Symbiosis (2024) 92:257–269
https://doi.org/10.1007/s13199-024-00971-x

ORIGINAL PAPERS

Monocropping and Intercropping of Maize with Six Food Legumes at Malkerns in Eswatini: Their Effects on Plant Growth, Grain Yield and N_2 Fixation, Measured using the ^{15}N Natural Abundance and Ureide Techniques

Zanele D. Ngwenya[1] · Mustapha Mohammed[2,3] · Felix D. Dakora[2]

Received: 28 September 2023 / Accepted: 1 January 2024 / Published online: 22 January 2024

Abstract
Intercropping of legumes and cereals has many benefits to both plant partners. In this study, the effect of legume-maize intercropping on plant growth, grain yield and N_2 fixation of six legumes was assessed using the ^{15}N natural abundance and ureide techniques. For this, a field experiment involving six legume species and two cropping systems was established at the Malkerns Research Station, Eswatini during the 2017/2018 cropping season. Based on the ^{15}N isotopic and ureide analysis, the six test legumes respectively obtained 39.06 – 70.19% and 16.46 – 55.79% of their N nutrition from symbiosis. The amounts of N-fixed ranged from 12.66 to 66.57 kg ha^{-1}. In general, high amount of N-fixed by legumes correlated strongly with greater shoot dry matter accumulation ($r = 0.7981$; $p < 0.001$) and high grain yield ($r = 0.5905$; $p < 0.001$), indicating the importance of N_2 fixation in plant growth and reproduction. Legumes grown under monocropping recorded higher plant growth, symbiotic performance and grain yield when compared to those grown in mixed culture with maize. However, shoot %Ndfa was much higher under intercropping than sole cropping due to competition by cereal and legume for soil N. Components of maize yield were similar for the two cropping systems. The %N derived from fixation and %relative ureide-N abundance were significantly correlated ($r = 0.4005$; $p < 0.001$), indicating that the ^{15}N natural abundance technique and the ureide method were complementary in measuring N_2 fixation in the test legumes. These results have provided some insights on the impact of cropping system on plant growth, symbiotic performance and grain yield of six selected legumes.

Keywords Nitrogen fixation · Ureide · %Ndfa and grain yield

1 Introduction

Cereal-legume intercropping plays a central role in smallholder farming in developing countries, especially where water resources are limited (Tsubo et al. 2005). In Southern Africa, smallholder farmers generally grow drybean as an intercrop with maize, the main staple crop. When associated with soil bacteria called rhizobia, legumes form root nodules, and the bacteria inside these structures are able to reduce atmospheric N_2 into NH_3 for their own use. Although, the excess NH_3 is available for plant use, it can also be excreted from the nodules into the soil for uptake by associated plants (Dahmardeh et al. 2010). One of the earliest studies found that an intercropped legume could contribute as much as 40 kg N ha^{-1} to an associated maize crop (Willey 1979). Legumes are therefore a sustainable source of N in cereal-legume intercropping systems (Shen and Chu 2004). Several studies have shown that legume-maize intercropping can produce greater yields than either species grown alone (Li et al. 1999; Dahmardeh et al. 2010; Manasa 2020; Nasar et al. 2020).

✉ Zanele D. Ngwenya
NgwenyaZD@tut.ac.za

Mustapha Mohammed
imustaph@gmail.com

Felix D. Dakora
DakoraFD@tut.ac.za

[1] Department of Crop Sciences, Tshwane University of Technology, Private Bag X680, Pretoria 0001, South Africa

[2] Department of Chemistry, Tshwane University of Technology, Private Bag X680, Pretoria 001, South Africa

[3] Department of Crop Science, University for Development Studies, P.O. Box 1350, Tamale, Ghana

The inclusion of legumes in cropping systems also has the advantage of making extra N available to subsequent cereal crops from biological N_2 fixation (Ghanbari-Bonjar 2003; Dahmardeh et al. 2010). Furthermore, legumes can directly benefit associated cereal crops in low-N soils through N transfer from nodules to the cereal (Shen and Chu 2004), thus decreasing the N fertilizer requirements of cereals in an intercrop (Shen and Chu 2004). Additionally, intercropping is known to achieve higher grain yield than monoculture through improving the efficient use of water, light, nutrients, and other resources (Li et al. 2011). In fact, most intercropped crop species are complementary, as they generally use a given resource differently based on time, space, and growth characteristics (Zhang et al. 2014). Maitra et al. (2020) also found that success in a maize-legume intercropping system was largely dependent on factors such as the choice of crops, their maturity, density, and time of planting.

Currently, there are several methodologies for quantifying N_2 fixation by nodulated legumes and these include the N balance, N difference, ^{15}N isotope dilution, ureides concentration and acetylene reduction techniques (Peoples et al. 1989; Herridge and Giller 2016). Nitrogen balance compares the total N of a plant-soil system on two separate occasions, with any increase attributed to N_2 fixation after other possible inputs and outputs of N have been accounted for. On the other hand, the N difference method compares total N accumulated by the N_2-fixing plants with that of neighbouring non N_2-fixing plants, with the difference assumed to be due to N_2 fixation (Unkovich et al. 2008). The ^{15}N and ureide methods provide estimates of the percentage of total N of the legume crop that is derived from N_2 fixation (%Ndfa) (Herridge and Giller 2016) whereas the acetylene reduction technique assays the activity of nitrogenase, the enzyme catalyzing N_2 fixation (Peoples et al. 1989:2; Herridge and Giller 2016).

In the current study, the ^{15}N natural abundance and ureide techniques were employed to quantify the biological nitrogen fixation of selected test legumes. Despite the many benefits of intercropping, little information currently exists on maize-legume mixed culture in Eswatini, where maize intercropping is dominant. The aim of this study was to evaluate the effect of maize and legume intercropping on plant growth, symbiotic performance, and grain yield of six selected grain legumes in Eswatini.

2 Materials and methods

2.1 Study site

A field experiment was conducted at the Malkerns Research Station (26°33'S, 31°10'E), located in the Middleveld Agro-ecological zone of Eswatini. The area has a mean annual rainfall ranging from 800 to 1000 mm and an annual mean temperature of 7 °C to 26.6 °C (Edje and Ossom 2009; Dlamini et al. 2021). The daily temperatures recorded during the cropping season (December to May) ranged from 12.1 °C to 18.3 °C for minimum and 25.3 °C to 28.9 °C for maximum in the 2017/2018 cropping season (Table 1). The total rainfall recorded during the study period was 2986 mm in 2017, and 1189.2 mm in 2018. However, the total rainfall from planting to harvest was 816.4 mm during the 2017/2018 cropping season. The soil type at the Malkerns Research Station is classified as Deep Red Loam using the Malkerns series (Kunene et al. 2019). Prior to planting, soil samples were collected from plots across the field, and bulked for analysis of soil chemical and physical properties. The bulk soil had pH 5.15 and contained 19.63 cmol(+) kg^{-1} CEC, 1.14% SOC, 0.075% N, 14 mg kg^{-1} P, 54 mg kg^{-1} K, 0.91 mg kg^{-1} Cu, 461 mg kg^{-1} Ca, 204 mg kg^{-1} Mg, 0.91 mg kg^{-1} Co, 452.80 mg kg^{-1} Fe and 1.69 mg kg^{-1} Zn.

