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Associative nitrogen fixation linked with three perennial bioenergy grasses in field and greenhouse experiments

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Abstract

Associative nitrogen (N_2)-fixation (ANF) by bacteria in the root-zone of perennial bioenergy grasses has the potential to replace or supplement N fertilizer and support sustainable production of biomass, but its application in marginal ecosystems requires further evaluation. In this study, we first combined both greenhouse and field experiments, to explore the N_2 fixation effects of three temperate feedstocks *Miscanthus* × *giganteus* (giant miscanthus, Freedom), *Panicum virgatum* (switchgrass, Alamo), and *Saccharum* sp. (energycane, Ho 02-147). In field studies across three growing seasons, plant and soil pools of candidate feedstocks were partially composed of N derived from the atmosphere (Ndfa). Energycane, giant miscanthus, and switchgrass were estimated to derive >30%, %Ndfa. Greenhouse studies were also performed to trace isotopically labeled $^{15}N_2$ into plant biomass and soil pools. Evidence for Ndfa was detected in all three feedstock grasses (using reference ^{15}N of soil, chicory, and sorghum, $\delta^{15}N$~+7.0). Isotopically labeled $^{15}N_2$ was traced into biomass (during grass elongation stage) and soil pools. Extrapolation of rates during the 24 hr labeling period to 50 days estimated 30%–55% of plant Ndfa, with the greatest Ndfa for energycane. The findings of the field natural abundance and greenhouse $^{15}N_2$ feeding experiments provided complementary evidence that perennial bioenergy grasses have the potential to support relatively high rates of ANF, and accumulate diazotroph-derived N into biomass when grown on non-fertilized soil.

KEYWORDS

$^{15}N_2$, bioenergy grasses, biomass yield, marginal soils, nitrogen fixation, stable isotope

Jayani J. Wewalwela and Yuan Tian contributed equally to this work and should be considered co-first authors.

1 | INTRODUCTION

Perennial grasses can serve as a renewable feedstock to produce biofuels that help reduce demand for petroleum and other energy sources (Lee et al., 2018). The grasses require little management (e.g., fertilization) and have the potential to grow productively on non-fertilized or marginal lands (Blanco-Canqui, 2016; Jones, Finnan, & Hodkinson, 2015). This avoids competition for space with food crops (Carlsson, Mårtensson, Prade, Svensson, & Jensen, 2017; Dauber et al., 2012) while supporting the agricultural economy. Thus, assessing the potential for feedstock grasses to productively grow with low nutrient supply, such as low availability of N, can be used to create an integrated biofuel cropping system.

Diazotrophic bacteria associated with bioenergy grasses can fix atmospheric N_2, but there is scant research into the extent that N_2 fixation can support temperate grass growth in low N environments. The highest rates of associative nitrogen (N_2)-fixation (ANF) have been measured in sugarcane (e.g., CB 45-3, sp 70-1143), supplying up to 70% of the N requirement equivalent to 150 kg N ha^{-1} $year^{-1}$ (Boddey & Dobereiner, 1995; De Souza et al., 2016). In this regard, the bulk of the research related to ANF has centered on sugarcane and a few tropical forage grasses including *Brachiaria humidicola*, *B. decumbens*, *Paspalum notatum*, and *Panicum maximum* (Boddey & Dobereiner, 1995; Boddey, Sá, Alves, & Urquiaga, 1997; Keymer & Kent, 2014). In several cases, these grasses have been shown to incorporate 10%–40% of tissue N derived from N_2-fixation (Boddey & Knowles, 1987; Keymer & Kent, 2014). In addition, temperate perennial grasses have also been shown to support ANF and nitrogenase activity but estimates of their contribution to plant nitrogen derived from the atmosphere (Ndfa) have tended to be low and variable (Ma et al., 2018), with most rates being <10–20 kg N ha^{-1} $year^{-1}$. More recent studies have estimated rates >35 kg N ha^{-1} $year^{-1}$ associated with Switchgrass (Roley et al., 2018). These studies nevertheless indicate that a number of different grasses can support root-zone diazotrophs (Keymer & Kent, 2014; Van Deynze et al., 2018), yet the significance of ANF to support the N needs for multiple feedstock grasses remain an open question (Jessup, 2009).

Energycane is an F_1 hybrid of *Saccahrum* sp. and *Saccahrum spontaneum*. The interbreeding of these two plants has increased the cold tolerance and survivability of energycane compared to sugarcane (Wang et al., 2008). It has retained the ability to resprout from stolons following winters that have killed several sugarcane varieties on the same field plots and has been shown to have ~30% more aboveground biomass growth compared to switchgrass and giant miscanthus. The abilities to survive cold winters, outgrow other perennial grasses, and the potential to associate with N_2-fixing bacteria to meet much of its N needs, suggest it may be a strong alternative and sustainable feedstock for biofuel production for some regions. Switchgrass is a highly adapted and genetically diverse native grass that can grow productively in many regions of the United States, and other worldwide climates. Moreover, along with giant miscanthus, it has the potential to associate with N_2 fixers that can supply N for plant growth in temperate zones.

Both ^{15}N enrichment using $^{15}N_2$ incorporation and changes in ^{15}N natural abundance of plant biomass can provide semi-quantitative estimates of N_2-fixation (Bai et al., 2012; Munroe & Isaac, 2014; Robinson, 2001). Isotope labeling experiments can be used to confirm ANF and provide estimates of N_2-fixation across relatively short periods (hours and days). It is difficult to continuously utilize $^{15}N_2$ enrichment to confirm and estimate N_2-fixation over months or years of growth. The ^{15}N natural abundance method can help in this regard, to determine ANF under natural conditions for extended periods (Bai et al., 2012). Natural abundance isotopic studies were first implemented 70 years ago to identify symbiotic N_2-fixation (Burris & Miller, 1941). Biological N_2-fixation, determined using labeled $^{15}N_2$, provided evidence of "non-symbiotic" fixation (Delwiche & Wijler, 1956). Subsequently, there has been more of this type of activity but there are still relatively few published ^{15}N natural abundance studies and enriched ^{15}N-labeled N_2 experiments that investigated N_2-fixation by bacteria (e.g., *Azospirillum* sp.) in the root-zone of Gramineae (e.g., Boddey & Knowles, 1987; Buckley, Huangyutitham, Hsu, & Nelson, 2007; Roley et al., 2018). As with any isotope study, turnover and gas transformations of the isotope may contribute to the underestimation of Ndfa. When N_2-fixation is expected to occur, natural isotopic change can provide useful shifts or estimates of N_2-fixation across several years (Urquiaga et al., 2012; Williams, Rice, & Owensby, 2006). However, there is still controversy about the extent of N_2-fixation under natural field scenarios, and especially associated with temperate grasses (Ma et al., 2018; Roley et al., 2018). Research is thus critically needed to investigate the potential that N_2-fixation plays in grass feedstocks, as well as the global N cycle.

