Soil Biology and Biochemistry 129 (2019) 90–98

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Isotopic evidence for episodic nitrogen fixation in switchgrass (*Panicum virgatum* L.)

Sarah S. Roley[a,b,*], Chao Xue[c,d], Stephen K. Hamilton[a,b,e], James M. Tiedje[a,c,f], G. Philip Robertson[a,b,f]

[a] Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, MI, USA
[b] W.K. Kellogg Biological Station, Michigan State University, Hickory Corners, MI, USA
[c] Center for Microbial Ecology, Michigan State University, East Lansing, MI, USA
[d] Jiangsu Provincial Key Lab for Solid Organic Waste Utilization, National Engineering Research Center for Organic-based Fertilizers, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, Nanjing Agricultural University, Nanjing, China
[e] Department of Integrative Biology, Michigan State University, East Lansing, MI, USA
[f] Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI, USA

ARTICLE INFO

Keywords:
Associative nitrogen fixation
Diazotroph
Rhizosphere
Stable isotopes
Switchgrass

ABSTRACT

Perennial grasses can assimilate nitrogen (N) fixed by non-nodulating bacteria living in the rhizosphere and the plant's own tissues, but many details of associative N fixation (ANF) remain unknown, including ANF's contribution to grass N nutrition, the exact location of fixation, and composition of the associated microbial community. We examined ANF in switchgrass (*Panicum virgatum* L.), a North American perennial grass, using ^{15}N-enriched N_2 isotopic tracer additions in a combination of *in vitro*, greenhouse, and field experiments to estimate how much N is assimilated, where fixation takes place, and the likely N-fixing taxa present. Using *in vitro* incubations, we documented fixation in root-free rhizosphere soil and on root surfaces, with average rates of 3.8 μg N g root^{-1} d^{-1} on roots and 0.81 μg N g soil^{-1} d^{-1} in soil. In greenhouse transplants, N fixation occurred only in the early growing season, but in the field, fixation was irregularly detectable throughout the 3-month growing season. Soil, leaves, stems, and roots all contained diazotrophs and incorporated fixed N_2. Metagenomic analysis suggested that microbial communities were distinct among tissue types and influenced by N fertilizer application. A diverse array of microbes inhabiting the rhizosphere, and possibly aboveground tissues, appear to be episodically contributing fixed N to switchgrass.

1. Introduction

Biological N fixation has been well-documented in leguminous and actinorhizal plants and also occurs in non-nodulating species (Baldani et al., 1997; Santi et al., 2013). In grasses, fixation occurs via associations with endophytic or rhizosphere bacteria (Van Dommelen and Vanderleyden, 2007). This type of fixation, known as associative nitrogen fixation (ANF; (Bottomley and Myrold, 2015)), can account for up to 60% of sugar cane's annual N requirements (Herridge et al., 2008; Urquiaga et al., 2011). ANF has been primarily studied in tropical species of agricultural importance, but it can also occur in temperate grasses and trees (Morris et al., 1985; Bormann et al., 1993; Dalton et al., 2004), where mesocosm studies have inferred ANF contributions of up to 50 kg N ha^{-1} yr^{-1} (Chapman et al., 1949; Bormann et al., 1993).

Many details of ANF remain unclear, however, leading some to challenge the magnitude of ANF's contributions to overall plant N nutrition (Binkley et al., 2000; James, 2000). A first source of uncertainty is the indirect manner in which most N fixation estimates have been derived. Studies of ANF in temperate systems have employed a mass balance (budget) approach (Chapman et al., 1949; Bormann et al., 1993), microcosm experiments from a single sampling time (Tjepkema and Burris, 1976; Morris et al., 1985; Brejda et al., 1993; Dalton et al., 2004) or inferred rates from nitrogenase activity (Tjepkema and Burris, 1976; Brejda et al., 1993; Dalton et al., 2004). While these approaches have provided evidence for ANF, their rate estimates are challenged by large uncertainties due in part to assumptions associated with methodological limitations (Baptista et al., 2014; Roper and Gupta, 2016).

