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The effect of water availability on the carbon and nitrogen isotope composition of a C_4 plant (pearl millet, *Pennisetum glaucum*)

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Abstract

To evaluate the potential utility of isotope ratios in plant materials as an archaeological proxy for past crop water status, relationships between water availability and stable isotope ratios in C_4 species must be established. This study quantified the isotopic values ($\delta^{13}C$ and $\delta^{15}N$) of pearl millet (*Pennisetum glaucum*) seeds and leaves in response to varying degrees of water stress. Under greenhouse conditions, we exposed five strains of pearl millet to three different watering treatments. Pearl millet seed $\delta^{13}C$ values (mean and SD = -13.9 ± 0.5 ‰, n = 48) and leaf $\delta^{13}C$ values (mean and SD = -14.8 ± 0.7 ‰, n = 75) were positively correlated with water availability across 75 plants from five strains. The magnitude of the relationship for seeds (0.24 ± 0.04 ‰ per 0.1 m^3 m^{-3} increase in soil moisture) and leaves (0.24 ± 0.06 ‰) was similar. The five strains showed differences in bulk carbon isotope ratios but had indistinguishable responses to water availability. Water availability had no discernible effect on $\delta^{15}N$ in any of these strains. These results suggest that, while in some cases sensitive to water availability, the differences in the isotope ratios of pearl millet seeds and leaves across treatments were not of sufficient magnitude for reconstructing past crop water status without additional information.

Key Words: African millet, greenhouse experiment, palaeoenvironmental reconstruction, stable isotope analysis, water restriction

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Fig. 1 (color, fits 1.5 columns)

Fig. 2 (color, fits 2 columns)

Fig. 3 (color, fits 1.5 columns)

Fig. 4 (color, fits 1 column)

1. Introduction

1.1 Background

Strong relationships have been identified between water availability and the carbon and nitrogen isotope values ($\delta^{13}C$ and $\delta^{15}N$) of plant materials (e.g. Stewart et al., 1995; Handley et al., 1999; Schuur & Matson, 2001; Weiguo et al., 2005). For carbon isotopes in particular, these relationships have been integral to identifying past shifts in regional precipitation and aridity (e.g. Kohn, 2010; Kress et al., 2010; Schubert et al., 2011; Kress et al., 2014). Paleoclimate reconstructions have relied on the well-established negative correlation between $\delta^{13}C$ values of plant material and water availability in C_3 plants (Farquhar et al., 1989). In archaeology, this relationship can be applied to identify the presence of artificial water management practices (e.g. irrigation systems). Higher-than-expected water availability is consistent with human manipulation of water resources and is reflected in plant isotope ratios. As such, stable carbon and nitrogen isotope analyses of archaeobotanical remains have been employed to investigate crop water status in ancient times (e.g. Stokes et al., 2011; Bogaard et al., 2013; Wallace et al., 2013, Styring et al., 2017). Quantification of the relationships between water availability and isotope ratios for C_4 species, including pearl millet (*Pennisetum glaucum*), provides an important reference for investigations into archaeological use of these cereals by people in the past. In this study, we conducted a greenhouse watering experiment to establish relationships between water availability and carbon and nitrogen isotope ratios in the seeds and leaves of pearl millet.

The reliable identification of past water management is essential to our understanding of agricultural innovation and its environmental adaptation in the past. This is particularly true in a range of arid environments where early civilizations first developed (e.g. Steward, 1955; Adams, 1981). Numerous studies with an archaeological focus have sought to refine the relationship between water availability and stable carbon isotope ratios for crop species and to establish the limitations of this method for inferring past crop water status (e.g. Araus et al., 1999; Flohr et al., 2011; Wallace et al., 2013). In order to use stable isotope values measured in ancient plant materials as proxies for past water management practices, it is critical that the relationship between isotope ratios and water availability is calibrated for specific crops and regions.

C_4 crops are important to human diets globally. Maize and sugarcane rank among the 12 most profitable crops worldwide, and other C_4 crops such as sorghum and varieties of millet are among the United Nations Food and Agricultural Organization's top 150 crops produced

globally. C_4 grasses also provide essential, indirect support to human nutrition as the primary forage grasses for livestock in warm climates (Sage & Zhu, 2011). C_4 photosynthesis began appearing in the geologic record *c.* 30-35 million years ago, when atmospheric CO_2 levels were near-present and global climate change produced dry, highly seasonal subtropical and temperate regions that favored C_4 evolution (Sage, 2003). Within the last *c.* 30 million years, C_4 photosynthesis has independently evolved numerous times; 61 distinct evolutionary lineages of C_4 photosynthesis have been identified (Sage, 2016). Modern C_4 plants have particular significance on a regional level, providing hardy and resilient food crops in the arid and semiarid tropics due to their ability to withstand high temperatures and erratic rainfall patterns.

"Millet" describes a variety of C_4 taxa originating from several continents, from genus such as *Panicum, Setaria, Sorghum, Echinochloa, Eleusine, Pennisetum* (e.g. Weber, 1998). Millet crops share common ecological features, including a short summer growing season and modest water requirements, which made them vital food resources in arid environments (Lightfoot et al., 2018). We elected to examine pearl millet (*Pennisetum glaucum*) in this study. Pearl millet is the most geographically expansive millet crop being cultivated today, archaeologically important both in Africa and South Asia, and one of the key cereals across the Indian Ocean in the context prehistoric food globalization (e.g. Serba & Yadav, 2016, Manning et al. 2011, Fuller et al. 2011). Pearl millet was possibly domesticated in the southern edge of the Sahara up to 4,500 years ago and was subsequently cultivated throughout the African continent, driving its adaptation to a variety of environments including semi-desert zones, savannas and equatorial rainforests (e.g. Manning et al., 2011; Burgarella et al., 2018). It is an essential staple cereal of sub-Saharan Africa and parts of India. Scholars noted that pearl millet is the only African cereal that existing archaeobotanical evidence is adequate for quantitative assessment of its cultivation history and domestication process, and charred pearl millet grains are abundant in a series assemblages particularly in west Africa as well as across sub-Saharan Africa and India (Manning et al. 2011, Fuller 2007,) (Boivin and Fuller, 2009; Fuller and Hildebrand, 2013; Boivin et al., 2014). In the modern day, pearl millet is a staple crop for millions of rural communities in the hottest and most arid regions of Africa and the Indian subcontinent (Serba & Yadav, 2016), and its resilience endows pearl millet with particular relevance in planning for future food security.