The objective of this study was to characterize, using both greenhouse-based enriched $^{15}N_2$ feeding studies and field studies (using natural abundance $\delta^{15}N$) to estimate Ndfa in three perennial bioenergy grasses—*Miscanthus* × *giganteus* (giant miscanthus, Freedom), *P. virgatum* (switchgrass, Alamo), and *Saccharum* sp. (energycane, Ho 02-147). We hypothesized that (a) N in plant roots and shoots of perennial bioenergy grasses and root-zone soil would be partially derived from recently fixed atmospheric N_2; (b) energycane, a hybrid progeny of sugarcane, was expected to have the highest Ndfa, similar to sugarcane.

2 | MATERIALS AND METHODS

2.1 | Greenhouse experiment (Experiment 1)

2.1.1 | Sample collection and experimental setup

Soil was collected to a depth of 20 cm on field edges of the plots used for Experiment 2 (see below). Soil was then sieved through mesh (4.75 mm) to homogenize and remove gravel, roots, and detritus. About 2,500 g of soil (~20% w/w water content) was mixed 1:1 with sand, and the resulting root-zone media was placed into pots and packed to a target bulk density of 1 kg m^3. Prior to planting, soil properties, such as water content and particle density were recorded.

Three bioenergy grasses used for this greenhouse experimentation included energycane (*Saccharum* sp., Ho 02-147), giant miscanthus (*Miscanthus* × *giganteus* Freedom®) and switchgrass (*P. virgatum*, Alamo). In this current study as well as previous studies, sweet sorghum (*Sorghum*, M81-E), displayed no detectable Ndfa and was thus used as a negative control (Isopi, Fabbri, & Del Gallo, 1995). Rhizomes (~5 cm length) of similar size were collected and germinated inside a seed incubator with one shoot planted into separate pots; total weight of the pots and rhizomes were recorded. Pot mesocosms were spaced in the greenhouse using six replicates arranged with a randomized block design. Similarly, three replicate pots without plants were prepared identically to provide soil that had been incubated similarly, and could act as a possible reference in the unlikely event that soil ^{15}N of the planted but non-$^{15}N_2$ exposed soils were not significantly different from those of the $^{15}N_2$ fed plants. Pots were watered as needed, approximately every 2 and 6 days for plants and no-plant pots, respectively. An enriched $^{15}N_2$ tracer ($^{15}N_2$ feeding) study was conducted to determine Ndfa of the feedstocks (relative to sorghum reference) over a 24 hr cycle of growth during E4; Concurrently, *nifH* of root and rhizosphere bacterial diazotrophs were amplified to support proof if nitrogen fixation was detected.

2.1.2 | Isotope $^{15}N_2$ feeding experiment

Following ~10 weeks of growth, $^{15}N_2$ was injected into the pots. This was based on switchgrass maturation to the E4 elongation phase, which was represented by the formation of six leaves (Moore et al., 1991). Pots were covered with Teldar bags (Huskey, CFHK0610C) fitted with three rubber stoppers sealed into the sides of the pot Figure S1a. The intersection of the bag surrounding the pot approached the plant shoots that were banded and coated with vacuum grease to maintain a sealed atmosphere in the pot-soil system. The bags maintained gas under mild pressure and thus would have inconsequential loss over a 24 hr period. Neon gas was injected as a quantitative tracer (Hamme & Emerson, 2004). Neon (99.9%; Sigma Aldrich 601691) and isotopic $^{15}N_2$ (98%; Sigma Aldrich 364584) were diluted to arrive at 5% ^{15}N enrichment by mixing with atmospheric air (Figure S1b) to a final volume of 1 L in gas sampling bags (SKC, Inc). A total of 60 ml of the mixture was injected at three equally spaced locations along the vertical side of the pot. Rubber septa were pierced using the 60 ml syringe holding a $23^{1/4}$ gauge (0.06 cm), 7.6 cm luer-lock needle (Becton Dickinson). Plants expected to be non-fixing and soil without plants provided a reference showing that no added ^{15}N was found in those systems despite having been exposed to enriched $^{15}N_2$, indicating that no contaminants were found in the gases (Dabundo et al., 2014). Gases in the system were vigorously mixed following injection by pumping the syringe slowly four to five times over 30 s intervals.

Particle and bulk density of potted soil were used to determine the total pore space. Total gas phase volume was calculated from % water content and total gas filled pore space volume (842.5 ml) before injection. Furthermore, headspace above the soil of the Tedlar bags were also determined and included in the calculations of the expected isotope concentrations of the N_2 in the pot mesocosms. About 24 hr before and after feeding, needles were again inserted and sample gases were taken from within the pots. Three 10 ml syringes were used at both times and gases were stored in 10 ml vacuum tubes to be analyzed for Ne gas. Recovery was then calculated to estimate the potential for gas losses from the system. Greater than 84% of the Ne was recovered in all cases, and values were not different between time points. The change from initial injection was not significantly different from that measured following 24 hr, and so no alterations were needed to account for gas loss other than that from the initial injection. Observed recovery for Ne was assumed to equal that for $^{15}N_2$ for calculations. Soils were separated from the whole plants and shoots. Mass of these pools was measured. This information was used along with the isotope values to provide accurate weighted averages of each fraction to determine total plant Ndfa. Total dry matter yield was obtained by drying plants at 60°C for 3 days.

2.1.3 | Calculations for determining Ndfa for the isotope $^{15}N_2$ feeding experiment

Equations to calculate %Ndfa and total Ndfa were conducted according to methods used previously (Boddey, Polidoro, Resende, Alves, & Urquiaga, 2001; Buckley et al., 2007; Warembourg, 1993). The natural abundance of atmospheric N_2 was taken as 0.3663 atom% ($\delta^{15}N_{air} = 0$). The following equation was used to determine $\delta^{15}N$ (‰):

$$\delta^{15}N(‰) = 1,000 \times \frac{\text{atom\%}(^{15}N \text{ sample}) - 0.3663}{0.3663}, \quad (1)$$

where $\delta^{15}N$ is used to provide a description of relatively small changes in the Ndfa of soils and plants compared with that of using atom% ^{15}N. δ refers to the change in ^{15}N relative to a standard, which is atmospheric N_2 containing 0.3663% ^{15}N. ^{14}N makes up the balance of the stable N isotopes (99.6337%). Hence, δ notation is useful for displaying small changes in an environment dominated by large concentrations of ^{14}N. Changes in the enriched $^{15}N_2$ feeding experiments were calculated using the following equation:

$$\%\text{Ndfa} = 100\frac{\left(\delta^{15}N_{ref} - \delta^{15}N_{fix}\right)}{\left(\delta^{15}N_{ref} - B\right)}, \quad (2)$$

For the $^{15}N_2$ feeding experiments the $\delta^{15}N_{ref}$ was derived from the natural abundance of $\delta^{15}N$ of identically treated plants that received unenriched atmospheric N_2 ($\delta^{15}N_{air} = 0$). $\delta^{15}N_{fix}$ was derived from $\delta^{15}N$ of plants exposed to an enriched $^{15}N_2$ soil atmosphere. For the feeding experiments, $\delta^{15}N$ of the atmosphere (B) was the isotope value of the enriched $^{15}N_2$ gas injected following mixing of the gas with that enclosed with Tedlar in the airspace of the belowground potted soil, including soil pore space. For plants receiving atmospheric unenriched $^{15}N_2$, the value for B is 0 (Denton, Pearce, & Peoples, 2013; Frankow-Lindberg & Dahlin, 2013).