2.2 Experimental design and planting

The field experiment involved intercropping maize with six selected legumes. The maize and legumes were planted as sole crops or intercropped in a randomised complete block design, with 13 treatments, and replicated four times. The treatments consisted of maize (cv. SC 403) and six legumes [namely, Bambara groundnut, cowpea (Mtilane local), drybean (cv. Kranskop), groundnut (cv. Natal common), jack bean (Accession 493) and soybean (Nukwa local)] planted as sole crops or intercropped with maize (Supplementary Table S1). Nitrogen was applied to the field as a blanket treatment at a single rate of 10 kg N ha^{-1} using NPK (2:3:2). The experimental plots measured 4.5 m by 5 m (22.5 m^2) with 1 m separating adjacent plots and blocks. For sole maize, two seeds were planted per hole at a depth of 5 cm and later thinned to one seedling at a spacing of 0.9 m by 0.25 m. For sole-planted legumes, two seeds were sown per

Table 1 Climate monthly data for the cropping season (2017/2018)

	Temperature (°C)		Rainfall (mm)	Relative humidity (%)	
2017/2018	Min	Max		Min	Max
December	16.9	26.8	228.6	66.6	95.8
January	16.9	28.9	100	55.6	95.5
February	18.3	28.3	175	64	96.8
March	16.8	28.5	200	62.4	97.8
April	15.7	26.7	76.6	62.6	97.8
May	16.92	25.3	36.2	51.9	96.7
Mean	**16.9**	**27.4**	**136.1**	**60.5**	**96.7**

hole at 5 cm depth and thinned to one plant with 0.9 m by 0.15 m spacing. With intercropping, Bambara groundnut, cowpea, drybean, groundnut, jack bean and soybean seeds were planted in between two rows of maize with 45 cm spacing from the maize plants. Weeds were controlled manually using hand hoe, and pests controlled chemically using a mixture of Chlorpyrifos and Beef oil. Ploughing of the experimental field was done mechanically and harrowing done with a disc plough. Bulk soil samples were collected across the field at a depth of 30 cm, pooled and air-dried at room temperature, sieved (2.0 mm sieve) and analyzed at the Agricultural Research Council, Pretoria and at the Department of Agriculture, Elsenburg, Western Cape, for pH, soil organic carbon, cation exchange capacity and mineral nutrients.

2.3 Sampling for nodulation and plant biomass

Four legume plants per plot were sampled at early podding stage by digging them up and separating into shoots and nodulated roots. The shoots were stored in labelled brown paper bags and the roots with intact nodules were kept in plastic Ziploc bags, and transported to the laboratory. The roots were then gently washed to remove debris, and the nodules detached, counted and stored in silica gel prior to bacterial isolation and use in bacterial diversity studies. The shoot samples were each oven-dried at 65 °C to a constant weight to determine shoot dry matter yield. The shoots were then finely ground (0.85 mm) for isotopic analysis to assess N_2 fixation of the selected test legumes. The stems plus petioles of Bambara groundnut, cowpea, drybean, jack bean and soybean, which belong to the tribe Phaseoleae, were weighed and ground for ureide analysis as an additional measure of N_2 fixation.

To measure soil N uptake by the legumes, non-legume plant species growing within the experimental plots were sampled concurrently as the test legumes, and the shoots processed for $^{15}N/^{14}N$ isotopic analysis. These non-legume plants were used as reference plants to measure soil N uptake by the test legumes.

2.4 *Shoot $^{15}N/^{14}N$ analysis*

^{15}N isotopic analysis was performed at the Stable Light Isotope Laboratory, University of Cape Town, South Africa. Briefly, 2.5 mg of ground legume samples or reference plant samples were weighed into tin capsules and fed onto a Carlo Erba NA1500 Elemental Analyzer coupled to a Finnigan MAT 252 Mass Spectrometer (Finnigan MAT GmbH, Bremen, Germany) via a Conflo II Open-Split Device. An internal standard of *Nasturtium* spp. was included after every five runs of the plant samples to correct for machine error associated with the isotopic analysis. The $\delta^{15}N$ values of each of the six test legumes were calculated using the equation (Unkovich et al. 2008):

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

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

The %N in shoot was obtained directly from the mass spectrometer, and the shoot N content calculated as the product of %N and shoot dry matter.

The proportion of legume N derived from atmospheric N_2 fixation (%Ndfa) was calculated as (Unkovich et al. 2008):

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

where, $\delta^{15}N_{ref}$ is the ^{15}N natural abundance of the reference plant, $\delta^{15}N_{leg}$ is the ^{15}N natural abundance of the legume, and the *B*-value is the ^{15}N natural abundance of the test legume solely dependent on N_2 fixation for its N nutrition.

2.5 Determination of B-value

The B values used for Bambara groundnut, cowpea, drybean, groundnut and soybean were obtained from literature as -1.40, -1.61, -2.16, -0.88 and -1.83‰ respectively (Unkovich et al. 2008; Mohale et al. 2014). However, the B value of jack bean used in this study was experimentally determined in the glasshouse. For this, seeds of jack bean were surfaced-sterilised in 95% ethanol, followed by soaking in 3% sodium hypochlorite solution. The jack bean seeds were then rinsed five times in sterile distilled water and planted in autoclaved sand contained in plastic pots with sterile non-absorbent cotton covering the top to avoid contamination. Three pots were used for each of the isolates. The germinated seeds were inoculated with 1 mL broth suspension of rhizobial isolates earlier obtained from the root nodules of the jack bean. The plants were watered with N-free nutrient solution (Broughton and Dilworth 1971).

Sixty days after planting, the seedlings were harvested and separated into shoots, roots and nodules. The shoots and roots were oven-dried (65 °C) for 48 h and weighed. Shoots and roots of each plant were separately ground and analyzed for $^{15}N/^{14}N$ isotopes using mass spectrometry. The mean $\delta^{15}N$ value (-0.77‰) was calculated and used as the B-value to estimate the proportion of N derived from atmospheric N_2 fixation by jack bean..

2.6 Amount of N-fixed in shoots

The N-fixed by each test legume species was calculated as:

$$N - fixed = \frac{\%Ndfa}{100} \times Shoot\ content\ of\ legume$$

2.7 Soil N uptake

Soil N uptake was determined as the difference between legume total N and the amount of N-fixed.

2.8 Analysis of ureides and nitrate in stems and petioles

2.8.1 Solute extraction from ground stems and petioles

A 0.5 g ground stem plus petiole sample was weighed and transferred into 100 mL Erlenmeyer flask, and 25 mL distilled water added to each sample and boiled for 2 min in a hot water bath. The hot samples were filtered into a 50 mL Erlenmeyer flask using 15 cm Whatman No. 40 filter paper. The residue left was washed with distilled water, allowed to cool and the volume brought to 50 mL with distilled water. The extract was then stored at -20 °C in small vials prior to analysis of N solutes. Ureide and nitrate analyses were performed as described by Unkovich et al. (2008).

2.8.2 Ureide analysis

The concentration of ureides in plant extracts was determined colorimetrically as described by Young and Conway (1942). A standard curve of optical density (OD) plotted against the concentrations of allantoin standards (Fig. 1A) was used to extrapolate the concentration of ureide in the test sample. Briefly, 0.5 mL volume of the plant extract was pipetted into duplicate test tubes and made up to 2.5 mL with distilled water. Also, 2.5 mL of control (water) blanks and 2.5 mL of each of the five ureide standards (0, 0.01, 0.02, 0.04 and 0.10 mM) were included and 0.5 mL of 0.5 M NaOH added to each extract and standard. The test tubes with samples were placed in boiling water for 10 min, and removed from the water bath to cool on the bench at room temperature. Thereafter, 1.0 mL HCl / phenyl hydrazine chloride solution (100 mL 0.65 M HCl + 0.33 g phenyl hydrazine in 100 mL distilled water) was added to each tube and vortexed. The samples were then boiled for 2 min in a hot water bath and immediately placed in ice for 15 min, followed by adding 2.5 mL 10 M HCl / potassium ferricyanide solution and mixing thoroughly for colour to develop. Absorbance readings were taken after 10 min of colour development. The optical density (OD) was read at 525 nm on a UV–Visible Spectrophotometer (JENWAY 7300, Bibby Scientific Ltd, Stone, Staffs). The equation from the standard curve generated (Fig. 1A) was used to compute the concentration of ureides (mM) in the plant extracts and expressed as µg ureide-N. mL^{-1} of plant extract.