Controlled experiments constraining the relationship between carbon isotope discrimination and water availability are more abundant in the literature for C_3 plants than for C_4 plants (e.g. Condon et al., 1992; Li, 1999; Clay et al., 2001; Zhao et al., 2004). Models and some existing data suggest that isotope ratios in C_4 plants should be less sensitive to water availability than C_3 plants (e.g. Farquhar et al.,1989; Van de Water et al., 2002; Swap et al., 2004; Weiguo et al., 2005). Previous studies have quantified carbon isotope ratios in C_4 species along regional precipitation gradients, finding both positive (Schulze et al., 1996; Murphy & Bowman, 2009; An et al., 2015) and negative (Weiguo et al., 2005) correlations in $\delta^{13}C$ with respect to water availability (precipitation). In a controlled experimental set-up, $\delta^{13}C$ values in maize (*Zea mays L.*) have been shown to be positively correlated with water availability (Dercon et al., 2005). Additionally, a positive relationship between carbon isotope ratios and water availability was found recently in foxtail millet (*Setaria italica*) varieties exposed to different watering regimes in a growth chamber experiment (Lightfoot et al., 2020).

Existing data for pearl millet indicate that cumulative annual precipitation and temperature together account for 48% and 34% of the variability in seed $\delta^{13}C$ and $\delta^{15}N$ values, respectively (Reid et al., 2018). In this study, we sought to quantify previously observed relationships between water availability and pearl millet isotope ratios. Our results bear on the potential utility of stable isotopes in archaeological pearl millet seeds as a proxy for past water management practices. Isotopic analyses of a variety of modern regional accessions (genetically unique plant specimen added to an existing collection) also provide a useful baseline for detecting environmentally-induced traits, if differences among accessions are distinct enough to be discernible in archaeobotanical material.

1.2 Carbon isotopes and C_4 plants

Pearl millet fixes carbon through the Hatch-Slack pathway (Moser et al., 2004). The distinctive feature of this pathway is a carbon concentrating mechanism that delivers CO_2 to the carbon-fixing enzyme ribulose-1,5,-bisphosphate carboxylase/oxygenase (Rubisco) (Hatch, 2002). One consequence of this carbon-concentrating mechanism is that carbon isotope fractionations exhibited by C_4 plants are generally smaller than those observed in C_3 plants (Farquhar, 1989). C_4 plants, including most warm season grasses and arid-adapted dicots, have

been found to be more enriched in ^{13}C relative to C_3 plants. This relative enrichment is expressed using $\delta^{13}C$, defined (in ‰) as:

$$\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 10^3$$

$\delta^{13}C$ values for C_4 plants are understood to be *c.* -12.5 ± 1.1 ‰, while C_3 plants have lower $\delta^{13}C$ values of *c.* -26.7 ± 2.3 ‰ (Cerling et al., 1997).

The carbon isotope fractionation observed in any plant can be influenced by environmental conditions, including variations in temperature, light, and atmospheric CO_2 concentration. For example, higher intracellular CO_2 concentrations (due to high atmospheric CO_2 levels or high rates of stomatal diffusion) result in larger fractionations (Farquhar et al., 1982; Farquhar & Richards, 1984; Farquhar et al., 1989). In C_3 plants, decreased water availability results in lower rates of stomatal diffusion, producing smaller fractionations (Farquhar et al., 1989). However, in C_4 plants, the relationship between water availability and the $\delta^{13}C$ values of plant material is more complex. Prior to carbon fixation, atmospheric CO_2 first equilibrates – both chemically and isotopically – with intracellular dissolved bicarbonate. This bicarbonate is incorporated into the C_4 acid oxaloacetate, which is subsequently transported to bundle-sheath cells and decarboxylated, releasing CO_2 that is fixed by Rubisco (Sage, 2003). Carbon isotope fractionation between CO_2 and the final photosynthetic product can be positive, negative, or zero depending on the ratio of intercellular to ambient partial pressure of CO_2 ($\frac{p_i}{p_a}$), the temperature, and the leakiness (φ) of the bundle sheath cells (Farquhar et al., 1989; Williams et al., 2001).

1.3 Nitrogen isotopes and C_4 plants

Plant nitrogen isotope values integrate a range of environmental and physiological processes, but reflect largely the $\delta^{15}N$ value of the soil in which they are growing. The ratio of nitrogen isotopes in plant material depends on the nitrogen isotope composition of their soil, as well as isotopic fractionation during assimilation (Evans, 2001). In an archaeological context, soil nitrogen isotope ratios are influenced by land use history such as manuring (Peukert et al., 2012; Fraser et al., 2011; Bogaard et al., 2013; Styring et al., 2019). In addition, a positive correlation between aridity (low precipitation and high evapotranspiration) and $\delta^{15}N$ values has been documented in various soil and vegetation studies (e.g. Handley et al., 1999; Amundson et al., 2003; Craine et

al., 2009; Hartman & Danin, 2010). This is likely related to the 'openness' of the nitrogen cycle – the extent to which N is in excess to plant demand and can therefore be lost through volatilization (Austin & Vitousek, 1998). However, this simplified model is complicated by fractionating physiological mechanisms within the plant, which remain an ongoing topic of discussion.

Differences in N-isotope fractionation between C_3 and C_4 plants are not well established. Relative to C_3 plants, C_4 species exhibit more efficient nitrogen use (Brown, 1977; Schmitt & Edwards, 1981; Sage & Pearcy, 1987; Makino et al., 2003), which may cause $\delta^{15}N$ to differ between the C_3 and C_4 pathways (Murphy & Bowman, 2009). Though experimental data are limited, a previous observational study illustrated a positive relationship between *P. glaucum* seed $\delta^{15}N$ values and cumulative annual precipitation (Reid et al., 2018).

2. Methods

2.1 Experimental design and plant growth

We obtained pearl millet (*Pennisetum glaucum*) seeds from the collection of the United States Department of Agriculture (USDA). Our strains (and USDA accession numbers) were: Ghana (326520), Kenya (521624), Morocco (517022), Nigeria (286833), and South Africa (263540). We chose to examine five accessions in an effort to balance optimal sample size with space limitations in the greenhouse. For our experiment, we exposed five individuals of each of the five accessions to three different water treatments, for a total of 75 plants overall.