To ensure that we had a proper non-fixing reference, sorghum M-81E was used and shown to be unenriched with ^{15}N when exposed to enriched $^{15}N_2$ compared to that of plants receiving atmospheric levels of $^{15}N_2$. Similarly, soil without plants also received enriched $^{15}N_2$ to assess the potential for contaminants within the isotopically enriched gas, and/or the potential occurrence of detectable background levels of soil N_2 fixation. Though described in the results, it is noted here that no detectable levels of contaminants or fixed nitrogen was observed in our reference plant or unplanted soil.

The $\delta^{15}N$ isotope values of the shoots, roots (including those of rhizomes), and soils were determined. Whole plant isotope values in Table 1 were determined from a weighted average of shoots and roots to calculate total and %Ndfa. When samples (soil, root, shoot) were significantly enriched in ^{15}N compared to reference controls, the %Ndfa was calculated as described above. The N_2 fixation calculation for the whole plant accounted for the total N mass of each pool (root and shoot) and expressed per unit of dry mass. Total %Ndfa was thus also calculated based on weighted averages of the roots and shoots to arrive at values for the whole plant:

$$\delta^{15}NP_w = (\delta^{15}\text{N root} \times (M_p)) + (\delta^{15}\text{N shoot} \times (M_p)), \quad (3)$$

where M_p is the proportional mass of the plant part, where shoot and roots add up to a proportional total of 1, or 100% of the plant mass, and P_w is the whole plant. The total mass of each plant could then be used to arrive at total mg plant Ndfa per pot system. Note that for calculations involving plant mass (M_p), unsurprisingly, there were no significant differences in mass between labeled and unlabeled plants. Because these plants were considered as part of the same population, with the only difference being the receipt of enriched and unenriched $\delta^{15}N_2$, the plant mass of the two treatments were combined for each pool, roots, and shoots. Thus, the isotope values and not subtle differences in plant mass described variation in %Ndfa.

2.1.4 | Processing of samples for $\delta^{15}N$

The soil separated from the roots and shoots was sieved using a number 4 (4.75 mm) sieve. Subsamples were dried for 24 hr at 60°C, ground in a pestle and mortar for homogenization and finally passed through a 100 mesh (150 μm) sieve. Representative treatment soil was weighed (30–40 mg) into tin cups (5 × 9 mm; Costech #041077), which were then folded, sealed, and analyzed using Continuos Flow Isotope Ratio Mass Spectrometry (CFIRMS) whereby a dry combustion NA 1500 NC analyzer (Carlo Erba) was coupled to an Isoprime mass spectrometer (Micromass).

For plant materials, roots were washed with distilled water several times to remove soil particles. Shoots and roots (following washing) were dried for 24 hr at 65°C, weighed, ground with liquid N_2, and passed through a 60 mesh sieve (150 μm). Both root and shoot materials were weighed (5–6 mg) into tin cups (5 × 9 mm; Costech #041077) and analyzed for N and $\delta^{15}N$ with the CFIRMS instrumentation PDZ-Europa 20/20 Isotope Ratio Mass Spectrometer (Agilent, Oregon State University, Department of Crop and Soil Sciences).

2.2 | Field experiment (Experiment 2)

2.2.1 | Site description and field plot settings

This study was conducted at the Agronomy Unit 1 of the Leveck Animal Research Center located at Mississippi State University, Mississippi, United States (33°28′N and 88°47′W) and arranged as a randomized block design for analysis. The soil at this site is mapped as a Catalpa (fine, smectitic, thermic fluvaquentic hapludolls) and has been used for pasture or hay production. For the 2 years preceding plot installation, the area was fallow. The soil, a silty clay loam, had a 1:2 (soil: deionized water) pH of 6.5 for the 0 to 10 cm sampled depth. Soil C and N in the top 10 cm were 1.6 and 0.11%, respectively. The three perennial bioenergy crops were established in a randomized block design with four replicated plots (0.75 × 0.52 m) in May 2010. Energycane (Ho 02-147) was planted in two row plots with three stalks per row. Switchgrass (*P. virgatum*, Alamo), giant miscanthus (*Miscanthus* × *giganteus* Freedom®) and sweet sorghum (*Sorghum bicolor*, M81-E) seeds were planted, similarly in two row plots. Greenhouse studies indicated that this variety

TABLE 1 Greenhouse experiment using $^{15}N_2$ labeling. Soil, root, and shoot $\delta^{15}N$, N content, and yield per pot for $^{15}N_2$ enriched and non-enriched energycane, giant miscanthus, switchgrass, and negative control sorghum

	$\delta^{15}N$		N (mg/g)		Dry matter yield (g)		%Ndfa
Grass	Labeled	Unlabeled	Labeled	Unlabeled	Labeled	Unlabeled	(mg Ndfa)
Energycane							
Soil	5.2	4.6	1.27	1.14			
Roots	5.6*	2.8	12.6	13.2	10.0	8.80	
Shoots	3.8*	2.6	10.9	10.5	11.2	10.0	
Plant	4.7*	2.7					1.10 (2.57)
Switchgrass							
Soil	5.6	4.9	1.13	1.12			
Roots	4.6*	2.4	9.28	11.9	10.1	8.50	
Shoots	3.6	3.4	10.0	9.9	11.1	10.5	
Plant	4.1*	2.9					0.66 (1.16)
Miscanthus							
Soil	5.3	4.8	1.07	1.08			
Roots	6.3*	4.5	10.8	10.7	9.51	9.20	
Shoots	5.4	4.8	11.7	10.3	9.42	9.00	
Plant	5.8*	4.7					0.65 (1.37)
Sorghum							
Soil	5.1	4.6	1.01	1.09			
Roots	7.5	7.3	5.81	5.75	6.12	6.21	
Shoots	7.2	6.8	7.88	6.63	6.37	7.58	
Plant	7.3	7.1					0 (0)

Note: Mean soil, root, and shoot $\delta^{15}N$ values in switchgrass, giant miscanthus, energycane, and sorghum of $^{15}N_2$ labeled and unlabeled pots. Unlabeled plants were used for $\delta^{15}N_{ref}$ in Equation (2).