In addition, most ANF studies lack associated information about microbial N-fixers; in almost all cases N-fixing taxa were identified in

* Corresponding author. School of the Environment, Washington State University, Richland, WA, 99354, USA
E-mail address: sarah.roley@wsu.edu (S.S. Roley).

https://doi.org/10.1016/j.soilbio.2018.11.006
Received 5 July 2018; Received in revised form 8 October 2018; Accepted 6 November 2018
Available online 10 November 2018

the absence of rate estimates or fixation was measured without associated microbial information. As a result, important ecological information is missing. It's unclear which microbial taxa are present when fixation occurs or even where in the plant or rhizosphere fixation occurs. In sugarcane, fixation occurs by bacteria in rhizosphere soil (Dobereiner, 1961) and by bacterial endophytes (e.g., Boddey, 1987; James, 2000). ANF is generally assumed to occur in roots and rhizomes, with N subsequently transferred to aboveground tissues (Depolli et al., 1977; Eskew et al., 1981), but diazotrophs have been found throughout many plants (Davis et al., 2010; Ker et al., 2014; Moyes et al., 2016), leading to the potential for N fixation's occurring in aboveground tissues, as well.

Thus, although ANF has been documented in some perennial grasses, the location of fixation, the amount of fixed N transferred directly to plant tissues (as opposed to cycling through soils and the microbial loop; James, 2000), and the identity of N-fixing microbes remain largely unknown. We examined these unknowns in switchgrass (*Panicum virgatum* L.), a temperate-region prairie grass that is thought to rely in part on ANF for its N nutrition. Switchgrass is a dominant native species in North American tallgrass prairie and savannas (Casler, 2012), a forage crop, and a leading candidate feedstock for the production of cellulosic bioenergy and bioproducts (Mitchell et al., 2012), making it an excellent model species for studying ANF in temperate grasses. Its productivity can be non-responsive to N fertilizer addition, even when the grass is harvested annually (Parrish and Fike, 2005; Ruan et al., 2016; Fike et al., 2017; Roley et al., 2018). In addition, diazotrophic endophytes have been found within switchgrass tissues (Ker et al., 2014; Xu et al., 2018), and laboratory studies have found evidence for N fixation (Tjepkema and Burris, 1976; Morris et al., 1985; Roley et al., 2018).

If N fixation is significant to the N nutrition of switchgrass and similar temperate grasses, there are important consequences for the sustainability of perennial grass cropping systems (Robertson et al., 2017). Perennial grasses are typically fertilized at lower rates than row crops, but even these N fertilizer additions result in greenhouse gas emissions and N leaching (Oates et al., 2016; Ruan et al., 2016). If switchgrass can fix N at agronomically significant rates, it would have lower N fertilizer requirements than current recommendations suggest, reducing the energy inputs and perhaps the greenhouse gas emissions and water quality impacts of bioenergy crop production.

Using a combination of *in vitro*, greenhouse, and field experiments, we characterized the seasonal pattern of ANF in switchgrass so as to estimate annual N inputs from fixation. We also characterized the microbe-plant relationship that underlies ANF by determining the location of fixation and identifying the associated microbes. In doing so, we tested the hypotheses that 1) switchgrass augments its N supply with associatively fixed N, and 2) fixation occurs mainly in rhizosphere soils and root tissues.

2. Materials and methods

2.1. Site description

We conducted measurements in unfertilized stands of switchgrass (Cave-in-rock variety) established in 2008 at the Great Lakes Bioenergy Research Center, part of Kellogg Biological Station's Long-Term Ecological Research (KBS LTER) site, in Hickory Corners, MI (42.39°N, −85.37°W). The unfertilized switchgrass is grown in two experiments: the Switchgrass Nitrogen Rate Experiment (Ruan et al., 2016), which has four replicate plots of unfertilized switchgrass (https://lter.kbs.msu.edu/maps/images/current-switchgrass-nitrogen-experiment.pdf), and the Biofuel Cropping System Experiment (BCSE) (Sanford et al., 2016), which has five replicate plots of unfertilized switchgrass (https://lter.kbs.msu.edu/maps/images/current-glbrc-kbs-bcse-map.pdf). Both experiments were seeded in 2008 and all plots have been harvested annually since 2010. Harvest occurs post-senescence, usually in October, when all aboveground biomass > 10 cm height is removed.