We planted seeds in 4" diameter pots in a climate-controlled greenhouse 50 days prior to the start of the experiment (temperature maintained at 26.8 ± 3.2°C and humidity maintained at 25.8 ± 9.8% Rh). 25 days after planting, we transplanted the seedlings into larger 6" diameter pots. To ensure a randomized distribution and eliminate the potential for unintended environmental contributions to results (for example, variation in sunlight received), we arranged the plants on the greenhouse bench using a random number generator.

Before the start of the experiment, all plants were watered with tap water daily to the soil saturation limit (*c.* 0.5 ± 0.1 L of water per pot). We worked with greenhouse staff to determine this to be an appropriate water quantity, and thus daily watering to the saturation limit was used as a reference point for non-water stressed conditions. Beginning on the 50th day after planting, we watered plants in each of the three designated treatment groups to saturation at different

scheduled intervals: the non-stressed treatment was watered daily, the moderately-stressed treatment was watered every other day, and the highly-stressed treatment was watered every three days (two days in between watering). Water treatment consisted of scheduled watering partnered with soil moisture measurement using a Decagon Pro-Check (EC-5) moisture sensor. Twelve days into our experiment, we reevaluated our experimental conditions to impose more severe water restriction; from that point forward, we watered the moderately-stressed treatment every three days and the highly-stressed treatment every four days (three days in between watering). Relative to the non-water stressed group, the moderately- and highly-water stressed groups experienced lower average soil moisture levels throughout the course of the experiment. On average, soil moisture levels for each treatment group were: 0.41 $m^3 m^{-3} \pm 0.09$ (non-stressed), 0.21 $m^3 m^{-3} \pm 0.15$ (moderately-stressed), and 0.16 $m^3 m^{-3} \pm 0.14$ (highly-stressed). The degree of water stress imposed by infrequent watering is represented in Fig. 1; intervals without watering allowed soil moisture to drop well below the saturation limit (Fig. 1). Water stress indicators, such as wilting and gray tinting of leaves, were used in our experiment to confirm the water stress imposed on our moderately- and highly-stressed treatments. Payne et al. (1992) reported that an average soil moisture level of 0.17 $m^3 m^{-3}$ sustained non-water stressed conditions for pearl millet in a growth chamber environment. However, differences in experimental design prohibit direct comparison with our soil moisture measurements: (1) their experimental design involved watering plants daily to maintain a designated moisture level, while water stress in our experiment was instead administered through infrequent watering, and (2) Payne et al.'s experiment utilized larger pots (0.035 m^2 area, relative to our 0.018 m^2 area), so the net water input needed to achieve a specific soil moisture level differs. We imposed water treatments for 73 days, and at the end of this time (123 days after planting) seed and leaf samples were collected. The date of panicle emergence (in days from initial planting) was recorded for plants when applicable, and plant height was measured at the time of harvesting.

From Day 82 to the end of the experiment we deployed a Picarro G2121i CO_2 analyzer to quantify the concentration and isotope composition of CO_2 in the greenhouse. When using crop $\delta^{13}C$ values to infer water status of archaeological crop remains, it is crucial to account for the change in the $\delta^{13}C$ value of atmospheric carbon dioxide ($\delta^{13}C_{air}$) over time. Present day $\delta^{13}C_{air}$ is significantly lower than it was in the past, largely due to the fossil fuel-derived CO_2 (the Suess effect; Keeling, 1979). We therefore calculated the average $\delta^{13}C_{air}$ value across the measurement

period in order to determine discrimination during photosynthesis. This allows the interpretation established from modern crop watering experiments to be applied to crop remains from different time periods in future research:

$$^{13}\varepsilon = (1-\alpha) \times 10^3$$

$$\text{where } \alpha = R_{plant}/R_{air} = (^{13}C_{plant}/^{12}C_{plant})/(^{13}C_{air}/^{12}C_{air})$$

As defined here, $^{13}\varepsilon$ is mathematically equivalent to the fractionation factor $\Delta^{13}C$ often utilized by archaeologists (Farquhar et al., 1982; O'Leary, 1988):

$$\Delta^{13}C = (\delta^{13}C_{air} - \delta^{13}C_{plant}) / (1 + \delta^{13}C_{air}/1000)$$

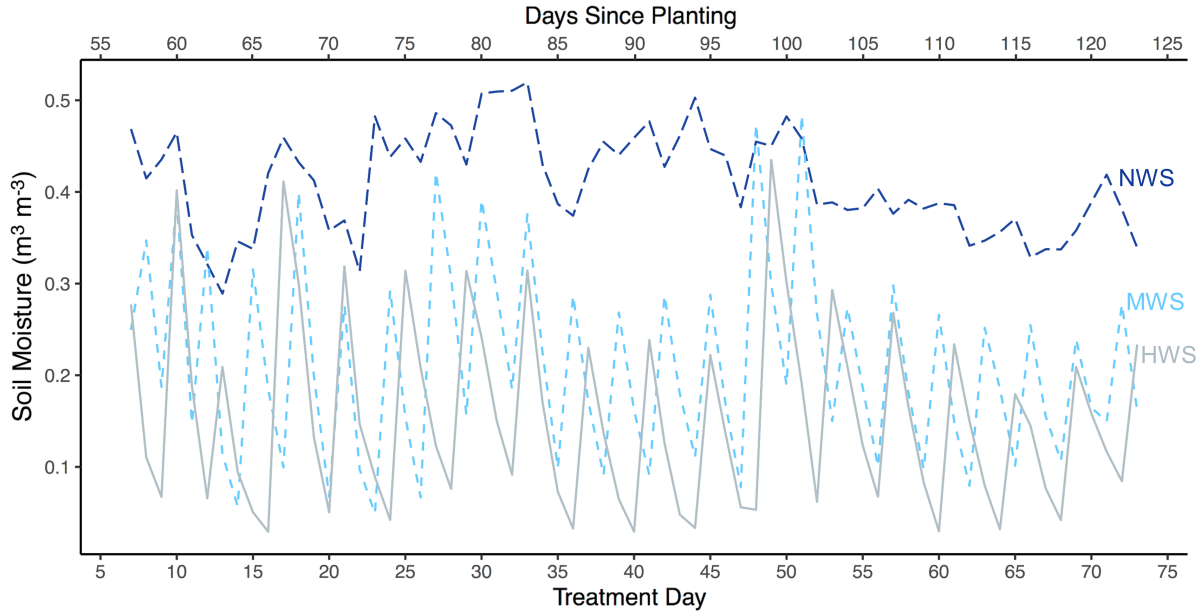


Fig. 1 Average moisture sensor records by treatment for the duration of the water restriction experiment. Treatment group designations: NWS = non-water stressed, MWS = moderately-water stressed, HWS = highly-water stressed.