Asterisk following the values indicates significant difference between labeled and unlabeled pots ($n = 3$; $p < .05$). Note that when both roots and shoots are pooled for each plant, labeled and unlabeled $\delta^{15}N$ were significantly different from each other for all three feedstocks, but not for sorghum.

of sorghum had a root-zone with low to no N_2 fixing bacteria, and no detectable incorporation of $^{15}N_2$ during the feeding study.

2.2.2 | Plant-soil collection and preparation

Field collection of soil samples were taken using a Hoeffer soil probe (2 cm diameter) at two depths as follows: 0–10 and 10–30 cm. Six to ten cores, at the subsurface and surface layers, respectively, were taken randomly from within a fixed circular growing area at the base of each plant, near to the root-zone (Batten, Scow, Davies, & Harrison, 2006). These soil samples (~500 g) were stored on ice in sealed Whirl-Pak bags during transport and subsequently stored at 4°C. Samples were collected from replicate plots prior to planting in May 2010, followed by collections every 5–7 months for 3 years at the beginning and end of the main growing season. Roots in the cores were separated from soil, and the soil was sieved using sieve number 4 (4.75 mm). Wet weights of both roots and soils were recorded. Then they were divided into two subsamples. One subsample was kept at 4°C for further processing (e.g., DNA extraction, PCR amplification) and a portion was stored at −80°C. The other subsample of root and soil were dried for 24 hr at 65°C to estimate water content, and then ground and homogenized with a pestle and mortar using liquid N_2.

At the end of the second and third growing seasons (January), total aboveground biomass was collected and the yield (dry weight) was determined for each plot. Because total biomass was not collected during the first establishment year, smaller subsamples from six to eight recently matured leaves (Ramos, Villatoro, Urquiaga, Alves, & Boddey, 2001) of aboveground shoots were taken. Roots were similarly sampled as part of the soil collections. Sample preparation for isotope analysis for soils, roots, and shoots were done identically to those described in Experiment 1.

2.2.3 | Calculations for determining Ndfa isotope in field experiment

The natural abundance of atmospheric N_2 was taken as 0.3663 atom% ($\delta^{15}N_{air} = 0$) and the same equation was used

to determine $\delta^{15}N$ (‰), as described in Experiment 1 of the feeding experiment (Equation 1).

Nitrogen derived from atmospheric N_2 in soils and plants of the natural abundance experiment was calculated using the same equation in Experiment 1, %Ndfa (Equation 2).

Sorghum M-81E was used as a reference ($\delta^{15}N_{ref}$) plant for comparison to each of the three perennial bioenergy grasses. Another sample of a field weed (chicory) was measured and found to have similar non-fixing isotope values to that of sorghum (Hogberg, 1997). For this natural abundance study, the value of *B* has been shown to vary from different species and with growth stage, but is close to $\delta^{15}N$ of 0 to −1 as with legumes (Denton et al., 2013; Frankow-Lindberg & Dahlin, 2013). Therefore, a conservative *B* value was taken ($\delta^{15}N = 0$).

Plant isotope values were determined the same way as that for the $^{15}N_2$ enriched study but were not estimated using the weighted average because only subsampling of roots was taken to calculate %Ndfa. When samples (soil, root, shoot, whole plant) were significantly enriched in ^{15}N compared to reference controls, the %Ndfa was calculated.

2.3 | Statistical analysis

The assumption of variance, homogeneity, and normality were met for all the data. One-way ANOVA was used to determine the statistical significance of differences in the response variables $\delta^{15}N$ and %Ndfa for soil, roots, shoots, and whole plants of greenhouse grown plants (Experiment 1). Two-way ANOVA of plant species, time (repeated measures command), and their interaction were used to determine the statistical significance of differences in the response variables $\delta^{15}N$, %Ndfa, and biomass for roots, shoots, and soil associated with the growth of grasses in the field (Experiment 2). The least significant difference test at $p < .05$ was used to assess the significance of statistical differences among treatment means. When calculating %Ndfa, only those plant parts that were found to be statistically significant were used. Statistical analysis was carried out using PROC MIXED and the repeated measures function in SAS version 9.3 (SAS 2010).

3 | RESULTS

3.1 | Experiment 1 (greenhouse)

3.1.1 | Comparisons of the N concentration and $\delta^{15}N$ in soils, roots, and shoots of grasses

Dry matter recovery of roots and shoots from pots was not significantly different between the feedstock grasses; however, feedstock grasses did have statistically greater dry matter recovery compared to sorghum (19.4 g vs. 13.1 g; Table 1). Plant N concentrations were not different; however, one outlier with a 3 *SD* difference from that of other values was removed. Nitrogen concentrations of roots and shoots for sorghum were significantly lower than the feedstock grasses. Yield was similar between roots and shoots across feedstock grasses, however, it was significantly greater than in sorghum ($p < .01$). Soil N concentrations were not significantly different across plants nor labeling treatment ($n = 3$; Table 1).

Each grass, except sorghum, was significantly enriched in $\delta^{15}N$ as a result of the $^{15}N_2$ exposure compared to the unlabeled pots (Table 1) as expected. Switchgrass and giant miscanthus, however, differed from energycane, where in this latter case there were significant differences in ^{15}N detected in both roots and shoots rather than roots only. This suggested movement of N from roots to shoots during the 24 hr feeding period. Not surprisingly, roots for switchgrass and giant miscanthus were relatively enriched with ^{15}N compared to shoots, a result consistent with isotope feeding and N_2-fixation in the rhizoplane or in the rhizosphere. The isotopic feeding experiment verified that plants had incorporated tracer $^{15}N_2$ via bacterial N_2 fixation in the root-zone in feedstock grasses, but not in the reference plant, as expected, for sorghum.

Following correction for the recovery of gas in the mesocosms, it was determined that %Ndfa by roots following the 24 hr feeding experiment was greatest for energycane than switchgrass and miscanthus, as expected (Table 1). The %Ndfa for energycane, switchgrass, and giant miscanthus, were 1.1, 0.65, and 0.66, respectively. Hence, after 24 hr of labeling at the V4 stage, and averaging across the grasses, up to 1 out of ~100 N atoms were derived from the process of bacterial N_2 fixation. If %Ndfa and plant N accrual were comparable across a 50 day period, these numbers would roughly translate into 55%, 32%, and 33% of plant Ndfa (Table 2), an assumption that is supported by a linear growth rate and steady N concentration during elongation (Frank, Berdahl, Hanson, Liebig, & Johnson, 2004; Garten et al., 2010; Lemus, Parrish,

TABLE 2 A simple extrapolation of the %Ndfa of three bioenergy grasses grown in the greenhouse with $^{15}N_2$-enriched gas from Table 1

Bioenergy grass	%Ndfa in labeled plants[1]
Energycane	55 a
Switchgrass	33 b
Giant miscanthus	32 b

Note: Means followed by the same letter (a, b) are not significantly different between species. $p < .05$ (LSD test).