Soils at the study site are primarily Alfisol loams (Kalamazoo series Typic Hapludalfs) formed from glacial outwash (Robertson and Hamilton, 2015). On average, surface soils contained 1% soil organic matter as carbon and 0.1% total N. The pH was close to neutral (range from 6.3 to 7).

2.2. Experiment 1: location of fixation

To determine where fixation is occurring, we exposed individual plant parts to ^{15}N-enriched N_2 *in vitro*, within airtight vials. We collected switchgrass leaves, stems, roots, and soil in late June from each of the four unfertilized switchgrass plots in the Nitrogen Rate Experiment. We sampled with a 2-cm diameter push corer, and the samples included both rhizosphere and bulk soil. Hereafter, we refer to soil samples beneath switchgrass as “switchgrass soil,” with the understanding that it refers to the mixture of bulk and rhizosphere soil present beneath plants. We passed the switchgrass soil through a 2-mm sieve and removed all roots. The sieving process removed most, but not all, of the soil adhering to the roots. Soil in direct contact with roots is likely to have the highest fixation rates because of its proximity to C-rich root exudates, and so this separation process may have decreased fixation rates. However, the addition of glucose (see following paragraph) may compensate for the loss of these C-rich soils. We rinsed all visible soil from roots with unchlorinated groundwater and then divided the root samples; half received a surface-sterilization treatment of 1.5% sodium hypochlorite (Miyamoto et al., 2004) and the other half remained washed but not sterilized. This allowed us to separate the contributions of surface-dwelling microbes from root endophytes. Our sample collection thus provided five sample types: soil, surface-sterilized roots, washed roots, leaves, and stems.

Following the methods of Gupta et al. (2014), we placed each replicate sample into duplicate 12-mL Exetainer vials (Labco, Lampeter, Ceredigion, UK) with screw caps and rubber septa. We added 3 mL of 4% (w/v) glucose solution to each vial to eliminate C limitation, and thereby assess N fixation potential. From each we removed 4 mL of headspace air with a syringe and then added 4.5 mL of 98% ^{15}N:^{15}N gas (hereafter called $^{15}N_2$; Sigma-Aldrich, St. Louis, MO, USA) to one vial of each duplicate (treatment sample) and the same volume of unenriched N_2 to the other vial (control sample). We placed root and soil samples in the dark and leaf and stem samples near a window, to mimic ambient light conditions. After 10 days, we removed the caps, measured the headspace volume of each vial via water displacement, and then measured the wet and dry mass and δ^{15}N of the tissues.

We calculated the ^{15}N atom excess of the headspace (AE_{atm}) of each vial by dividing the volume of $^{15}N_2$ added by the total N_2 in the headspace. Our AE_{atm} calculations are probably slight overestimates, because they do not account for potential changes in N_2 concentration in the vial as a result of denitrification. To determine if fixation occurred, we used Welch's one-tailed *t*-test (95% confidence interval, $\alpha = 0.05$) to compare the ^{15}N content of the treatment and control tissues. For samples that were significantly ^{15}N enriched relative to the controls, we calculated the percent of tissue or soil N derived from N fixation (%Ndfa) according to Warembourg (1993):

$$\%Ndfa_i = \frac{AE_i}{AE_{atm}} \times 100 \quad (1)$$

where AE_i is the ^{15}N atom excess of tissue *i*, relative to its control (Warembourg, 1993). We calculated the N fixation rate of individual plant parts or soil as:

$$\frac{(AE_i \times TN_i)}{(AE_{atm} \times t)} \quad (2)$$

(Warembourg, 1993), where t is the length of the incubation in days, and TN is total nitrogen content of tissue *i*, in g. We then divided by the dry mass of the sample to express fixation as μg N g^{-1} d^{-1}.

