Can ^{15}N- isotopic methods be used to estimate plant associated nitrogen fixation in hybrid perennial sorghum?

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Abstract
A redesign of current cropping systems that incorporates grain production from perennial species has been proposed as a method of sustainable staple grain production. Perennial sorghum is one such species under development as a grain crop, which has been suspected of hosting endophytic nitrogen fixing bacteria in its stems or crowns. A pilot study using isotope dilution methodology was used to measure the percent nitrogen derived from the atmosphere (%Ndfa) in perennial sorghum. Plants were grown in three metre columns over 90 days set in the field. Three treatments providing high (HN), low (LN) and zero (0N) N applied as urea were compared, with each treatment consisting of a perennial sorghum plant grown in the same column with *Lolium rigidum* and *L.perenne*. Additional nitrogen from urea significantly ($P< 0.05$) increased total shoot and root dry matter, compared to the 0N treatment. Interestingly, perennial sorghum plants in the 0N treatment had significantly ($P< 0.05$) longer roots and a higher root:shoot ratio ($P< 0.05$) compared to both additional N treatments. This suggests that N scarcity stimulated increased root allocation. Perennial sorghum showed no evidence of N_2 fixation under the HN and LN treatments at all sampling dates. However, over time in the 0N treatment there was a trend for increasing levels of biologically sourced N with an average of 30 %Ndfa at the final sampling date, although calculations varied with the reference species used. The current study provides evidence of N_2 fixation in perennial sorghum but highlights concerns about the adequacy of the reference plant δ^{15}N values.

Key Words
%Ndfa, perennial cereals, ^{15}N dilution

Introduction
Modern agricultural grain production relies on annual species and is characterised by soil disturbance either through the use of tillage and/or herbicides in the yearly establishment of crops. The requirement for resowing of annual species maintains grain cropping systems in a constant state of early plant succession and is responsible for much of the soil degradation experienced in these systems due to erosion, salinity, soil organic matter decline and nutrient leaching. Lifting grain production to a more advanced stage of ecological succession, through the development of perennial grain crops that resemble natural systems, has been proposed as a method to address the negative environmental consequences of reliance on annual crop production (Crews *et al.* 2016). Perennial sorghum is one such species under development as a grain crop and is derived from hybridisation between grain sorghum (*Sorghum biclor*) and either of two wild perennial grasses *Sorghum halepense* or *S. propinquum*. This crop has potential to be grown in diverse landscapes and provide livestock feed, biofuel production and grain for human consumption (Cox *et al.* 2018).

In any cropping system a source of nitrogen (N) is important for production. Soil N can be low in grassland soils and could conceivably become depleted in perennial grain crops as the stand ages, without external inputs of fertiliser or endogenous sources of N. Perennial sorghum has been suspected of hosting endophytic nitrogen fixing bacteria in its stems or crowns. Previous nitrogenase activity has been identified in perennial sorghum and S. *halepense* through the acetylene reduction technique (Crews pers com.). This technique is indicative of N_2 fixation however, cannot accurately quantify the amount of N_2 fixed. To further examine N_2 fixation in perennial sorghum and attempt to estimate the quantity of N fixed, a pilot study was conducted to answer two research questions:

a) Can isotope dilution methodology be used to measure biological nitrogen fixation in perennial sorghum?
b) Are C_3 *Lolium sp*. suitable as reference plants?

Methods

The pilot study was conducted at The Land Institute, Salina, Kansas, USA in 2016. Plants were grown in three metre PVC columns that were 250mm in diameter and set in the field. Each column was filed with a growing media called Turface® which is an inert granular product and contains no nitrogen. The Turface was prepared for each PVC column by spraying with 0.05g labeled urea (9.9% atom excess) mixed with water. Sufficient phosphorous (P), potassium (K) to support plant growth (2g P, 50g K per column) was added to the media. Also each column received 0.5g of a micronutrient mix (Ca 6%, Mg 3%, Fe 17%, Mn 2.5%, Cu 1%, Mo 0.05%, and Zn 1%) A small amount of soil (2% total mixture by weight) collected from a previous perennial sorghum field was added to improve inoculation with endophyte. Three nitrogen treatments were imposed: High (HN), calculated as the non-limiting N requirement for a perennial sorghum plant (50g N), Low (LN) which was 40% of the High N amount (20g N) and no added N (0N). The N treatments were applied as normal urea (48% N). All components for each treatment were blended in the cement mixer to achieve uniform distribution and loaded into individual PVC columns. Treatments were randomly assigned to one of four replicates. The mix was allowed to settle for six weeks before planting. During this period seedlings of a breeding line of perennial sorghum, developed by The Land Institute, were established in the glasshouse in potting mix in small trays. At the same time seedlings of *Lolium perenne* and *L. ridgidum* were also established to be used as reference plants. One seedling of perennial sorghum and one each of the reference species was planted into each PVC tube in the first week of June. The experiment ran for 90 days.