Abbreviation: LSD, least significant difference.

[1]Values assume that plant %Ndfa is the same for 50 days of plant growth. It is typical for growth to occur for 160 days or more.

& Abaye, 2008). Though a simplistic calculation, switchgrass can grow for >160 days and up to 200 days/year in the southern United States (Lee et al., 2018; Sanderson & Reed, 2000; Teshager, Gassman, Schoof, & Secchi, 2016), and so the potential for 50 of those days receiving adequate spring and summer rainfall to support plant growth and nitrogen fixation seem reasonable. This assumes favorable water status and water-limited root carbon flow in soil results in greater N demand in support of N fixation. This would seem to be further supported because April–September there is a daily 40% chance of rain of more than 0.25 cm. Each spring and summer month averages 10–13 cm rainfall (https://www.ncdc.noaa.gov/cdo-web/datatools/normals). Though this is a simplistic exercise, the logic seems to bear out the potential for >32% of yearly plant N coming from bacterial nitrogen fixation.

3.2 | Experiment 2 (field)

3.2.1 | $\delta^{15}N$ in root-zone soils, shoots, and roots of the plants

Soil $\delta^{15}N$ prior to planting averaged +7.1 for the 0–30 cm depth with relatively low variability between plots (Figure S2). Overall, the $\delta^{15}N$ of the surface (0–10 cm depth) soil was relatively stable, but significantly less variable than the subsurface (10–30 cm depth), which tended to be depleted compared to the surface by ~0.6 per mil. Root-zone soil $\delta^{15}N$ was temporally dynamic across the three growing seasons in the subsurface, varying by ~1 per mil. Shoot $\delta^{15}N$ tended to vary more than roots with season (Figure 1). Similarly, there were dynamics in soil N due to plant species but no clear evidence for increased N from that of the beginning of the experiment (Figure S3).

Roots and shoots could not be sampled the first year during plant establishment without compromising the future growth and success of the perennial feedstock grasses. Mean $\delta^{15}N$ of the root, shoot, and whole plant at the end of the establishment year (December 2010) and for the remainder of the experiment in May and December are shown in Figure 1. Because of establishment, root foraging across a larger soil volume than in a pot study, and relatively low N demand, it was expected that there would be less or no evidence for atmospheric N_2 movement into plants in the first growing season. The $\delta^{15}N$ values for the roots, however, particularly in energycane (Figure 1b) suggest the potential for active N_2 fixation. Following the third but not the second growing season (December 2010), more significant changes in the $\delta^{15}N$ of the feedstocks were observed (Figure 1c), consistent with changes that would occur with incorporation of Ndfa. Also, as expected, $\delta^{15}N$ of energycane was significantly lower than switchgrass and giant miscanthus, which in turn were significantly lower than the whole-soil and reference plant values (Table S1). Thus, there is evidence of diazotroph-derived N_2 incorporated into plants detected using the natural abundance technique.

The $\delta^{15}N$ of shoots (Figure 1a) were dynamic and significantly greater in spring (May) compared to winter (December) for both years. It should be noted that the sampling of roots in 2010 was likely more variable due to the lower mass of available roots. Variation between sampling

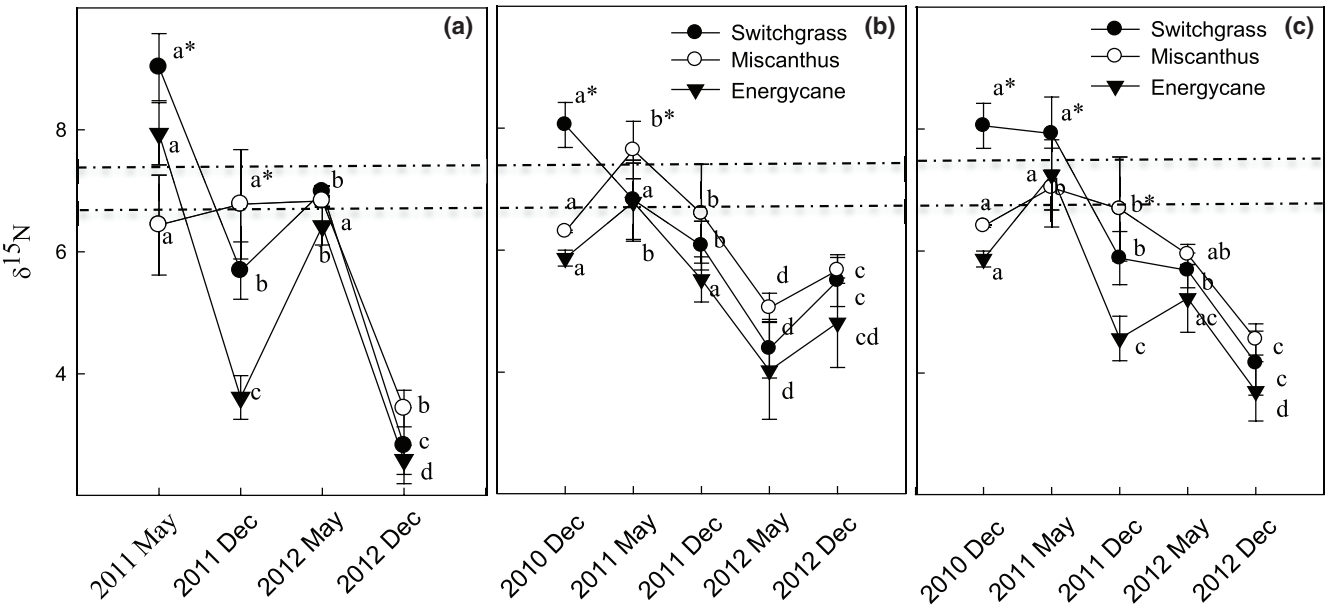

FIGURE 1 Mean $\delta^{15}N$ of (a) shoot, (b) root, and (c) whole plant of switchgrass, giant miscanthus and energycane grown from seedling/rhizome over 3 years. Letters (a, b, c, d) denote significant differences between the time points within the same grass species and error bars represent standard error ($n = 4$; $p < .05$). Asterisks indicate significant differences between grasses at a specific sampling time ($p < .05$). The dashed lines represent the 95% confidence interval for the $\delta^{15}N$ of the sorghum and chicory reference plants.

within the annual cycle is consistent with the translocation of aboveground to belowground tissues.