2.3. Experiment 2: whole-plant fixation in greenhouse mesocosms

To determine if fixed N was taken up and incorporated into plant tissues, we incubated whole plants with $^{15}N_2$ in the greenhouse. On each of two dates (27 May and 1 July 2016), we collected four plants with surrounding soil from the replicated unfertilized plots. In May, the plants were in the tiller stage and in July, they were in the stem elongation stage. The incubations included plants from replicates 2–5 of the BCSE. For controls, we collected an additional plant from each of two unfertilized plots. We placed the plants in large nursery pots (29 cm diameter x 25 cm height) lined with pea gravel and placed all plants in the greenhouse. The greenhouse temperature was between 24 and 26 °C for both incubations. Soil moisture varied between incubations but was in the range appropriate for plant growth; during the May (tiller stage) incubation, average post-incubation soil gravimetric moisture was 17% ± 2% SE, and during the July incubation (stem elongation stage), gravimetric soil moisture averaged 22% ± 1% SE.

After a 2–5 day acclimation period, we placed four polyethylene gas delivery tubes and a nylon gas sampling tube into the root zone of each of the pots. Each gas delivery tube was perforated along 6 cm from a sealed end and was inserted into the soil at a unique angle (i.e., each tube occupied its own section of soil) to deliver gas to the full soil pore space. The gas sampling tube was placed into soil midway from the bottom of the pot. We added another gas sampling tube to the headspace (Fig. 1).

We then enclosed each pot in a large nylon bag (True Liberty, Windsor, CA, USA) and sealed the bag below the pot. As an additional protection against gas loss and exchange with the outside air, we placed the bag opening under water and also added water to the inside of the bag to a level just below the top of the pea gravel (Fig. 1). Gas diffuses much more slowly through water than through air, and preliminary tests found that a tracer gas (methane, CH_4) remained in the bag with no measurable leakage over 2 days.

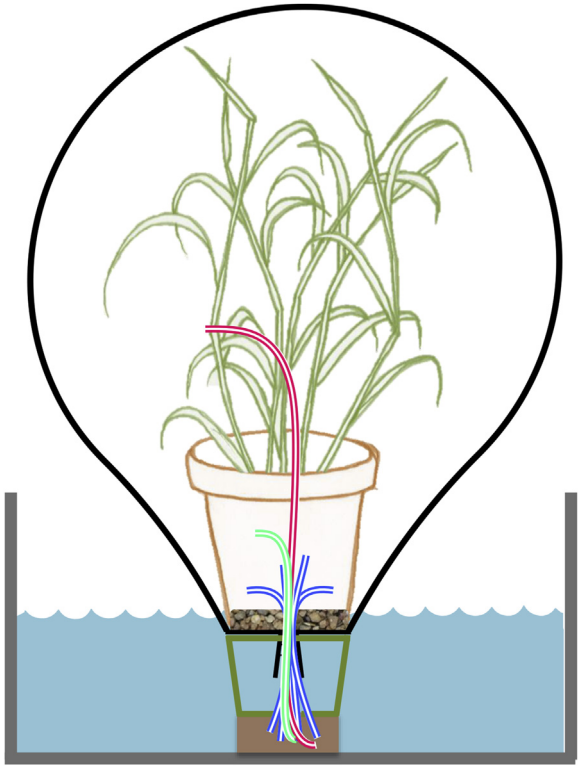


Fig. 1. Experimental design to expose a whole switchgrass plant to $^{15}N_2$ using an airtight bag in the greenhouse. The magenta tube was used to sample the headspace, the green tube was used to sample gas in the soil, and the blue tubes were used to add the $^{15}N_2$ gas mixture. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

After sealing each bag, we added 1450 mL of a mixture of 78% N_2 + 22% O_2, with treatment plants receiving $^{15}N_2$ and control plants receiving unenriched ambient N_2. We distributed the gas evenly among the 4 gas addition tubes per pot. We added gas by attaching a syringe to a tube, and keeping the tube opening and syringe under water while slowly pushing the gas mixture into the soil. We distributed the gas evenly, adding 362.5 mL to each tube.