2.2 Stable isotope analyses

We prepared samples for isotopic analysis by washing seeds with deionized water and oven-drying both seeds and leaves at 60ºC for 48 hours. Seed samples (5-10 seeds) were ground and homogenized in an agate mortar and pestle and leaf samples were homogenized using a rock tumbler before being weighed into 5x9 mm tin capsules. Stable carbon and nitrogen isotope analyses were performed using an elemental analyzer (EA) coupled to a Delta V Plus continuous

flow isotope ratio mass spectrometer located in the Department of Earth and Planetary Sciences at Washington University in St. Louis. We calibrated $\delta^{13}C$ and $\delta^{15}N$ values to VPDB and AIR scales with two-point calibration using USGS 40 ($\delta^{13}C$ = -26.39 ± 0.04 ‰, $\delta^{15}N$ = -4.5 ± 0.1 ‰) and USGS 41 ($\delta^{13}C$ = 37.63 ± 0.05 ‰, $\delta^{15}N$ = 47.6 ± 0.2 ‰). We monitored instrument performance using IU acetanilide #1 ($\delta^{13}C$ = -29.53 ± 0.01 ‰, $\delta^{15}N$ = 1.18 ± 0.02 ‰) and a well-characterized internal standard (BR millet: Bob's Red Mill millet flour; $\delta^{13}C$ = -13.18 ± 0.06 ‰, $\delta^{15}N$ = 3.28 ± 0.13 ‰). Using the equations presented in Appendices F and G of Szpak et al. (2017), we determined the total analytical uncertainty to be ± 0.08 ‰ for $\delta^{13}C$ and ± 0.16 ‰ for $\delta^{15}N$ based on the calibration and check standards (Notes A.1). Our measurements also included pseudoreplicate samples of both leaf and seed material, selected at random; ultimately, this yielded 6 seed $\delta^{13}C$, 4 seed $\delta^{15}N$, 41 leaf $\delta^{13}C$, and 3 leaf $\delta^{15}N$ pseudoreplicate measurements.

2.3 Statistical analyses

We conducted all statistical analyses in R (R Development Core Team, 2019; Notes A.2). We evaluated the distribution of our $\delta^{13}C$ and $\delta^{15}N$ results by performing Shapiro and Levene's tests for normality and equality of variance, respectively. Specific pairwise comparisons between treatments were made using Tukey's Honest Significant Difference (HSD) test. We used generalized linear models (GLMs) to evaluate the effects of soil moisture, water treatment, and accession on $\delta^{13}C$ and $\delta^{15}N$ values and used AIC model selection to identify best-fit models. Linear regression analysis was performed against both soil moisture and water treatment to account for variability in water uptake (and thus soil moisture) within plants of the same treatment group. We assessed model assumptions graphically by plotting standardized residuals versus fitted values to check for homogeneity and verified the normality of the residuals with a QQ-plot.

3. Results

The full dataset is available in the Supplementary Information (Tables A.1 and A.2).

3.1 Plant growth

At the end of the experiment a total of 48 plants representing all five accessions had produced seeds, including 7 of the Ghanaian accession, 6 of the Kenyan accession, 11 of the

Moroccan accession, 10 of the Nigerian accession, and 14 of the South African accession (Table A.1). The other 27 plants did not produce seeds by the conclusion of the experiment. Of the plants that developed panicles, the average number of days to panicle emergence was 92 days for the Ghanaian accession, 99 days for the Kenyan accession, 80 days for the Moroccan accession, 98 days for the Nigerian accession, and 75 days for the South African accession. Average plant height varied across accessions from 1.80 ± 0.41 m for the Nigerian accession to 2.11 ± 0.41 m for the South African accession.

3.2 Carbon isotopes

P. glaucum seed $\delta^{13}C$ values ranged between -14.8 and -12.9 ‰ (mean and SD = -13.9 ± 0.5 ‰, n = 48) and leaf $\delta^{13}C$ values ranged between -16.9 to -13.3 ‰ (mean and SD = -14.8 ± 0.7 ‰, n = 75) (Table A.2). Considering all five accessions collectively, both seed and leaf $\delta^{13}C$ values differed among water treatments ($F_{2,45} = 5.61$, $P = 0.007$; $F_{2,72} = 9.00$, $P < 0.001$, respectively). Seeds produced under the non-stressed water treatment had $\delta^{13}C$ values that were higher than both the moderately- and highly-water stressed treatments, but the $\delta^{13}C$ values of seeds from the moderately- and highly-water stressed treatments could not be distinguished. Plants in the highly-water stressed group produced leaves with $\delta^{13}C$ values that were lower than from the non-stressed and moderately-water stressed treatments; non-stressed and moderately water stressed groups did not differ (Fig. 2a,c). Based on AIC model selection, the best-fit model for seed $\delta^{13}C$ values was a GLM that included both soil moisture and accession as explanatory variables (Table 1; Fig. 3); the best-fit model for leaf $\delta^{13}C$ values included water treatment and accession (Table 1). When data from all accessions were analyzed together, these models indicated that seed $\delta^{13}C$ values are positively correlated with water availability (Fig. A.1a,c), such that for each 0.1 $m^3\ m^{-3}$ increase in average soil moisture, seed $\delta^{13}C$ values increase by 0.24 ± 0.04 ‰. Likewise, for leaf $\delta^{13}C$, a GLM with soil moisture and accession included as explanatory variables showed that leaf $\delta^{13}C$ values increase by 0.24 ± 0.06 ‰ per 0.1 $m^3\ m^{-3}$ increase in soil moisture. Both seeds and leaves showed distinct, but relatively small, differences in $\delta^{13}C$ values among accessions ($F_{4,43} = 3.49$, $P = 0.015$; $F_{4,70} = 3.05$, $P = 0.022$); overall variability in seed and leaf $\delta^{13}C$ values was within a range of 1.9 ‰ and 3.6 ‰, respectively. We found no significant interactions between water treatment and millet accession, indicating that responses to water treatment did not differ among accessions. Seed $\delta^{13}C$ values across all five