2.8.3 Nitrate assay

Nitrate–N in the plant extracts was analyzed using the salicylic acid method (Cataldo et al. 1975), as described by Unkovich et al. (2008). A standard curve was constructed of optical density versus known nitrate concentrations (Fig. 1B), and the absorbances of extracts read from the standard curve. Duplicate tubes were used for each nitrate concentration and the control (water). Briefly, 0.05 mL

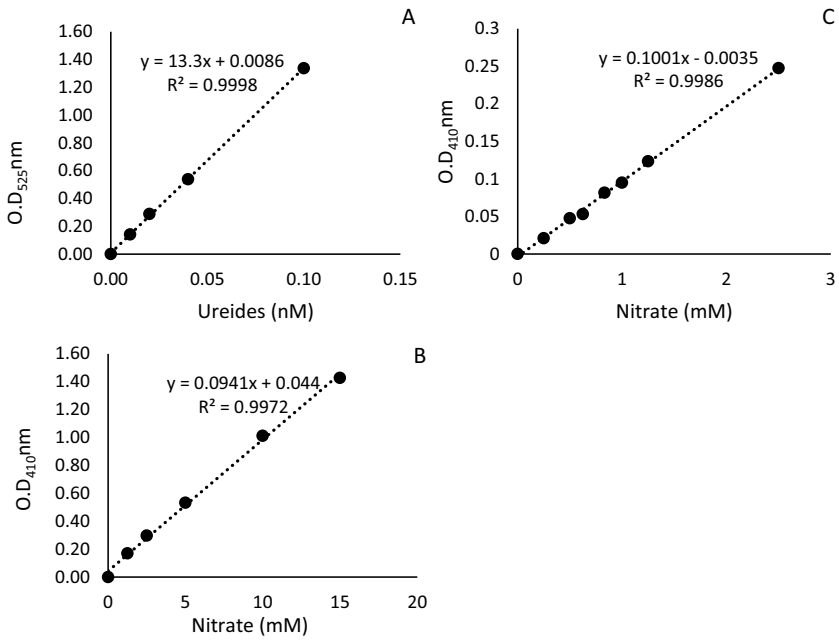

Fig. 1 Standard curves showing the response of O.D. to increasing concentrations of A) ureides and B and C) nitrate

of each extract, standard or water (control) was pipetted into duplicate test tubes, and 0.20 mL salicylic/sulphuric acid added, vortexed and left to stand on the bench at room temperature for 20 min, then 4.75 mL 2 M NaOH was added, and left to stand on the bench at room temperature for 10 min for a yellow colour to develop. The optical density was quickly read at 410 nm using the UV–Visible Spectrophotometer (JENWAY 7300, Bibby Scientific Ltd, Stone, Staffs). The nitrate concentration in each test sample was calculated using the equation from the standard curve (Fig. 1B). The nitrate concentration in the samples was expressed as µg nitrate–N. mL^{-1} of plant extract.

Percent relative ureide-N abundance.

The percent relative ureide-N abundance (RU–N) was calculated as:

$$RU - N(\%) = [(4a)/(4a + b)] \times 100$$

where a and b are the molar concentrations of ureide-N (ureides contain four N atoms per molecule) and nitrate–N, respectively (Unkovich et al. 2008).

2.8.4 Grain yield determination

At physiological maturity, two inner rows per plot were harvested for both maize and each legume crop (Bambara groundnut, cowpea, drybean, groundnut, jack bean and soybean) to determine the grain yield. The pods were separated from the plants, air-dried to 15% moisture content and shelled to obtain the seeds. The maize cobs were air-dried to 12.5% moisture content and five cobs per plot shelled manually. The seeds were then weighed, and grain yield expressed per hectare based on the plant population. Additionally, 100-seed weight was determined for each test plant species.

2.9 Statistical analysis

The data collected were tested for normality before being subjected to a 2-way analysis of variance using Statistica (version 10.1). Duncan's multiple range test was used to separate means that showed significant differences at $p \leq 0.05$. Correlation analyses were performed to assess the relationship between measured parameters.

3 Results

3.1 *Shoot $\delta^{15}N$ values of reference plants*

The combined mean $\delta^{15}N$ value of all the reference plants collected from the experimental plots was used to calculate soil N uptake (+7.79‰) by the six legumes (Supplementary Table S2).

3.2 Plant growth, symbiotic parameters and grain yield

3.2.1 Main effect of species

Of the six legumes tested, jack bean produced much larger amount of shoot DM, followed by soybean, groundnut, and then cowpea (Table 2), while drybean recorded the lowest shoot biomass. Shoot %N was however higher in soybean, followed by jack bean, and lowest in drybean (Table 2). Shoot N content was also much higher in jack bean, followed by soybean, and lowest in drybean. Shoot $\delta^{15}N$ values were lowest in Bambara groundnut, and much higher in soybean (Table 2). As a result, percent N derived from fixation was highest in Bambara groundnut, followed by groundnut, and lowest in soybean. However, the amount of N-fixed was much greater in jack bean due to its bigger shoot DM, and lowest in drybean which recorded the least shoot biomass. Soil N uptake ranged from 13.4 to 90.7 kg ha^{-1}, and was highest in jack bean. Grain yield was highest in jack bean, followed by Bambara groundnut and groundnut, and lowest in cowpea (Table 2).

3.3 Main effect of cropping system

Shoot biomass, N content, $\delta^{15}N$, amount of N-fixed and soil N uptake were all significantly higher in monocropping compared to intercropping (Table 2). Percent N derived from fixation was however much greater in mixed culture relative to monocropping. But shoot N concentration and grain yield were similar for sole and mixed culture (Table 2).

3.4 Species x cropping system interaction

Species x cropping system interaction was significant for shoot DM, shoot %N, N content, $\delta^{15}N$, percent N derived from fixation, amount of N-fixed, soil N uptake and grain yield (Table 2). Shoot %N was similar for sole and mixed cropping in Bambara groundnut, cowpea, drybean, groundnut and jack bean, but greater in monocultured soybean than mixed culture (Fig. 2A). Shoot N content was however higher under monocropping than intercropping for cowpea, drybean, groundnut, jack bean and soybean, but lower in sole crop relative to mixed cropping for Bambara groundnut (Fig. 2B). Shoot $\delta^{15}N$ values were greater in monocultured Bambara groundnut, cowpea, drybean and groundnut than mixed culture, except for jack bean which recorded similar values in the two cropping systems and soybean which had the highest shoot $\delta^{15}N$ in mixed

Table 2 Plant growth and symbiotic parameters of six legume crops grown at Malkerns in the middle-veld of Eswatini in 2017/18 cropping season. At planting all plots received a single dose of mineral N at 100 kg ha^{-1} [NPK (2:3:2 (48)]

Treatment	Shoot DM	%N	N content	δ ^{15}N	Ndfa	N fixed	Soil N uptake	Grain yield
	g plant^{-1}		mg plant^{-1}	‰	%	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}
Species								
Bambara groundnut	30.52 ± 0.64c	2.64 ± 0.05c	802.46 ± 16.35c	1.34 ± 0,11d	70.19 ± 1.21a	41.82 ± 1.30b	17.62 ± 0.62d	4513.67 ± 1416.57b
Cowpea	37.62 ± 1.74b	2.25 ± 0.04d	849.42 ± 45.66c	3.27 ± 0.15b	48.13 ± 1.54c	29.59 ± 0.90c	33.33 ± 2.66c	893.25 ± 223.55b
Drybean	19.20 ± 0.68d	1.83 ± 0.04e	351.77 ± 15.63d	2.88 ± 0.16c	49.39 ± 1.60c	12.66 ± 0.36d	13.40 ± 0.96d	2459.01 ± 503.46b
Groundnut	38.15 ± 2.94b	2.34 ± 0.03d	891.84 ± 68.26c	2.82 ± 0.13c	57.36 ± 1.53b	36.78 ± 1.97b	29.28 ± 3.14c	4122.45 ± 801.57b
Jack bean	69.27 ± 2.92a	2.92 ± 0.04b	2020.51 ± 81.66a	3.47 ± 0.06b	43.42 ± 3.28d	66.57 ± 5.28a	90.66 ± 9.41a	20,887.47 ± 3535.41a
Soybean	41.18 ± 3.60b	3.36 ± 0.07a	1410.93 ± 144.29b	4.03 ± 0.13a	39.06 ± 1.33e	42.63 ± 5.40b	61.89 ± 5.38b	1837.71 ± 310.59b
Cropping System (CS)								
Intercropping	32.22 ± 1.89b	2.51 ± 0.07a	843.76 ± 64.79b	2.73 ± 0.15b	54.52 ± 1.84a	33.78 ± 2.81b	29.80 ± 3.00b	4057.59 ± 1227.43a
Monocropping	46.43 ± 2.78a	2.60 ± 0.08a	1265.22 ± 97.81a	3.21 ± 0.12a	47.99 ± 1.68b	42.90 ± 3.02a	52.26 ± 5.59a	7513.60 ± 1978.16a
F statistics								
Species (S)	713.97***	176.21***	685.56***	181.25***	30.71***	22.84***	70.02***	28.84***
Cropping system (C)	779.14***	6.93*	541.67***	74.05***	59.52***	57.41***	81.48***	9.13**
S x C	90.43***	4.45**	86.77***	31.71***	7.73***	13.55***	6.84***	2.06*