The sorghum reference plant tissue and associated soil was shown to have no detectable shift in $\delta^{15}N$ following growth in both field and greenhouse studies. These results support the main criteria for a suitable reference plant, which is to derive its N from a representative soil pool of bioavailable N and not from the atmosphere via root associations with diazotrophs. Overall, the reference plants were deemed suitable to provide estimates to calculate plant Ndfa (Table S1). It is notable that $\delta^{15}N$ for sorghum were about 0.7 per mil greater in the greenhouse than values in the field. This could have been the result of soil sampling at a depth of 0–20 cm rather than 0–10 cm. Similarly, collected weed samples from the field also had similar $\delta^{15}N$ than those of sorghum, and to soil, indicating that reference plants were useful for calculating %Ndfa with the natural abundance techniques in the field. It is also notable that bacteria attached to the roots are visible in the feedstock grasses (Figure S4), but not in the root of sorghum. These data, together, with the *nifH* electrophoretic results, revealed that very few to no bacteria were visualized for sorghum but are more apparent for the feedstock grasses (Figure S5).

Using the isotopic values derived at the end of the third growing season, an estimate of plant %Ndfa was calculated (Table 3). Both switchgrass and energycane roots had greater %Ndfa compared to giant miscanthus. Overall, the field estimates of %Ndfa were broadly similar across plants, however, miscanthus had the lowest values and was broadly similar to outcomes of Ndfa in the greenhouse.

3.2.2 | %N in shoots and roots of the plants

Shoot %N had similar temporal dynamics to that of $\delta^{15}N$ (Figure 2a). There were discernable valleys and troughs in %N in shoots with the greatest values during the early growing season and lowest during the late growing season. This pattern reflected plant demand for N associated with growth (dilution effect) during the growing season. Root N dynamics were less variable (Figure 2b), but shifts along with shoot N dynamics were likely the result of translocation between shoot and belowground rhizomes and roots, changing with the growing season. In general, N concentrations of the

TABLE 3 Ndfa percentage (0–10 and 10–30 cm depth) for root, shoot, and soils derived from three perennial bioenergy grasses sampled following dormancy in the second (December 2011) and third growing season (December 2012)

	2011			2012		
	Switchgrass	Giant miscanthus	Energycane	Switchgrass	Giant miscanthus	Energycane
Roots	17b	4.8a	24cd	14b	6.7a	24c
Shoots	7.1a	9.0ab	14b	48d	37cd	52d
Total plant	12b	6.9a	19bc	31cd	21c-	38cd
Soil 0–10 cm depth	6.8	4.5	12[1]	4.4	1.6	7.6[1]
Soil 10–30 cm depth	4.3	3.2	6.1	−2.8	−2.1	4.5

Abbreviation: LSD, least significant difference.

[1]For soil, a symbol following the value indicates a significant difference from 0. Plant root values are inclusive of rhizomes and stolons. Means followed by the same letter (a, b, c, d) are not significantly different. All the data were compared using LSD test at $p < .05$.

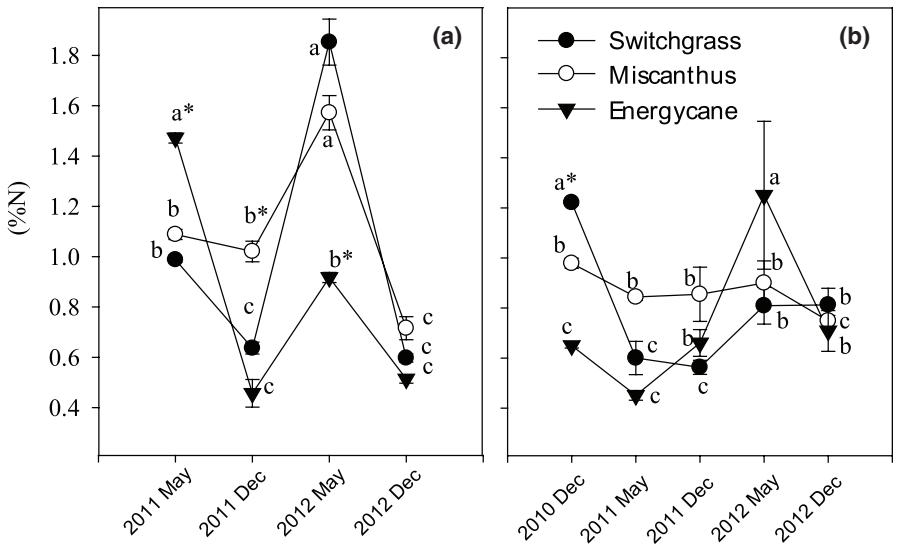

FIGURE 2 Mean %N of (a) shoot and (b) root in switchgrass, giant miscanthus, and energycane grown over 3 years. Letters (a, b, c) denote significant difference between the time points within the same grass species and error bars represent standard error ($n = 4$; $p < .05$). An asterisk following the values indicates significant difference within shoots of each time point of different grass species ($p < .05$)

plants were low relative to that reported in the literature. This was expected to some extent and supports the limitation of N on non-fertilized land. It also favors high N demand that would support the need for N_2 fixation to meet plant demand (Boddey, 1995). Yield was similar between years, averaging 14.1, 13.2, and 12.0 Mg/ha for energycane, switchgrass, and miscanthus, respectively, indicating high N demand.

4 | DISCUSSION

The results of the greenhouse and field studies were highly complementary to one another and supported the hypothesis that temperate feedstock grasses can derive a significant agronomic N benefit through association with root-zone diazotrophs. Though it is not possible to come to firm conclusions about the precise amounts of N fixed and made available to plants, conservative Ndfa amounts of ~30% of plant N were estimated. Roley et al. (2018) estimated somewhat smaller values, but their experiments for switchgrass were carried out on excised plant tissues grown in the field. These results thus support the need for further investigation into the potential for feedstock grasses to obtain atmospheric N_2 through association with diazotrophic bacteria when unfertilized and in relatively low N soils. Breeding of plants for strong diazotrophic associations with high N_2 fixing rates could help to improve the productivity of feedstocks growing on non-fertilized or marginal lands.