accessions showed somewhat higher variability in the non-stressed treatment group relative to the highly water-stressed treatment group (evaluated via the standard deviation from mean values for an accession within each treatment group); the difference in standard deviation was on the order of 0.1-0.2 ‰. The moderately-stressed treatment group showed seed $\delta^{13}C$ variability intermediate to that of the non-stressed and highly-stressed groups. On the other hand, the magnitude of variability in leaf $\delta^{13}C$ values with respect to water treatment was inconsistent across the five accessions. For the Ghanaian, Kenyan, and Nigerian accessions, $\delta^{13}C$ values were more variable for the non-stressed group relative to the highly-stressed group (on the order of 0.2, 0.6, 0.1 ‰, respectively). However, the Moroccan accession showed higher variability in the highly-water stressed group (on the order of 0.2 ‰), and the South African accession showed approximately the same magnitude of variability between the two groups. For the Moroccan, Nigerian, and Kenyan accessions, the highest magnitude variability in $\delta^{13}C$ values was observed in the moderately-stressed treatment group. Variability in the moderately-stressed group was notably high for the South African accession (0.8 ‰ higher than variability in the non-stressed and highly-stressed groups).

To examine additional factors potentially contributing to variability in $\delta^{13}C$ values, we assessed linear relationships between GLM deviance residuals and physiological variables (plant height, time of panicle emergence). For seed $\delta^{13}C$ values, a weakly negative linear relationship existed between plant height and the GLM residuals ($F_{1,45} = 5.14$, $P = 0.028$) (Fig. A.2). However, a similar relationship was not present for the leaf $\delta^{13}C$ data.

The average $\delta^{13}C$ value of the CO_2 in the greenhouse (-3.7 ± 1.8 ‰) allowed us to determine $\Delta^{13}C$ (isotopic discrimination) for *P. glaucum* seeds as described under Materials and Methods. Seeds had a mean $\Delta^{13}C$ value 10.2 ± 0.5 ‰ (range 9.2 ‰ to 11.1 ‰) (Fig. 4). As $\Delta^{13}C$ is based on a transformation of $\delta^{13}C$, isotopic discrimination also differed among water treatments and accessions.

3.3 Nitrogen isotopes

P. glaucum seed $\delta^{15}N$ values ranged between 0.8 and 2.9 ‰ (mean and SD = 1.7 ± 0.5 ‰, n = 48), and leaf $\delta^{15}N$ values ranged between -0.9 to 2.3 ‰ (mean and SD = 1.0 ± 0.8 ‰, n = 68) (Table A.2). In contrast with $\delta^{13}C$ values, there were no discernable differences in the $\delta^{15}N$ values of leaves and seeds between the non-stressed and highly-stressed treatment groups. Seed

347 $\delta^{15}N$ values did not differ at all among treatments ($F_{2,45} = 0.08$, $P = 0.928$). Leaf $\delta^{15}N$ values only
348 had discernable differences between the non-stressed and moderately-stressed treatments ($F_{2,65} =$
349 3.44, $P = 0.038$); the non-stressed and highly-stressed groups were statistically indistinguishable
350 (Fig. 2b,d). The best-fit model for seed $\delta^{15}N$ was a GLM with accession as the lone explanatory
351 variable, while the best-fit model for leaf $\delta^{15}N$ was a GLM with both water treatment and
352 accession included as explanatory variables (Table 1). Unlike the $\delta^{13}C$ models, there was no
353 isotopic signal associated with increasing soil moisture; the direction of the relationship between
354 $\delta^{15}N$ and water availability varied among accessions and was indistinct when all accessions were
355 considered together (Fig. A.1b,d). Both seeds and leaves showed differences in $\delta^{15}N$ among
356 accessions ($F_{4,43} = 3.99$, $P = 0.008$; $F_{4,63} = 5.86$, $P < 0.001$); however, no single accession was
357 consistently more or less enriched in ^{15}N (relative to other accessions) across both the seed and
358 leaf data. The relationships between GLM deviance residuals and physiological variables for leaf
359 $\delta^{15}N$ data showed a positive relationship between GLM residuals and panicle emergence time
360 ($F_{1,52} = 16.85$, $P < 0.001$), but not plant height (Fig. A.3). No linear relationships exist between
361 GLM residuals and seed $\delta^{15}N$ values.
362

Table 1 Comparison of GLM ability to account for $\delta^{13}C$ and $\delta^{15}N$ variability in pearl millet seeds and leaves, with best-fit model italicized.

Model	Residual Deviance	Residual DF[a]	AIC[b]	ΔAIC[c]
Seed $\delta^{13}C$				
Treatment	7.94	45	57.85	29.01
Soil Moisture	5.73	46	40.23	11.39
Accession	7.49	43	59.04	30.20
Soil Moisture + Accession	*3.83*	*42*	*28.84*	*0*
Treatment + Accession	4.83	41	42.04	13.20
Soil Moisture * Accession	3.74	38	35.74	6.91
Treatment * Accession	4.01	33	49.08	20.24
Leaf $\delta^{13}C$				
Treatment	31.02	72	154.64	7.39
Soil Moisture	30.91	73	152.35	5.10
Accession	33.02	70	163.32	16.07
Soil Moisture + Accession	26.50	69	148.80	1.55
Treatment + Accession	*25.27*	*68*	*147.25*	*0*
Soil Moisture * Accession	23.99	65	149.35	2.10
Treatment * Accession	21.68	60	151.74	4.49
Seed $\delta^{15}N$				
Treatment	10.92	45	73.13	10.97
Soil Moisture	10.79	46	70.56	8.40
Accession	*7.99*	*43*	*62.16*	*0*
Soil Moisture + Accession	7.94	42	63.88	1.72
Treatment + Accession	7.90	41	65.61	3.45
Soil Moisture * Accession	7.25	38	67.50	5.34
Treatment * Accession	5.59	33	65.04	2.88
Leaf $\delta^{15}N$				
Treatment	37.00	65	159.59	17.20
Soil Moisture	40.88	66	164.37	21.98
Accession	29.82	63	148.91	6.52
Soil Moisture + Accession	29.29	62	149.69	7.30
Treatment + Accession	*25.54*	*61*	*142.39*	*0*
Soil Moisture * Accession	26.74	58	151.50	9.11
Treatment * Accession	21.89	53	147.89	5.50