Values (means±SE of dissimilar letters in a column are significantly different at*p≤0.05, **p≤0.01, ***p≤0.001, DM=dry matter, N=nitrogen

culture (Fig. 2C). As a result of the higher shoot δ^{15}N values under monocropping, the percent N derived from fixation was significantly lower in the shoots of all test species under sole cropping, except for soybean which derived more N from fixation in monoculture than mixed culture (Fig. 2C). The amount of N-fixed was much higher in sole cropped cowpea, groundnut and soybean than mixed culture. In contrast, N-fixed was greater in mixed cultured Bambara groundnut, and similar for drybean and jack bean in the two cropping systems (Fig. 2E). Soil N uptake was significantly increased in shoots of monocultured cowpea, drybean, groundnut, jack bean and soybean compared to the mixed culture, but similar for the two cropping systems in Bambara groundnut (Fig. 2F). Grain yield was generally higher in all six test species grown under monoculture, but significant for only Bambara groundnut, groundnut, jack bean and soybean (Fig. 3).

3.5 Growth and yield components of maize intercropped with legumes

Maize intercropped with Bambara groundnut recorded the highest shoot DM, followed by sole maize and maize intercropped with groundnut (Table 3). Cob length per plant, seed rows per cob, cob diameter, seed dry weight per plant, 100-seed weight and grain yield were similar for both sole and intercropped maize (Table 3).

3.6 Tissue ureide and nitrate concentrations

In the ureide analysis, the absorbance of samples were higher for soybean and cowpea when compared to the other legumes, followed by drybean, and lowest in jack bean. As a result, ureide concentration in stem + petiole was also markedly greater in soybean and cowpea and lowest in jack bean (Table 4). The optical density of nitrate in stem and petiole extracts was highest in soybean and lowest in Bambara groundnut (Table 4). As a result, nitrate levels were also much greater in soybean, and lowest in Bambara groundnut. Percent relative ureide-N abundance was highest in Bambara groundnut, cowpea and drybean, and lowest in jack bean (Table 4).

3.7 Main effect of cropping system

Optical densities of stem + petiole extracts, ureide and nitrate concentrations, as well as percent relative ureide-N abundance were all unaffected by cropping system (Table 4).

3.8 Species x cropping system interaction

Species x cropping system interaction was significant for ureide concentrations, nitrate levels in stem and petioles, as well as for the percent relative ureide-N abundance

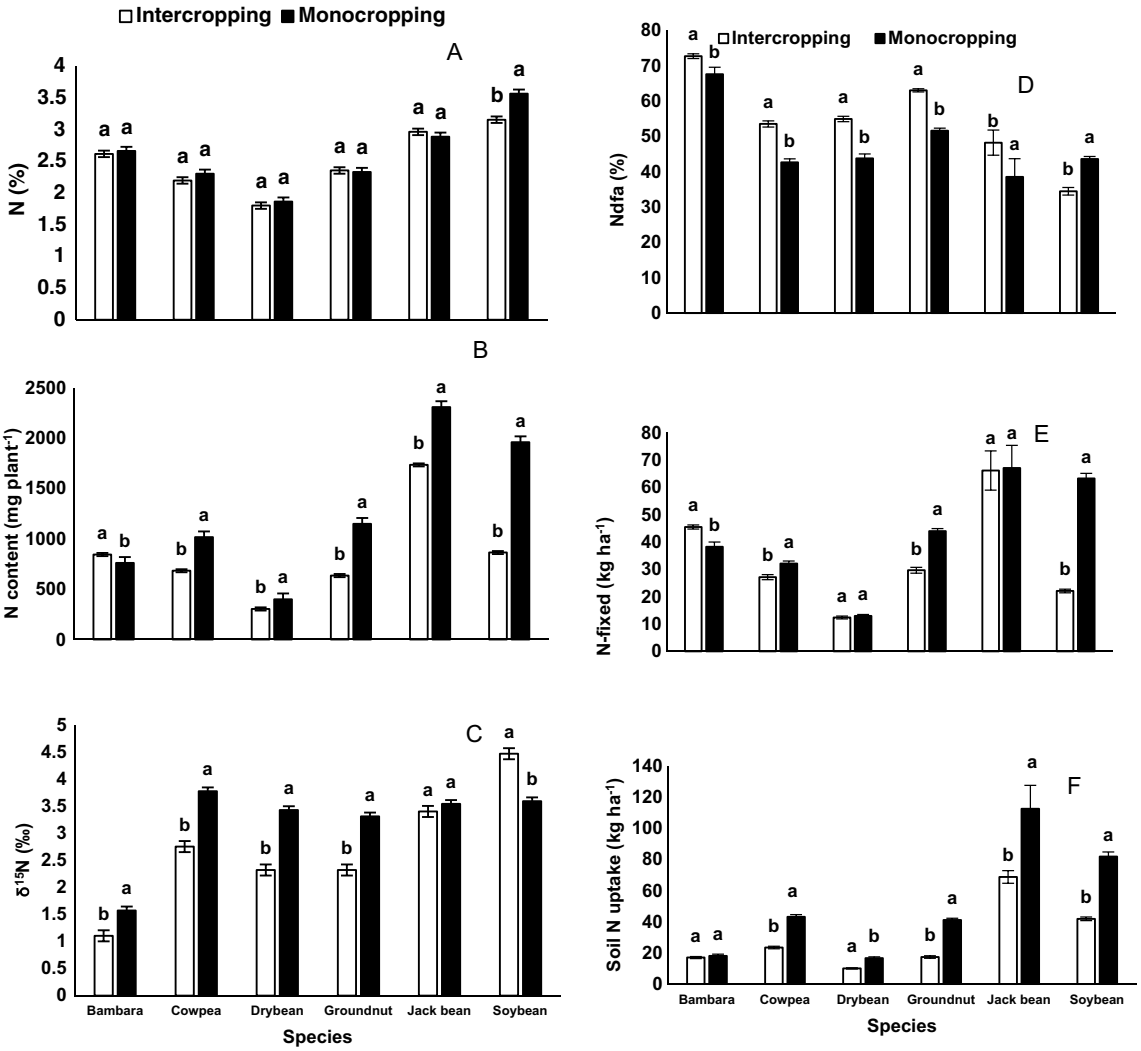

Fig. 2 Interaction effect of species x cropping system on A) %N, B) N content, C) $\delta^{15}N$, D) Ndfa, E) N-fixed and F) soil N uptake of six legumes planted at Malkerns Research Station, Eswatini during the 2017/18 cropping season. In each species, bars with dissimilar letters are significantly different at $p \leq 0.05$ and the error bars represents standard error (SE)