At 21 day intervals the third fully expanded leaf from the main stem of each plant species was sampled. Leaf samples were dried and then ground in a ball mill. $\delta^{15}N$ of each sample was determined using automatic N and carbon analysis interfaced to a stable isotope mass spectrometer. The percentage of biological N derived from the atmosphere (%Ndfa) was calculated using the formula: %Ndfa = (1 - ^{15}N% atom excess in perennial sorghum leaves / ^{15}N% atom excess of reference plant leaves) x 100. At the final sampling all plant species were removed from the PVC columns and the roots washed from the growing media. Plant height and root length (crown to deepest root tip) of perennial sorghum were recorded. The biomass removed from each treatment was dried at 65 °C in a dehydrating oven for 48 hours and weighed to determine above and below ground biomass. Biomass data were analysed using a general analyses of variance with replicate as a blocking structure and N level as the treatment structure (Genstat version 17; VSN International Ltd). NxDate was added to the treatment structure when analysing %Ndfa. All data was analysed at the 95% significance level ($P = 0.05$).

Results

Shoot and root production from the pilot study is listed in Table 1. Perennial sorghum harvested from the HN and LN treatments had similar amounts of shoot dry matter and were ~12-fold greater than the 0N treatment ($P = 0.05$). Root dry matter production was 89% less in the 0N treatments compared with both the added N treatments ($P = 0.05$). Plant height was similar between all nitrogen treatments but root length was 6.9% and 10.9% longer in the 0N treatment compared with the HN and LN treatments, respectively ($P = 0.05$). This corresponded to a 96.4% increase in root:shoot ratio in the 0N treatment compared to the added N treatments. There was a large disparity in dry matter production between sorghum plants and the references species (Table 2). Averaged across all N treatments perennial sorghum produced 100-fold more shoot dry matter than either annual or perennial rye.

Table 1: Plant Height, Root length, Shoot dry matter, root dry matter and rot shoot ratio of perennial sorghum grown under three nitrogen levels

	Shoot Dry Matter (g)	Root Dry Matter (g)	Plant Height (mm)	Root Length (mm)	Root:Shoot ratio
High Nitrogen	398	93	1429	3618	0.244
Low Nitrogen	380	90	1648	3488	0.253
Zero Nitrogen	32	10	1010	3867	0.489
l.s.d (P=0.05)	242.5	71.5	ns	248.3	0.165

Table 2: Comparison of shoot dry matter for Annual Rye, Perennial Rye and Perennial sorghum averaged across Nitrogen treatments at the final sampling date.

	Annual Rye	Perennial Rye	Perennial Sorghum	*l.s.d* (P=0.05)
Shoot Dry Matter (g)	2.0	2.0	270.0	79.8

There was no evidence of biological nitrogen fixation where urea was added (data not shown) with the perennial sorghum plants having higher $\delta^{15}N$ values than the references species. In the 0N treatment there was increasing levels of fixation over the course of the sampling period, particularly when calculated using *L. perenne* as the reference species, (Figure 1). At the early sampling times in June and July there was negligible N fixation, depending on the reference species used in the calculation. By the later sampling dates the average calculated %Ndfa between *L, perenne* and *L. ridgidum* equalled 12% and 28% for August and September, respectively.