[a] Residual degrees of freedom, [b] Akaike Information Criterion, [c] Difference in AIC from best model

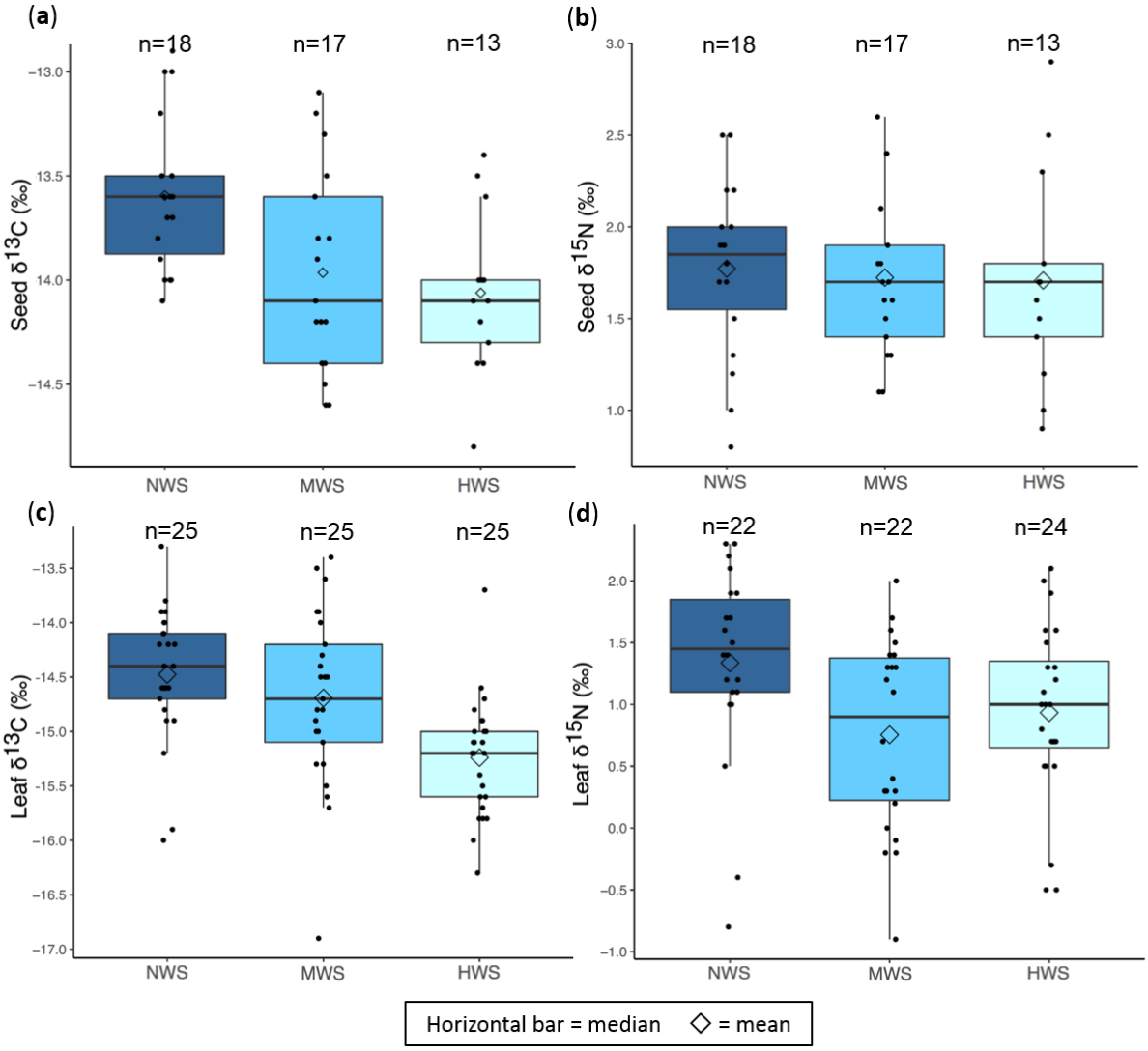
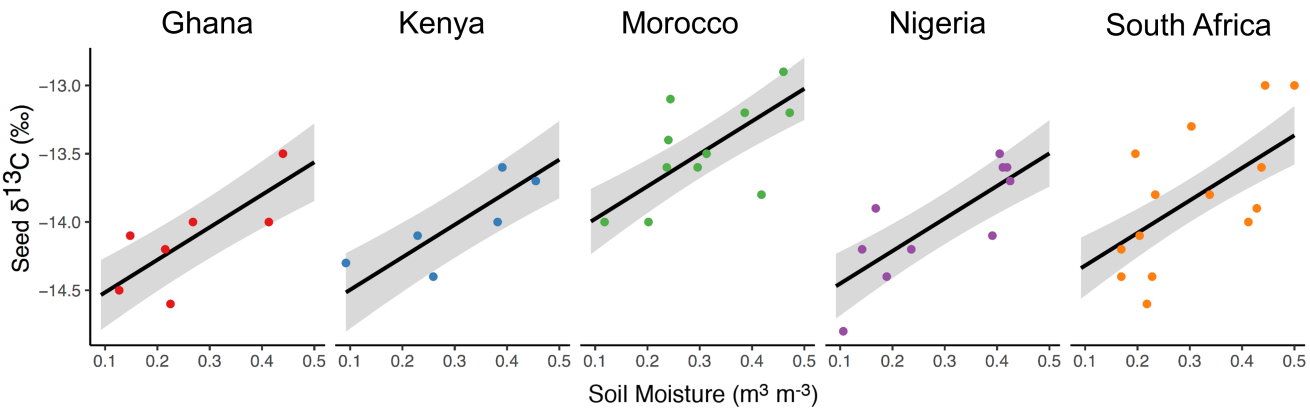