We left the bags closed for 2 days, and over this period took gas samples six times from each of the two gas sampling ports (i.e., one headspace and one soil pore space sample). At each sampling time we attached a 5 mL syringe to the tubing underwater, removed and discarded 2 mL of tubing dead space air, and then removed 4 mL of sample and injected it into a 6-mL Exetainer (Labco, Lampeter, UK) containing air. All tubing, syringes, and vials were kept underwater for the entire sampling period to avoid contamination with ambient air.

The first sample (T0) was taken immediately before gas addition, T1 at 1–2 h after gas addition, T2 at 3–6 h, T3 at 8–10 h, T4 at 23–25 h, T5 at 32–34 h, and T5 was taken immediately before taking down the experiment (48 h). We took six replicate samples at each sampling event from both the soil and the headspace, with three replicates stored upside down in a centrifuge tube of water for later analysis of $^{15}N_2$, and three replicates analyzed for CO_2 immediately on a GC equipped with a thermal conductivity detector (TCD) (Shimadzu GC14A, Columbia, MD, USA). If the CO_2 dropped below 400 ppmv, we added CO_2 of sufficient quantity to bring the concentration to 400 ppmv based on the headspace volume. For most replicates, we only needed to add CO_2 once, during the first day. After that, soil respiration was sufficient to keep CO_2 above 400 ppmv.

We determined headspace volume by adding 100 mL of 10% CH_4 balanced with argon to each bag at T0. After 1 h, we removed replicate 5-mL samples of headspace and measured its CH_4 concentration on a Shimadzu GC14A gas chromatograph (Columbia, MD, USA) equipped with a flame ionization detector. We calculated the headspace volume from the dilution of added CH_4 within the headspace, assuming any uptake by soil methanotrophs is undetectable relative to the CH_4 concentration and the incubation time.

At the end of the incubation period we opened the bags and immediately separated the plants into leaves, stems, and roots. We passed all soil through a 4-mm sieve and picked out all visible roots, then washed the roots over a 1-mm sieve to remove soil. We measured the total fresh and dry weight of each plant. We compared the ^{15}N enrichment of the plants to two control plants that had each been incubated in the same way, but with ambient (unenriched) N_2.

We calculated %Ndfa and fixation as in equations [1] and [2]. In addition, we used weighted atom excess (WAE) to calculate the fixation rate of the whole plant (Warembourg, 1993):

$$WAE = \frac{[(AE_{leaves} \times TN_{leaves}) + (AE_{stems} \times TN_{stems}) + (AE_{roots} \times TN_{roots})]}{TN_{leaves+stems+roots}} \quad (3)$$

We used the WAE values to calculate whole plant fixation as:

$$Whole\ plant\ fixation = \frac{(WAE \times TN_{plant})}{(AE_{atm} \times t)} \quad (4)$$

which has units of mg N plant^{-1} d^{-1} (Warembourg, 1993). Because weights varied greatly among replicates, we also divided by total plant weight to express fixation in mg N g plant^{-1} d^{-1}.

2.4. Experiment 3: N fixation in situ

To document ANF in the field, we added $^{15}N_2$ and the conservative tracer sulfur hexafluoride (SF_6) to the root zones of individual switchgrass plants *in situ*. We completed numerous additions, spanning two

growing seasons. In 2013, we added $^{15}N_2$ to the root zone beneath two plants, with the first receiving $^{15}N_2$ in mid-June, during the stem elongation stage, and the second in early July, as switchgrass was entering the boot stage. In 2015, we included additional replicates and added $^{15}N_2$ gas during the tiller stage (n = 3), the stem elongation stage (n = 4), and at peak biomass, when the switchgrass had completed flowering and was setting seeds (n = 3). All switchgrass stages were determined according to Sanderson (1992).