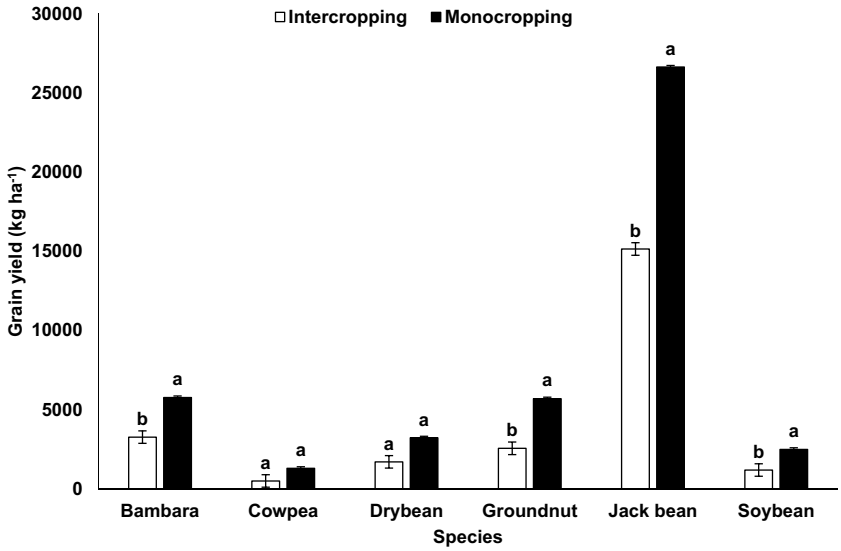

Fig. 3 Interaction effect of species x cropping system on grain yield of six legumes planted at Malkerns Research Station, Eswatini during the 2017/18 cropping season. In each species, bars with dissimilar letters are significantly different at $p \leq 0.05$ and the error bars represents standard error (SE)

Table 3 Grain yield of maize grown at Malkerns in the middle-veld of Eswatini in 2017/18 cropping season. At planting all plots received a dose rate of mineral N at 100 kg ha^{-1} [NPK (2:3:2 (48)]

Treatment	Shoot DM	Cob length	Seed lines	Cob diameter	Seed dry weight	100-seeds weight	Grain yield
	g plant^{-1}	cm	per cob	cm	g plant^{-1}	g	kg ha^{-1}
Maize	44.27 ± 0.82b	209.38 ± 2.91a	13.20 ± 0.28a	4.58 ± 0.18a	221.30 ± 15.80a	31.65 ± 1.27a	9835.33 ± 702.35a
Maize + Bambara	49.77 ± 1.61a	204.06 ± 4.55a	12.40 ± 0.40a	4.50 ± 0.08a	194.01 ± 7.97a	28.19 ± 1.12a	8622.60 ± 354.22a
Maize + cowpea	35.07 ± 1.73c	189.63 ± 8.19a	13.20 ± 0.43a	4.68 ± 0.15a	206.71 ± 25.79a	29.64 ± 2.25a	9187.04 ± 1146.27a
Maize + drybean	27.49 ± 0.57d	202.75 ± 5.66a	12.30 ± 0.41a	4.60 ± 0.08a	201.88 ± 11.70a	29.12 ± 1.51a	8972.58 ± 520.18a
Maize + groundnut	41.84 ± 1.33b	202.19 ± 6.20a	12.55 ± 0.28a	4.41 ± 0.21a	188.27 ± 12.45a	28.17 ± 2.43a	8367.44 ± 553.16a
Maize + jack bean	31.40 ± 1.37c	202.56 ± 6.00a	12.80 ± 0.32a	4.74 ± 0.03a	207.44 ± 6.43a	28.88 ± 0.81a	9219.53 ± 285.67a
Maize + soybean	33.43 ± 0.62c	208.13 ± 5.06a	13.60 ± 0.49a	4.92 ± 0.11a	230.77 ± 12.68a	32.38 ± 1.08a	10,256.61 ± 563.58a
F statistics							
Treatment	41.26*	1.263 ns	1.607 ns	1.587 ns	1.042 ns	1.042 ns	1.042 ns

Values (Means ± SE) of dissimilar letters in a column are significantly different at *$p \leq 0.05$, ns = not significant, DM = dry matter

(Table 4). An analysis of species x cropping system interaction revealed marked differences in species response to the cropping systems (Fig. 4). As shown in Fig. 4A-C, Bambara groundnut and cowpea recorded significantly greater absorbances and ureide concentrations with intercropping relative to monoculture, while soybean revealed the reverse. The same parameters in drybean and jack bean were however unaffected by cropping system (Fig. 4A-C). Intercropping increased tissue nitrate absorbances and concentrations in Bambara groundnut, and decreased it in monoculture, but these parameters were unaffected by cropping system in cowpea, jack bean and soybean (Fig. 4D-F). Except for drybean, which showed decreased percent relative ureide-N abundance with intercropping, this parameter was unaffected by cropping system in Bambara groundnut, cowpea, jack bean and soybean (Fig. 4G).

4 Discussion

The ability of bacteroids in the root nodules of legumes to reduce atmospheric N_2 to NH_3 has huge nutritional benefits for associated intercropped partners, as well as to following cereal crops in rotation (Shen and Chu 2004; Mafongoya et al. 2006; Dahmardeh et al. 2010; Rusinamhodzi et al. 2012). In this study, differences in plant growth, symbiotic functioning, and grain yield were evaluated in six grain legumes intercropped with maize at Malkerns Research Station, Eswatini. The results revealed significant differences in plant growth, symbiotic performance and grain yield due to species differences, phenology and growth characteristics. Whereas the levels of key elements such as N (0.075%), P (14 mg kg^{-1}) and K (54 mg kg^{-1}) were generally low in the test soil, the plants in this study benefited from the blanket application of NPK fertilizer to plots prior to planting. In most instances, high shoot dry matter (SDM) in the test legumes was associated with high shoot $\delta^{15}N$, %N and N content as evidenced by jack bean which had the highest SDM, a relatively high shoot $\delta^{15}N$ and %N as well as the highest N content (Table 2). Conversely, drybean had the lowest SDM, low $\delta^{15}N$ and the lowest shoot %N and N content (Table 2). These findings were supported by the significant positive correlations found between shoot DM and %N ($r = 0.5280$), shoot DM and N content ($r = 0.9582$) (Fig. 5A, B). Furthermore, intercropping with maize caused low legume SDM from competition with maize and low $\delta^{15}N$ from increased N_2 fixation triggered by competition from the associated cereal plant for soil N. Monocultured legumes however had high SDM, $\delta^{15}N$ and N content, an indication of reduced competition for water and mineral resources. The observed low shoot DM and low shoot $\delta^{15}N$ values associated with intercropping in this study are consistent with the findings of Kermah et al. (2018). It has been shown that low

Table 4 Symbiotic performance of five legume crops grown at Malkerns in the middle-veld of Eswatini in 2017/18 cropping season

Treatment	Ureide concentration	Nitrate concentration	RU–N
	µmol g^{-1}	µmol g^{-1}	%
Species			
Bambara groundnut	6.73 ± 0.63b	20.82 ± 1.65c	55.79 ± 0.69a
Cowpea	11.34 ± 1.03a	38.77 ± 4.82b	55.08 ± 1.86a
Drybean	7.37 ± 1.01b	24.41 ± 1.72bc	52.60 ± 3.17a
Jack bean	1.63 ± 0.12c	36.68 ± 2.80b	16.46 ± 1.15c
Soybean	12.23 ± 1.34a	77.55 ± 10.22a	40.47 ± 3.02b
Cropping system (CS)			
Intercropping	7.72 ± 0.73a	37.64 ± 2.36a	43.69 ± 2.67a
Monocropping	8.00 ± 0.93a	41.66 ± 6.06a	44.46 ± 2.82a
F statistics			
Species (S)	37.56***	30.01***	60.93***
Cropping system (C)	0.20 ns	1.19 ns	0.16 ns
S x C	16.13***	13.47***	2.60*

Values (means ± SE) of dissimilar letters in a column are significantly different at *$p \leq 0.05$, ns = not significant, *** $p \leq 0.001$, RU = relative ureide, N = nitrogen

shoot $\delta^{15}N$ is generally the result of greater %Ndfa legumes (Mokgehle et al. 2014; Beyan et al. 2018; Lengwati et al. 2020). This was also the case in this study, with legumes that recorded low $\delta^{15}N$ values exhibiting high %Ndfa, and vice versa. Bambara groundnut, for example, recorded the lowest shoot $\delta^{15}N$ and the highest %Ndfa, while soybean which had the highest shoot $\delta^{15}N$ recorded the lowest %Ndfa (Table 2). Although Kermah et al. (2018) found that the %Ndfa of legumes is not influenced by the cropping system but differs among legume species and study sites, this was not the case in this study as the cropping system had a marked effect on the %Ndfa of the test legumes (Table 2). Similar observations were made by Giller et al. (1991) who also found that intercropping of grain legumes with cereals generally resulted in the legume deriving a greater proportion of its N from symbiotic fixation than when grown in monoculture.