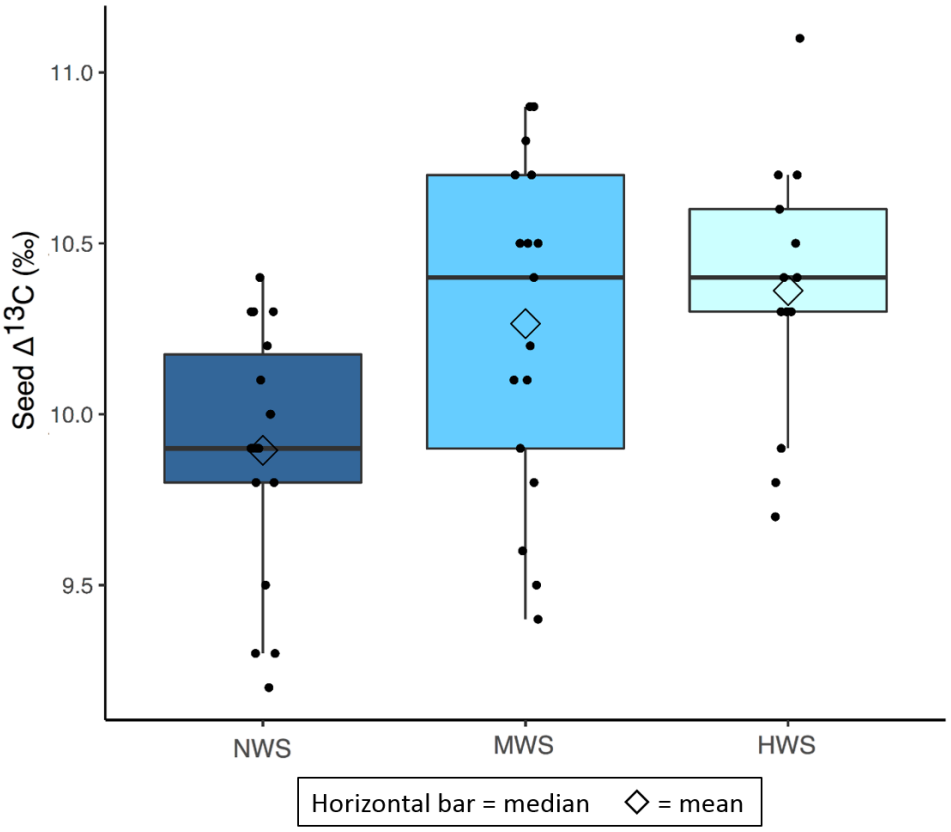
Fig. 2 Boxplots of pearl millet seed and leaf $\delta^{13}C$ and $\delta^{15}N$ values by water treatment. To avoid overplotting, data points within each discrete treatment group have been distributed randomly with respect to a set horizontal width. Treatment group designations: NWS = non-water stressed, MWS = moderately-water stressed, HWS = highly-water stressed.



374

375 **Fig. 3** Best-fit regression lines for each pearl millet accession, plotted against average soil
376 moisture; gray shaded region represents the standard error.

377



378

379 **Fig. 4** Boxplot of pearl millet seed $\Delta^{13}C$ by water treatment. To avoid overplotting, data points
380 within each discrete treatment group have been distributed randomly with respect to a set
381 horizontal width. Treatment group designations: NWS = non-water stressed, MWS =
382 moderately-water stressed, HWS = highly-water stressed.

4. Discussion

4.1 Carbon isotopes

Pearl millet seed and leaf $\delta^{13}C$ values are positively correlated with water availability, and this relationship is observed in all accessions in this study. We infer from the indistinguishable seed $\delta^{13}C$ values for the moderately- and highly-water stressed treatment groups that these two treatments did not differ dramatically in the degree of water restriction imposed; however, the non-water stressed treatment produced distinctly higher $\delta^{13}C$ values (Fig. 2a,c). Linear regression analyses of $\delta^{13}C$ values against average soil moisture further reinforce this finding, while accounting for disparities in average soil moisture within treatment groups. The magnitude of the positive relationship between $\delta^{13}C$ values and water availability is approximately equal in seeds and leaves, though pearl millet seeds do tend to have overall slightly higher (less negative) $\delta^{13}C$ values than leaves, consistent with the findings of An et al. (2015) for foxtail (*Setaria italica*) and common (*Panicum miliaceum*) millets. The positive correlation between water availability and $\delta^{13}C$ values is the opposite of the trend that is typically exhibited by C_3 plants.

This relationship has also been observed in other C_4 species, including foxtail millets (*Setaria italica*) (An et al., 2015; Lightfoot et al., 2020) and grasses of the Aristida genus (Schulze et al., 1996; Murphy & Bowman, 2009), and was previously reported for *P. glaucum* in an observational study (Reid et al., 2018). Previous studies have attributed this relationship to C_4 plant physiology, particularly to the equilibrium isotope effect between dissolved CO_2 and bicarbonate in mesophyll cells (e.g., Schulze et al., 1996). All carbon incorporated by Rubisco in C_4 plants derives from bicarbonate in mesophyll cells, which is enriched in ^{13}C relative to dissolved CO_2 (by 9 ‰ at 25 °C; Mook et al., 1974). When this carbon is released as CO_2 in bundle-sheath cells, it can be incorporated into photosynthate or leak away from the site of decarboxylation and back into the mesophyll. The expressed fractionation is highly sensitive to the flux of CO_2 that leaks (Farquhar et al., 1989), complicating the factors governing photosynthetic control on isotopic fractionation in C_4 plants. The overall carbon isotope fractionation during carbon fixation by a C_4 plant (ε_P) can be expressed using the equation established by Hayes (2001) after Farquhar (1983):

$$\varepsilon_p = \varepsilon_{ta} + \frac{p_i}{p_a}\left[\varepsilon_c - \varepsilon_{b/d} + \varphi(\varepsilon_f - \varepsilon_{tw}) - \varepsilon_{ta}\right]$$