4.1 | Greenhouse $^{15}N_2$ feeding experiment

Several studies have been conducted using $^{15}N_2$ incorporation into legumes, grasses, and sugarcane (Molero, Tcherkez, Araus, Nogués, & Aranjuelo, 2014; Ohyama et al., 2014; Rasmussen, Gylfadóttir, Dhalama, De Notaris, & Kätterer, 2019) to directly assess N_2 fixation and plant Ndfa. Yet, to our knowledge, no such experiments have been performed using natural abundance or enriched $^{15}N_2$ feeding techniques with the temperate bioenergy grasses energycane, switchgrass, and giant miscanthus and verfication under both field and greenhouse conditions. The greenhouse experiment herein provided strong evidence that feedstock grasses obtained significant amounts of N as a result of diazotrophic bacteria activity. The $\delta^{15}N$ of plant tissues also supported the use of chicory and sorghum ($\delta^{15}N$, 6.5–7.6) as useful reference plants to detect N_2 fixation using a ^{15}N natural abundance approach in the 3-year field experiment also described herein. As hypothesized, evidence for the greatest rates of N_2 fixation and plant Ndfa was associated with energycane, but biologically significant amounts of plant Ndfa were also clearly associated with switchgrass and giant miscanthus. Energycane, interestingly, also showed evidence for Ndfa in soil. Indeed, the relatively high amounts of atmospherically derived diazotrophic N_2 in the root-zone soil of this grass suggest the possibility that an even greater potential role may exist for diazotrophs than interpreted from plant Ndfa alone. In addition, this ^{15}N feeding study would have not detected fixation from leaf endophytes or some of the nitrogen that is fixed, but not accrued by the plant (De Souza et al., 2016; Li, Voigt, & Kent, 2016; Liu & Ludewig, 2019). Values of Ndfa do not provide perfect estimates of N_2 fixation, but during short term (24 hr) greenhouse exposures, the values may come relatively close to providing a representative estimate. However, the limitation of such a short-term study is the degree to which this snapshot can be used to estimate biologically relevant fixation across weeks and growing seasons. Measurements across numerous plant growth stages and environmental contexts will help to better resolve the role that N_2 fixation can play in support of feedstock N demand. Though always limited in capacity to extrapolate to real field scenarios, greenhouse feeding studies are a critical means to understanding the potential role that bacterial N_2 fixation has in support of growing feedstock grasses for biofuel production (dos Santos, Chaves, da Silva Ribeiro, Alves, & Reis, 2019; Wei et al., 2014).

Many $^{15}N_2$ incorporation studies have been conducted in controlled environment growth chambers (Chalk et al., 2014; Ito, Cabrera, & Watanabe, 1980). In this study, a novel cost-effective method of directly estimating N_2-fixation was tested by measuring the incorporation of isotopic $^{15}N_2$ in soil and plant pools following direct injection and vigorous mixing into the belowground portion of the pot. It also utilized a neon tracer gas to aid estimation of gas leakage from the system, thus helping to provide realistic estimates of N_2 fixation. It was concluded that the belowground injection method was a very effective means to follow the flow of $^{15}N_2$ into plant and soil.

Rate estimates across a 24 hr $^{15}N_2$ feeding study were from ~0.65%–1.1% of the plant N derived from diazotrophic fixation. If these estimates were reliable indicators of field scale N_2 fixation across ~50 days, then 33%–55% of plant N could be derived from the atmosphere. Numerous assumptions about growth rate, and % N underly these estimates as described in the results, but they are supported by growth measures described in the literature (Frank et al., 2004; Garten et al., 2010; Lemus et al., 2008); and thus, provide some guidance on the possible levels of N_2 fixation that could occur in 50 of the total >160 days growing season. One explanation backing this assertion comes from the need for favorable water status driving water-limited root carbon flow in soil that then results in greater N demand in support of N fixation. In the growing season months there is a daily 40% chance of rain of more than 0.25 and 10–13 cm of rainfall typical per month (https://www.ncdc.noaa.gov/cdo-web/datatools/normals). Rainfall could thus create the moist soil conditions needed to support periods of high nitrogen fixation, assuming water in addition to N limitation controls levels of fixation. The Ndfa extrapolations are somewhat high, but broadly in agreement with estimates based on the field study.

The numbers should be used primarily as a working model from which future studies further test the hypothesis that diazotrophic N_2 fixation can provide agronomically significant levels of N to feedstocks when grown without fertilization.

The results serve to illustrate the potential of these grasses to accumulate significant amounts of bacterially fixed N_2. It is notable that while the system was set up to have ideal aeration and soil water content, O_2 levels could have dropped during the experiment when the belowground biomass in the pots was sealed. This could have increased N_2 fixing activity. This would not be unusual, however, under field conditions, following rainfall, high microbial consumption of oxygen, or movement of water from deeper in the soil to surface via plant-driven hydraulic lift. Greater water contents would be a key driver of N_2 fixation in the field because of the potential for lower O_2 concentrations in the high microbial activity of the rhizosphere and rhizoplane coupled with low diffusion of O_2 to replace that utilized by microbial heterotrophs. The results nevertheless serve to illustrate the potential for detectable levels of N_2 fixation in the root zone in a moist environment. Grasses can also have endophytes in their leaves, and thus even more N_2 fixation may occur than from our belowground estimates. Overall, these results suggest that all feedstock grass mesocosms were supported by N_2 fixation, and with the greatest atmospheric N_2 incorporated into energycane. These results also supported the use of Sorghum M-81E as a reference plant, and its use in the calculation of Ndfa.

Isotopically labeled $^{15}N_2$ was incorporated into plants relatively rapidly (24 hr), and though most of this N remained in the roots, there was considerable flow from roots to shoots in energycane. This result may reflect differences in N translocation traits between the grasses, or the location of N_2 fixers as endophytes, in the rhizosphere and rhizoplane. The greater flow of isotope to energycane relative to the other feedstock grasses also was expected because it is a hybrid derived from sugarcane, and the latter is well known for its ability to associate with diazotrophs that reduce atmospheric N_2 that is then incorporated into plant tissues (Wei et al., 2014). Though not clear in our measurements of plant biomass, sugarcane, a parent to energycane, begins to grow at a relatively faster rate than other grasses during elongation. If this was the case in our study, the greater N demand could have been coupled with the transition into the greater relative growth and thus foster the greater movement of labeled N into the energycane relative to switchgrass and giant miscanthus (Morris, Zuberer, & Weaver, 1985).

4.2 | Field-based ^{15}N natural abundance experiment

This 3-year field study, as hypothesized, also provided evidence that three perennial feedstock grasses derived N_2 from atmospheric fixation by diazotrophs. Like that of the greenhouse experiment, energycane, as expected, showed evidence for the greatest amounts of Ndfa, followed closely by switchgrass, and then giant miscanthus. This latter point shows consistency between field and greenhouse studies suggesting that fixation served as a significant source of plant tissue N. Studies of sugarcane have demonstrated that 60%–80% (>150 kg N ha^{-1} $year^{-1}$) of plant N is derived from N_2 fixation (Baptista et al., 2014; Boddey et al., 1995). Other grasses such as elephant grass have also observed shifts in ^{15}N natural abundance that were interpreted to result from N_2 fixation (Videira et al., 2012). Studies on temperate perennial grasses have speculated and hypothesized that plant Ndfa may be greater than once thought (Boddey & Knowles, 1987; Keymer & Kent, 2014). However, paired greenhouse and field experiments help to corroborate the importance of N_2 fixation, as described herein, and thus provided another level of support to the idea that high productivity temperate feedstock grasses can associate with diazotrophs and derive agriculturally significant levels of N through bacterial N_2 fixation. These results support the need for more research into the potential of feedstock grasses and crop plants to form strong associations with diazotrophs that would be expected to aid sustainable biofuel crop productivity and vigor when grown on non-fertilized or marginal lands (Carlsson et al., 2017; Dauber et al., 2012).