Due to their higher shoot biomass, the monocultured legumes produced more symbiotic N than their intercropped counterparts (Table 2). These results were similar to those of Giller (2001) and Adeleke and Haruna (2012), who also reported greater shoot DM with monoculture leading to increased amounts of fixed-N when compared to the mixed culture. Egbe et al. (2013) as well as Kermah et al. (2018) also found high amounts of N-fixed in Bambara groundnut, cowpea, groundnut and soybean monocultures than when intercropped with maize. In contrast, shoot percent N derived from N_2 fixation was much higher under intercropping than monocropping due to intense competition for soil N by two partners in the former system. As a result, soil N uptake by legumes was lower under mixed culture than monoculture. Independent of the cropping system, the amount of N-fixed was significantly higher in jack bean, possibly due to its huge shoot biomass, which required more N for growth and development. In general, the amount of N-fixed and soil N uptake were higher in monoculture than mixed culture. As a result, grain yield was also greater under monocropping than intercropping. At species level, jack bean produced more grain, followed by Bambara groundnut and groundnut. In general, monocultures of the six legumes produced more grain yield than mixed cultures (Table 2).

In Southern Africa, smallholder farmers generally practice maize-legume intercropping in order to reduce total crop failure under monoculture (Rusinamhodzi et al. 2012; Kermah et al. 2017). In this study however maize yields were similar for both sole cropping and mixed culture (Table 3). These results contradict an earlier report by Hassan et al. (2014) on maize yield components under intercropping. Generally, maize grain yield has been reported to be higher under legume-maize intercropping than sole maize (Mthembu et al. 2018). However, Alhassan and Egbe (2014) recorded higher maize grain yield in sole maize relative to intercropped maize, and in this study there were no differences in grain yield under monocropping and intercropping. These inconsistences can be attributed to a number of factors including the plant heights of the legume and maize, sequential planting dates of legume and maize, level of shadiness caused by the cereal, the rhizosphere dynamics of the intercropped partner, etc. More field experiments are needed between short stalk maize and legumes (less shadiness), as well as long stalk maize and legumes (more shadiness), in order to resolve the yield benefits of intercropping on the two partners.

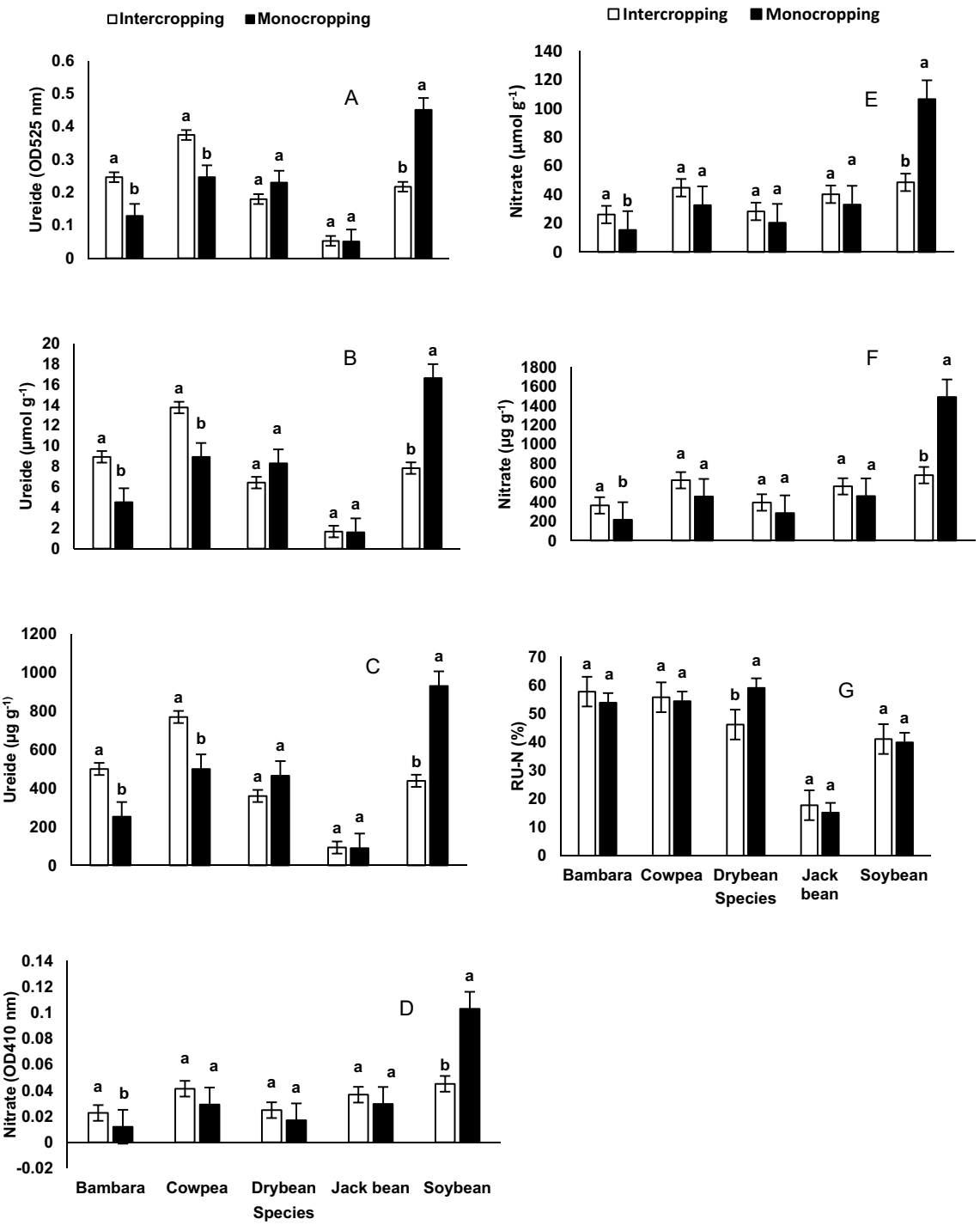

Fig. 4 Interaction effect of species x cropping system on A-C) Ureide concentration, D-F) Nitrate concentration and E) RU–N of six legumes planted at Malkerns Research Station, Eswatini during the 2017/18 cropping season. In each species, bars with dissimilar letters are significantly different at $p \leq 0.05$ and the error bars represents standard error (SE)