where ε_{ta} and ε_{tw} are the isotope effects associated with mass transport of CO_2 in air and water, respectively (4.4 and 0.8 ‰; Hayes, 2001), ε_c is the isotope effect associated with fixation of bicarbonate by phosphoenolpyruvate carboxylase (2.2 ‰, as per O'Leary, 1981), $\varepsilon_{b/d}$ is the equilibrium isotope effect relating bicarbonate and dissolved CO_2 (8.7 ‰ at 27° C as per Mook et al., 1974), ε_f is the isotope effect associated with the fixation of CO_2 (27 ‰ for land plants, as per Farquahar et al., 1989), $\frac{p_i}{p_a}$ is the ratio of partial pressures of CO_2 inside and outside carbon-fixing cells, and φ is the cellular 'leakiness' coefficient. Water availability directly alters the $\frac{p_i}{p_a}$ term, as plants respond to water stress by closing their stomatal pores, which results in a decrease in stomatal conductance and the internal CO_2 concentration (decreasing $\frac{p_i}{p_a}$). The relationship between $\delta^{13}C$ and water availability depends on whether the 'leakiness' of bundle-sheath cells is above or below a threshold value dictated by the other fractionations taking place during carbon fixation. If the cellular 'leakiness' is below this threshold value, then $\delta^{13}C$ of photosynthate increases (ε_P decreases) with increasing $\frac{p_i}{p_a}$ (increasing water availability). Above the threshold value, the $\delta^{13}C$ of plant material decreases with increasing $\frac{p_i}{p_a}$. In our greenhouse conditions, using the model parameters defined above, this leakiness threshold (φ) is between 0.41 and 0.42. Given the positive relationship observed in our data, φ is below that threshold for *P. glaucum* under our experimental conditions. The $\delta^{13}C$ values measured in this experiment are more negative than the Hayes model's theoretical isotopic discrimination would allow. One caveat to this relationship is that we examine bulk leaf and seed materials, and not isolated primary photosynthate. The low $\delta^{13}C$ values here may suggest that secondary processing of photosynthate is producing an additional discrimination effect against ^{13}C.

Our data also show $\delta^{13}C$ values of pearl millet vary for strains with different origins (accessions). The strong linear relationship between GLM residuals and plant height for the seed $\delta^{13}C$ model suggests that differences in plant physiology (associated with regional accession) may help explain the observed differences in seed carbon isotope ratios among accessions. Physiological differences may contribute to different light-use or transpiration efficiencies that ultimately influence carbon isotope fractionation during photosynthesis. However, according to our best-fit model, the measured physiological variables are unable to explain $\delta^{13}C$ variability at the leaf level.

4.2 Nitrogen isotopes

We found no strong correlations between water availability and $\delta^{15}N$, and overall variability in both leaf and seed $\delta^{15}N$ values was relatively low (*c.* 2 ‰ in seeds and 3 ‰ in leaves). The absence of a relationship between soil moisture and $\delta^{15}N$ values differs from patterns observed globally. Our data are consistent with the interpretation that the globally observed trends are a consequence of environmental parameters affecting soil $\delta^{15}N$, rather than nitrogen isotope fractionation.

4.3 Isotopes as proxies for water management

While we were able to observe correlations between water availability and seed $\delta^{13}C$ values, the observed effects are small. Seed $\delta^{13}C$ values for each accession showed a trend toward decreasing variability (standard deviation from the mean) with increasing degree of water restriction, though this effect is too minor to influence isotopic interpretation (0.1-0.2 ‰); moreover, this trend was not observed in the leaf $\delta^{13}C$ data. The weak signals observed here within accessions are likely to be obscured by environmental and genetic individualities among plants, which differ between environmentally-controlled greenhouses and "real-life" agricultural set-ups. Our results are consistent with the recent findings of Lightfoot et al. (2020) for foxtail millet (*Setaria italica*). Differences in $\delta^{13}C$ values produced by different watering treatments are not large enough in magnitude to be distinguishable from the isotopic variability between millet accessions. With this precaution in mind, however, should contextual information be rich (*cf.* household storage), $\delta^{13}C$ values derived from archaeological grains could be useful for addressing issues such as water stress or context-specific water availability. Future research to address landrace-specific questions, including "real-life" field experiments based upon modern agricultural water supply parameters, is needed.

4.4 Isotopes as proxies for past landraces

Phenotypic variations resulting from environmental adaptation may account for some of the variability observed in isotope ratios, an observation that may be of use in ethnobotanical studies. We observed that physiological traits, such as plant height and panicle emergence time, may account for a small degree of variability in our seed $\delta^{13}C$ and leaf $\delta^{15}N$ best-fit models (no more than *c.* 1 ‰ and 2 ‰, respectively) (Fig. A.2 and A.3). Previous ethnobotanical studies

have documented morphological variations of African millet landraces (e.g. *Eleusine coracana)*, particularly in relation to seed size and shape, as well as environmental adaptations (e.g. Lule et al., 2012; Umar & Kwon-Ndung, 2014). For example, Brunken et al. (1977) describe a number of landraces of pearl millet that can be recognized on the basis of grain shape and plant morphology and have distinctive geographic ranges. Studies also note that farmers sometimes select landraces based on phenotypic characteristics, such as seed size and plant height, for various reasons (Tseshaye et al., 2006). Thus, it may be possible to use such measurements in combination with seed stable isotope values to identify specific landraces, if strong relationships between morphology and isotope ratios are established.

4.5 Conclusions

Water availability plays a small but detectable role in determining $\delta^{13}C$ in *P. glaucum* seeds and leaves. Based on established models of isotopic discrimination during C_4 carbon fixation, the positive relationship observed between $\delta^{13}C$ and water availability suggests that bundle sheath 'leakiness' was below a threshold value (calculated to be 0.42) for our experimental set-up, resulting in a positive relationship between $\delta^{13}C$ and $\frac{p_i}{p_a}$. The relatively low magnitude of variability (less than *c.* 2 ‰) in response to water stress suggests that millet $\delta^{13}C$ values are not a reliable proxy for past water management practices, as the isotopic signal associated with water availability is likely to be masked by the equally significant genetic variability among landraces.

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Declarations of Interest

None

504 **Author Contributions**

505 XL and REBR conceptualized the research initially. LHS and REBR designed the study. LHS
506 carried out the greenhouse experiment. LHS wrote the first draft of the manuscript, to which
507 REBR, ASB and XL contributed revisions.
508
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Appendix A. Supplementary Information

Fig. A.1 Scatter plots demonstrating the relationship between pearl millet seed and leaf $\delta^{13}C$ and $\delta^{15}N$ and average soil moisture for each of five accessions.

Fig. A.2 Scatter plots demonstrating the relationship between GLM residuals and plant height for pearl millet seed $\delta^{13}C$ values.

Fig. A.3 Scatter plot demonstrating the relationship between GLM residuals and time of panicle emergence for pearl millet leaf $\delta^{15}N$ values.

Table A.1 Summary of data for each accession.

Table A.2 Data collected from experimental plants.

Notes A.1 R Code for calculating total analytical uncertainty.

Notes A.2 R Code for analyzing C and N isotope data for pearl millet seeds and leaves.