Based on calculations of the aboveground plant material (14 Mg/ha yield; plant, 1.3% N) 183 kg N/ha would be contained in plant mass each year. If belowground mass and C flow were half these aboveground values, then 275 kg N/ha would be needed to support total plant biomass. For energycane, if 38% of this N was derived from bacterial nitrogen fixation, then it would represent 105 kg N/ha. This value, if directly translated into a measure of nitrogen fixation is substantially lower than the amounts reported for sugarcane, but agronomically nevertheless, very significant. Ndfa values are good measures of input from nitrogen fixation, but over long periods translating those values to rates of nitrogen fixation could both over- or underestimate rates of nitrogen fixation.

While the exact values of N_2 fixation, and the amount that benefits the plant is uncertain, the outcomes of both experiments suggest the potential for relatively high rates of nitrogen fixation associated with feedstocks. It is also worth noting that in the case of energycane, significant amounts of Ndfa were detected in the soil. This was not highly surprising given the high potential for bacterial-associated N_2 fixation with its parent sugarcane; however, because of the sampling design, and unknown amount of rhizosphere volume, it is difficult to use these soil data to estimate amounts of Ndfa associated with energycane. Given the relatively large background quantity of N in soil, alterations in $\delta^{15}N$ soil would represent significant amounts of Ndfa, conservatively estimating >5% of plant N.

The above Ndfa calculations were derived from shoots and roots of perennial bioenergy grasses that had significantly lower $\delta^{15}N$ relative to a non-ANF-associated reference plant sorghum M-81E and the weed chicory. To accurately determine Ndfa using the ^{15}N natural abundance method, the choice of reference plant is a major experimental consideration (Shearer & Kohl, 1986). Nevertheless, the choice of reference plant is almost never a perfect one. An example relevant to this study is related to root architecture, whereby annual grasses tend to have lower root biomass and more shallow root systems. This would alter their access to mineral N with depth and thus access to nitrogen with a different isotopic signature (Koteen, Baldocchi, & Harte, 2011). Greater rooting biomass and depth of perennial grasses compared to sorghum (Myers, 1980) would be expected to support the uptake of isotopically enriched N in the subsoil and shift $\delta^{15}N$ of plant tissue in a way that dilutes the effect of atmospherically derived N ($\delta^{15}N = 0$). This would reduce estimates of Ndfa in perennial grasses (Urquiaga et al., 2012). An ideal reference plant is one that provides the same root habitat and background measure of N derived exclusively from the soil, but such a reference does not yet exist. The results presented herein are compelling, nevertheless, because they are supported by greenhouse studies and utilize a reasonable set of reference plants. Expectations of a soil source of N are also consistent with isotopic values derived from other varieties of non-fixing sorghum (Lee, Wani, Yoneyama, Trimurtulu, & Harikrishnan, 1994; Urquiaga et al., 2012). Sorghum M-81E thus shows promise as a viable reference plant to calculate Ndfa for perennial diazotroph-associated grasses. It is notable that some varieties of sorghum, such as BRA 308 (Coelho et al., 2009), and *Sorghum halepense* (Rout & Chrzanowski, 2009) have been shown to support root-zone N_2 fixers and perhaps plant acquisition of Ndfa. Results from our *nifH* analysis, however, also supported the use of sorghum M-81E as a suitable reference plant because, unlike the feedstock grasses, *nifH* amplicons were not detectable. It is not known if this is a genomic trait of sorghum M-81, or whether environmental conditions, such as the lack of proper bacterial inoculants present in the soils could account for its lack of plant association with diazotrophs. It was concluded, nevertheless, that at least for the purpose of this study, sorghum M-81E meets the criteria as a suitable reference plant.

Perennial grasses translocate ~90% of their aboveground N to belowground stolons and rhizomes during shoot senescence at the end of the growing season (Heckathorn & Delucia, 1996). An effect of isotope discrimination during N translocation cannot be excluded. However, because of the high translocation efficiency, it was not considered to be a significant source of variation during sampling before rigorous aboveground growth in May, and after die-off of stems in December. These data do indicate the importance of sampling in both above- and belowground plant tissues to arrive at isotope values that are reflective of the plant, however. Isotopic fractionation tends to be relatively small compared to the 4–5 per mil shifts in plant tissues (Unkovich, Baldock, & Peoples, 2010). From the aboveground perspective, plant shoots were removed similarly to roots at the end of each growing season following senescence, a small pool of depleted $\delta^{15}N$ would have also been removed, and the net effect, if any, would be to underestimate N_2 fixation.

In the early periods of the field experiment, the relatively high $\delta^{15}N$ in the roots and shoots could be interpreted as the result of low colonization and activity of N_2 fixers in the root-zone and greater N acquisition from soil sources. Plant and root $\delta^{15}N$ values from our study were generally enriched at levels similar to the soil early in their growth, but by the third year showed evidence 4–5 per mil decline. The tissue $\delta^{15}N$ was depleted between 0 and 1.5 per mil compared to surface (0–10 cm) soil in 18 grasslands (Kahmen, Wanek, & Buchmann, 2008), perhaps indicating N_2 fixation, N source variation, and isotopic discrimination. Isotopic fractionation during N mineralization and subsequent plant uptake is one possibility (Denk et al., 2017; Kahmen et al., 2008), but many grasses have been suggested to obtain detectable amounts of N from fixation by diazotrophs, including cultivated maize (Van Deynze et al., 2018) and wheat (Wang et al., 2016). There is still controversy about the contribution of N_2 fixation associated with many types of grasses, especially temperate grasses, but the results herein, including reference plants for comparison, suggested under N-limited conditions that Ndfa may be agronomically significant for several temperate feedstock grasses. Moreover, high biomass yield and high N demand of these perennial bioenergy grasses created the conditions for high C flow belowground to support the growth and need for diazotroph-derived atmospheric N_2 (Rodrigues, Moon, Zhao, & Williams, 2017).

5 | CONCLUSIONS

A comparative analysis of the potential from several high productivity temperate grasses to attain Ndfa was conducted. Here it was shown that giant miscanthus, switchgrass, and energycane could associate with N_2-fixing microorganisms, and obtain N derived from atmospheric N_2 fixation. Energycane, as expected, showed evidence in both the greenhouse and field, for the greatest amounts of N derived from the atmosphere (38%–50%), followed closely by switchgrass, and giant miscanthus. These results support the notion that N_2-fixing bacteria support the growth and sustainability of feedstock grasses growing on low N soils, such as those described in marginal lands. Further research is needed, to both confirm and assess whether other feedstock grasses

are supported by N supplied through diazotroph N_2-fixation under field conditions.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings will be available at the Knowledge Network for Biocomplexity at https://knb.ecoinformatics.org/view/doi:10.5063/F1NP22TK following a 1 year embargo from the date of publication to allow for use of data for a companion publication.

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SUPPORTING INFORMATION

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