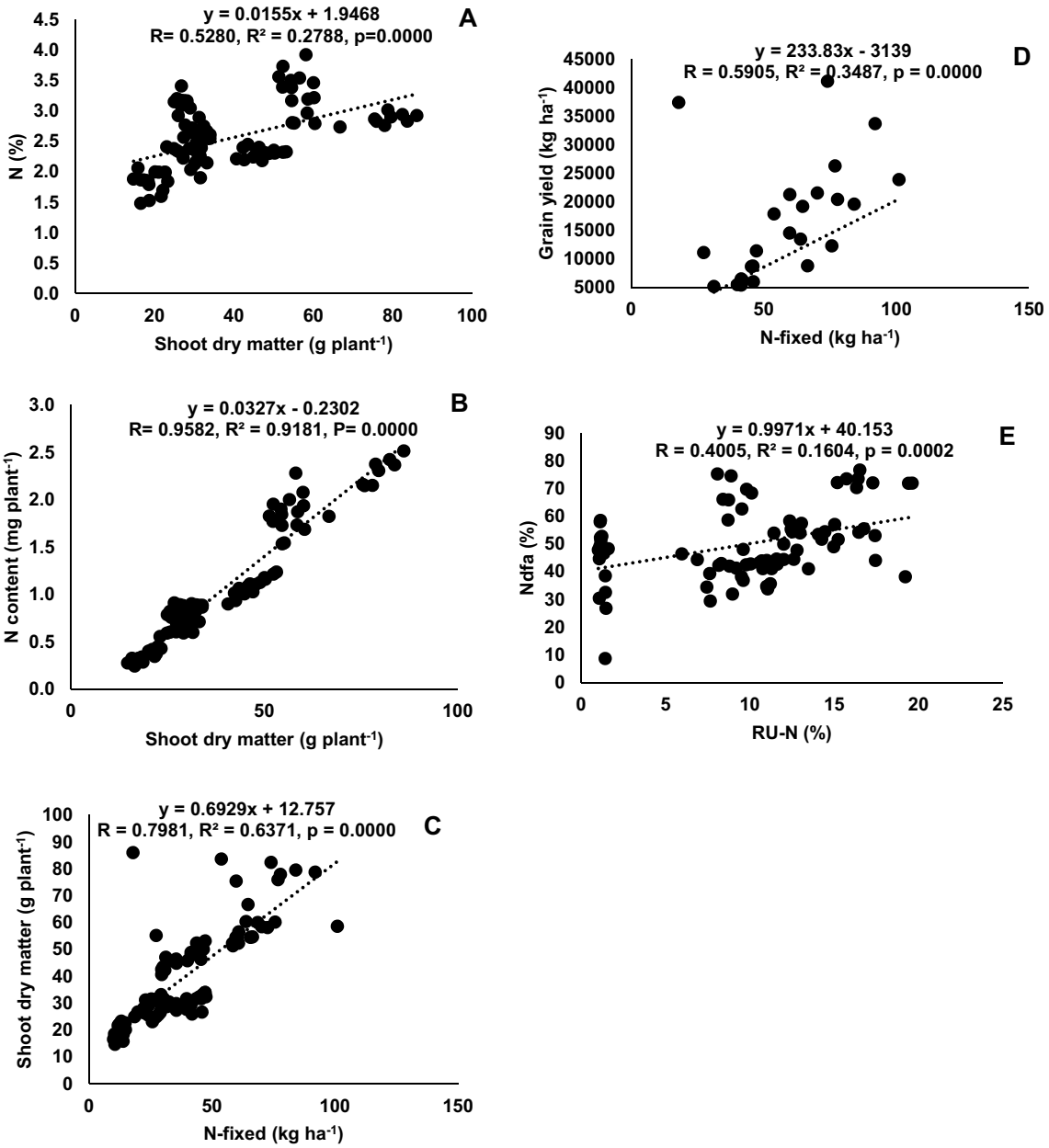

Fig. 5 Correlation and regression analysis between A) shoot dry matter and %N, B) shoot DM and N content **C**) N-fixed and shoot dry matter, D) N-fixed and grain yield and E) RU–N and %Ndfa of six selected legumes planted at Malkerns Research Station, Eswatini during the 2017/18 cropping season

5 N_2 fixation in five Phaseoleae legume species measured using the ureide assay vs. the ^{15}N natural abundance technique

In this study, the ureide and ^{15}N isotopic techniques were used to measure N_2 fixation in the five ureide-exporting test legumes, and the results showed that the legumes with high ureide-N concentrations generally recorded low tissue nitrate–N concentrations, and vice versa, albeit a few exceptions (Table 4). Jack bean, for example, showed low ureide-N concentration and high tissue nitrate–N, while cowpea in contrast revealed high ureide and low nitrate–N levels (Table 4). High nitrate concentrations in the stem and petiole extracts of the test legumes in this study suggested their greater reliance on soil N uptake than symbiosis for their N nutrition (Herridge and Peoples 1990), while the recorded high ureide concentrations in stem extracts indicate greater N_2 fixation (Hayat et al. 2008; Mohammed et al. 2022).

Consistent with the ^{15}N natural abundance data, Bambara groundnut, cowpea and drybean also recorded the highest percent relative ureide-N abundance (55.79, 55.08 and 52.60%, respectively), while soybean (40.47%) and jack bean (16.46%) revealed the lowest %RU–N in conformity with the %Ndfa values from ^{15}N isotopic analysis. Based on the two methods, Bambara groundnut, cowpea and drybean derived most of their N nutrition from N_2 fixation, while jack bean and soybean obtained most of their N from uptake in the soil (Table 2 and 4). Hayat et al. (2008) showed that legume N_2 fixation was positively correlated with shoot DM and grain yield, which is consistent with the results of this study (Fig. 5C, D). However, high relative ureide-N abundance did not translate into in high grain yield in some legumes, and vice versa (Table 2 and 4). For example, jack bean showed the lowest relative ureide-N and the highest grain yield, while cowpea recorded much higher relative-ureide-N but produced low grain yield. This can be attributed to the instantaneous nature of the ureide method, as it reflects N_2 fixation at the time of the sampling, and not over the entire growth period of the plant (Unkovich et al. 2008). However, the positive correlation found between %RU–N and %Ndfa (Fig. 5E) has confirmed the robustness of the ^{15}N abundance technique and the ureide assay in measuring N_2 fixation in members of the tribe Phaseoleae (Mohammed et al. 2022).

In conclusion, the results of this study showed that cropping system has an effect on plant growth, symbiotic performance and grain yield. Legumes grown under monocropping recorded higher plant growth, symbiotic performance and grain yield when compared to those grown in mixed cultures. However, shoot %Ndfa was much higher under intercropping than monocropping due to competition by cereal and legume for soil N which generally forces the legume to symbiotically fix more N_2. The components of maize yield were similar for the two cropping systems. The ^{15}N natural abundance technique and ureide method were complementary in measuring N_2 fixation in members of the tribe Phaseoleae.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13199-024-00971-x.

Acknowledgements We are grateful to the National Research Foundation grant number PDG21040959 3370, Tshwane University of Technology, and the South African Research Chair in Agrochemurgy and Plant Symbioses for financial support. The authors also appreciate support from Mr. Manana and all staff of the Malkerns Research Station in Eswatini for helping with field experiments.

Author contributions Z.D.N. collected and analysed data, and drafted the manuscript. M.M. took part data collection and analysis and drafted the manuscript with Z.D.N. F.D.D. was the post-doctoral host of Z.D.N., and approved the final manuscript.

Funding Open access funding provided by Tshwane University of Technology.

Data Availability Data are contained within the article.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Adeleke MA, Haruna IM (2012) Residual nitrogen contributions from grain legumes to the growth and development of succeeding maize crop. ISRN Agronomy 2012:1–5. https://doi.org/10.5402/2012/213729

Alhassan GA, Egbe MO (2014) Bambara groundnut/maize intercropping: Effects of planting densities in Southern guinea savanna of Nigeria. Afr J Agric Res 9:479–486

Beyan SM, Wolde-meskel E, Dakora FD (2018) An assessment of plant growth and N_2 fixation in soybean genotypes grown in uninoculated soils collected from different locations in Ethiopia. Symbiosis 75:189–203. https://doi.org/10.1007/s13199-018-0540-9

Broughton WJ, Dilworth MJ (1971) Control of leghaemoglobin synthesis in snake beans. Biochem J 125:1075–1080

Cataldo D, Haroon M, Schrader LE, Youngs VL (1975) Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun Soil Sci Plant Anal 6:71–80

Dahmardeh M, Ghanbari A, Syahsar BA, Ramrodi M (2010) The role of intercropping maize (Zea mays L.) and Cowpea (*Vigna unguiculata* L.) on yield and soil chemical properties. Afr J Agric Res 5:631–636. https://doi.org/10.5897/AJAR09.607

