie emerged as the cowpea genotype with better plant growth (Makoi *et al*. 2009) because of its greater C accumulation against the background of high plant density and mixed culture.

The water relations of crop plants are easily affected by both mixed culture and high plant density because of plant-to-plant competition for soil water and mineral nutrients. As with photosynthetic rates, cowpea and sorghum plants at low density or in monoculture exhibited greater WUE (photosynthate produced per unit water transpired) relative to their counterparts at high density or in mixed culture (Table 3). Although there were no differences in photosynthetic WUE between and among the five cowpea genotypes (Table 3), their $\delta^{13}C$ values were markedly different (Table 4). The $\delta^{13}C$ of C_3 plants is known to be an integrator of WUE (Farquhar *et al*. 1989), with the more negative $\delta^{13}C$ values indicating low WUE, and the less negative $\delta^{13}C$ values, high WUE. From that perspective, Sanzie exhibited high ^{13}C discrimination during photosynthesis (*i.e*. a more negative $\delta^{13}C$ value) and therefore showed the lowest WUE, followed by Omondaw and Bensogla, while ITH98-46 and TVu1509 had low ^{13}C discrimination (*i.e*. a less negative $\delta^{13}C$ value) which suggested high WUE (Table 4). Some earlier studies also found that low ^{13}C discrimination (or less negative $\delta^{13}C$ value) in wheat were related to high WUE (Farquhar and Richards 1984, Ehdaie and Waines 1993). The cowpea and sorghum plants from low-density treatment or monoculture also showed greater WUE relative to those at high plant density or in mixed culture (Table 4). High ^{13}C discrimination during photosynthesis is generally associated with a higher stomatal conductance, greater CO_2 reduction

and, to some extent, adequate availability of soil moisture. Thus, the increased photosynthetic C accumulation in the shoots of Sanzie is consistent with the notion of high ^{13}C discrimination. Whether measured isotopically or from gas-exchange studies (Table 5), sorghum (a C_4 species) was found to exhibit a significantly higher WUE compared with cowpea (a C_3 species), thus, indicating the superior water-use use efficiency of C_4 relative to C_3 species (Farquhar and Richards 1984, Ehdaie and Waines 1993).

In conclusion, the decrease in Chl concentration, photosynthetic rates, and C concentration/content of cowpea and sorghum shoots with intercropping or high plant density, as well as the reduction in the amounts of P, K, Ca, Mg, Fe, Cu, Zn, Mn, and B in shoots of the two species, could account for the lower crop yields under these practices in farmers' fields in Africa. This argument is re-enforced by the observation that, Sanzie, which showed a marked increase in shoot accumulation of P, K, Ca, Mg, Fe, Cu, Zn, Mn, and B, recorded the highest plant growth (Table 4) and grain yield (Makoi *et al*. 2009). However, the farmer-selected varieties (Sanzie, Omondaw and Bensogla) showed lower WUE relative to the improved cultivars (ITH98-46 and TVu1509), just as sorghum also showed a much higher WUE relative to cowpea.

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