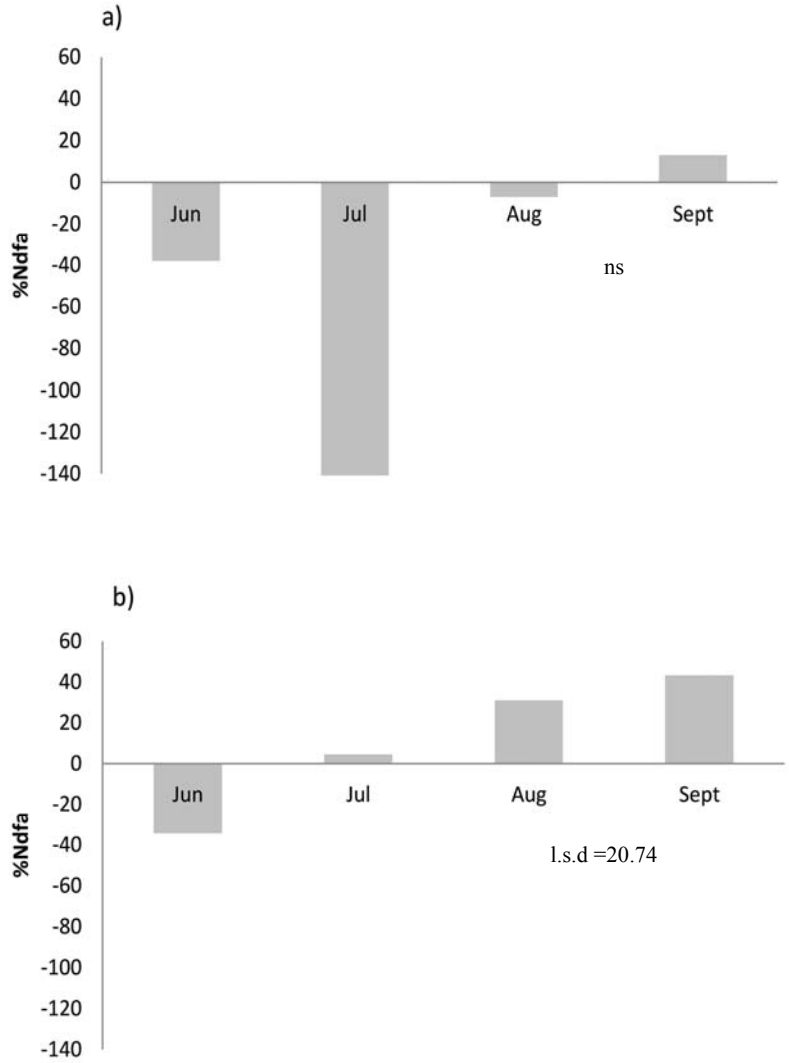

Figure 1: **The concentration of nitrogen derived from atmosphere (%Ndfa) in perennial sorghum grown with a) *Loilium ridgidum* and b) *L. perenne* as reference plants at 21 day intervals (ns = not significant).**

Discussion

This pilot study examined the use of the ^{15}N dilution technique to quantify N_2 fixation in perennial sorghum. Quantifying N_2 fixation from associated bacteria can be more difficult than in symbiotic systems as the amounts are generally low (Unkovich 2008). The selection of references species is a critical component in the technique and requires plants which have an absence of N_2 fixing ability. In the current study C_3 species where used as reference species as there was low confidence in identifying a C_4 species which would not fix nitrogen.

In the 0N treatment there was an indication of N_2 fixation in the perennial sorghum plants, although the overall calculated amount of N_2 fixed was generally low and would not sustain production. One of the assumptions for the ^{15}N dilution technique is that the reference plant has similar soil mineral N uptake and rooting depth to the fixing plant. This condition was not meet when using *L. ridgidum*, as the majority of N fixation calculations were negative over time and highly variable. In this instance the vigorous root system of perennial sorghum allowed greater ^{15}N enrichment in comparison to *L. ridgidum*. It is known that perennials prioritise more resources into root development and this may have allowed *L. perenne* to maintain a similar N uptake pattern to perennial sorghum. The early negative values when using *L. perenne* are potentially due to slow establishment of the N_2 fixing association. Negative values of N_2 fixation due to greater ^{15}N enrichment of the putative N_2 fixing plant are also reported in the literature (Sanginga *et al.* 1990). The %Ndfa calculation significantly increased across the sampling dates and by the last assessment reached 43% which is similar to values derived in sugarcane (Asis *et al.* 2002).

The High and Low N treatments showed no indication of N_2 fixation. It is known that increasing rates of nitrogenous fertiliser causes a reduction in nitrogenase activity and so the increased rates of available nitrogen from the added urea may have limited the ability of N_2 fixation in perennial sorghum in these treatments. It is also likely that the added nitrogen increased the vigour and competitive ability of the perennial sorghum plant, leading to greater disparity in N uptake patterns and dry matter accumulation compared to the reference species, as indicated in Table 2. This in turn may have reduced the sensitivity in detecting small amounts of N_2 fixation in the calculation. The addition of extra urea increased the biomass of perennial sorghum plants compared to the 0N treatment. It was interesting to observe an increased root:shoot ratio in the 0N treatment. This is indicative of optimal partitioning theory (Bloom *et al.* 1985) which suggests greater resource allocation to root production to enable acquisition of nutrients that most limit plant growth when they become scarce in the soil.

Conclusion

This pilot study was a first attempt to quantify N_2 fixation in perennial sorghum. While there was some evidence of N_2 fixation the calculation was potentially limited by the choice of reference species as perennial sorghum maintained higher levels of ^{15}N enrichment due to disparity in soil N acquisition pattern in most cases. Using individual pots for the reference species may improve N uptake of the references species by reducing competition. Alternatively, selecting reference species with root systems which better approximate the root development of perennial sorghum would improve sensitivity to N_2 fixation and the estimate of %Ndfa. Using other C_4 species as reference plants could play a role here; however their potential for N_2 fixation would need to be considered.

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