Dlamini ST, Jaiswal SK, Mohammed M, Dakora FD (2021) Studies of phylogeny, symbiotic functioning and ecological traits of indigenous microsymbionts nodulating Bambara groundnut (*Vigna subterranea* L. Verdc) in Eswatini. Microb Ecol 1:1–6

Edje OT, Ossom EM (2009) Crop science handbook. Blue Moon Publishers, Manzini, Swaziland

Egbe MO, Alhassan GA, Ijoyah M (2013) Nodulation, nitrogen yield and fixation by bambara groundnut (*Vigna subterranea* (L.) Verdc.) landraces intercropped with cowpea and maize in Southern Guinea Savanna of Nigeria. Agric Sci 1:15–28

Ghanbari-Bonjar A, Lee HC (2003) Intercropped wheat (*Triticum aestivum* L.) and bean (*Vicia faba* L.) as a whole-crop forage: effect of harvest time on forage yield and quality. Grass Forage Sci 58:28–36

Giller KE (2001) Nitrogen fixation in tropical cropping systems. CABI Publishing, Wallingford

Giller KE, Ormesher J, Awah FM (1991) Nitrogen transfer from *Phaseolus* bean to intercropped maize measured using ^{15}N-enrichment and ^{15}N-isotope dilution methods. Soil Biol Biochem 23:339–346

Hassan MR, Amodu JT, Muhammad IR et al (2014) Forage yield and quality of lablab (*Lablab purpureus* L. Sweet) intercropped with maize (Zea mays L.) with flooded irrigation system in the semi-arid zone of Nigeria. J Agric Sci 6:196–211. https://doi.org/10.5539/jas.v6n11p196

Hayat R, Ali S, Sohail Ijaz S et al (2008) Estimation of N_2-fixation of mung bean and mash bean through xylem ureide technique under rainfed conditions. Pak J Bot 40:723–734

Herridge DF, Giller KE (2016) Measurement of nitrogen fixation. In: Howieson JG, Dilworth MJ (eds) Working with rhizobia. Australian Centre for International Agricultural Research, Canberra, pp 187–220

Herridge DF, Peoples MB (1990) Ureide assay for measuring nitrogen fixation by nodulated soybean calibrated by ^{15}N methods. Plant Physiol 93:495–503

Kermah M, Franke AC, Adjei-Nsiah S et al (2017) Maize-grain legume intercropping for enhanced resource use efficiency and crop productivity in the Guinea savanna of northern Ghana. Field Crops Res 213:38–50. https://doi.org/10.1016/j.fcr.2017.07.008

Kermah M, Franke AC, Adjei-Nsiah S et al (2018) N2-fixation and N contribution by grain legumes under different soil fertility status and cropping systems in the Guinea savanna of northern Ghana. Agric Ecosyst Environ 261:201–210. https://doi.org/10.1016/j.agee.2017.08.028

Kunene TR, Masarirambi MT, Wahome PK, Oseni TO (2019) Influence of kraal manure, chicken manure and inorganic fertilizer on growth, yield and post-harvest quality of pepper (*Capsicum annuum* L.) in a Sub-tropical environment. Asian J Adv Agric Res 1–11. https://doi.org/10.9734/ajaar/2019/v11i130043

Lengwati DM, Mathews C, Dakora FD (2020) Rotation Benefits From N2-Fixing Grain Legumes to Cereals: From Increases in Seed Yield and Quality to Greater Household Cash-Income by a Following Maize Crop. Front Sustain Food Syst 4:1–16. https://doi.org/10.3389/fsufs.2020.00094

Li L, Yang S, Li X et al (1999) Interspecific complementary and competitive interactions between intercropped maize and faba bean. Plant Soil 212:105–114

Li QZ, Sun JH, Wei XJ et al (2011) Overyielding and interspecific interactions mediated by nitrogen fertilization in strip intercropping of maize with faba bean, wheat and barley. Plant Soil 339:147–161

Mafongoya PL, Bationo A, Kihara J, Waswa BS (2006) Appropriate technologies to replenish soil fertility in southern Africa. Nutr Cycl Agroecosyst 76:137–151. https://doi.org/10.1007/s10705-006-9049-3

Maitra S, Shankar T, Banerjee P (2020) Potential and advantages of maize-legume intercropping system. In: Hossain A (ed) Maize-production and use. Bangladesh Wheat and Maize Research Institute (BWMRI), Dinajpur, pp 103–116

Manasa P (2020) Yield attributes, yield, competitive ability and economics of summer maize-legume intercropping system. Int J Agric Enviro and Biotechnol 13:33–38. https://doi.org/10.30954/0974-1712.1.2020.16

Mohale KC, Belane AK, Dakora FD (2014) Symbiotic N nutrition, C assimilation, and plant water use efficiency in Bambara groundnut (*Vigna subterranea* L. Verdc) grown in farmers' fields in South Africa, measured using ^{15}N and ^{13}C natural abundance. Biol Fertil Soils 50:307–319

Mohammed M, Mbah GC, Sowley ENK, Dakora FD (2022) Cowpea genotypic variations in N_2 fixation, water use efficiency (δ^{13}C), and grain yield in response to *Bradyrhizobium* inoculation in the field, measured using xylem n solutes, ^{15}N, and ^{13}C natural abundance. Front Agron 4:1–24. https://doi.org/10.3389/fagro.2022.764070

Mokgehle SN, Dakora FD, Mathews C (2014) Variation in N_2 fixation and N contribution by 25 groundnut (*Arachis hypogaea* L.) varieties grown in different agro-ecologies, measured using ^{15}N natural abundance. Agric Ecosyst Environ 195:161–172. https://doi.org/10.1016/j.agee.2014.05.014

Mthembu BE, Everson TM, Everson CS (2018) Intercropping maize (*Zea mays* L.) with lablab (*Lablab purpureus* L.) for sustainable fodder production and quality in smallholder rural farming systems in South Africa. Agroecol Sustain Food Syst 42:362–382. https://doi.org/10.1080/21683565.2017.1393649

Nasar J, Shao Z, Arshad A et al (2020) The effect of maize–alfalfa intercropping on the physiological characteristics, nitrogen uptake and yield of maize. Plant Biol 22:1140–1149. https://doi.org/10.1111/plb.13157

Peoples MB, Faizah AW, Rerkasem B, Herridge DF (1989) Methods for evaluating nitrogen fixation by nodulated legumes in the field. Australian Centre for International Agricultural Research, Canberra

Rusinamhodzi L, Corbeels M, Nyamangara J, Giller KE (2012) Maize-grain legume intercropping is an attractive option for ecological intensification that reduces climatic risk for smallholder farmers in central Mozambique. Field Crops Res 136:12–22. https://doi.org/10.1016/j.fcr.2012.07.014

Shen Q, Chu G (2004) Bi-directional nitrogen transfer in an intercropping system of peanut with rice cultivated in aerobic soil. Biol Fertil Soils 40:81–87. https://doi.org/10.1007/s00374-004-0737-3

Tsubo M, Walker S, Ogindo HO (2005) A simulation model of cereal-legume intercropping systems for semi-arid regions: I. Model Development Field Crops Res 93:10–22. https://doi.org/10.1016/j.fcr.2004.09.002

Unkovich M, Herridge D, Peoples M, Cadisch G, Boddey R, Giller K, Alves B, Chalk P (2008) Measuring plant-associated nitrogen fixation in agricultural systems. Australian Centre for International Agricultural Research, Canberra

Willey RW (1979) Intercropping: its importance and its research needs, Part 2. Agron Relatsh Field Crop Abstracts 32:73–85

Young GBE, Conway CF (1942) On the estimation of allantoin by the Rimini-Schryver reaction. J Biol Chem 142:839–853

Zhang X, Huang G, Zhao Q (2014) Differences in maize physiological characteristics, Nitrogen accumulation, and yield under different cropping patterns and nitrogen levels. Chil J Agric Res 74:326–332. https://doi.org/10.4067/S0718-58392014000300011