To add and sample gases in the soil atmosphere, we installed three gas samplers and one gas injector beneath individual switchgrass plants. The gas injector consisted of a piece of 1.6 mm ID x 3 mm OD polyethylene tubing, perforated with small (1-mm) holes along the bottom 2.5 cm from a sealed end. The sealed end forced the gas to diffuse evenly through the perforations and thereby spread more evenly through the soil. The other end of the gas injector was attached to a Swagelok (Swagelok Company, Solon, OH, USA) fitting. Gas samplers consisted of a piece of 1.6 mm ID x 3 mm OD polyethylene tubing, open at one end, with the other end attached to a rubber septum.

To install the samplers and injector, we first pushed a solid aluminum rod (3 mm diameter) into the ground at a 45° angle to create a pilot hole. We then inserted the sampler or injector by pushing the tube into the pilot hole after first placing a metal wire within the tube to prevent it from clogging with soil. Once the tubing was in place, we removed the metal wire and replaced the rubber septum (samplers) or added a Swagelok fitting (injector). Finally, we sealed the soil surface around the tube with bentonite clay to inhibit gas diffusion through preferential flow up the side of the tubing. We placed the injector so that the bottom was directly under the plant, 10 cm deep. We aimed to enrich a 20-cm sphere of the rhizosphere and placed the three gas samplers on the edges of this sphere at three different depths: 10 cm deep at 10 lateral cm from the injection point, 18 cm deep directly below the injection point, and 5 cm deep at 10 lateral cm from the injection point (Fig. 2).

To add the gas, we attached the gas injector to a tube that received a mixture of diluted SF_6 and $^{15}N_2$ gas (final concentration was > 99% N_2). The SF_6 was used as a conservative tracer and a first check that a measurable amount of gas remained in the rhizosphere; if SF_6 was present, then we proceeded with plant and gas isotope analyses. The gases were mixed in a gas-tight bag (Zefon International, Ocala, FL, USA) and were drawn from the bag with a small battery-operated pump. A flow controller downstream of the pump kept flow rates at 5 mL min^{-1}. We added the gas for 4–15 h and left all systems in place for at least 2 h after shutting off the pump, to allow the gas remaining in the tubing to diffuse into the soil. The flow rate and incubation times were chosen as a balance between sufficient $^{15}N_2$ rhizosphere enrichment and conservation of $^{15}N_2$; lower flow rates and minimal exposure times require less $^{15}N_2$, which allows for more replicates but at the expense of raising the detection limit.

To check the degree of rhizosphere enrichment, we took gas samples from all gas sampling tubes prior to adding the gas, and periodically throughout the addition by piercing the rubber septum with a needle attached to a syringe. We discarded the first 2 mL as tubing dead space and then removed 20 mL for analysis. We used the SF_6 as a conservative tracer to ensure that the $^{15}N_2$ gas reached the rhizosphere, and analyzed those samples within a week. We stored the $^{15}N_2$ samples upside down in water-filled centrifuge tubes until analysis (details below), which occurred within 2 months of collection (previous tests have shown that samples are stable over that time period: (Hamilton and Ostrom, 2007)). Prior to and immediately after adding the gas, we took leaf samples from the treatment ($^{15}N_2$ addition) and control plant (no gas addition). We sampled the leaves by removing whole, actively-growing leaves with scissors, and each sample consisted of three whole leaves. If flowers had started to form, we also sampled flowers of the control and treatment plants. After the gas addition, we continued to sample leaves of the treatment and control plants for at least 1 week. We report here results from the first sampling time only, because there was no substantive change in the tissue ^{15}N concentrations during the week that followed. In 2015, we also took switchgrass soil, root, and whole-plant samples. The switchgrass soil and root samples were taken at least 2 h after the end of the gas addition by pushing a soil corer (2 cm diameter) directly beneath the plant to a depth of 20 cm. We took three samples from each plant, separated roots from soils, and analyzed them for ^{15}N content. After 1 week, we also harvested the whole plant by clipping stems at the soil surface.

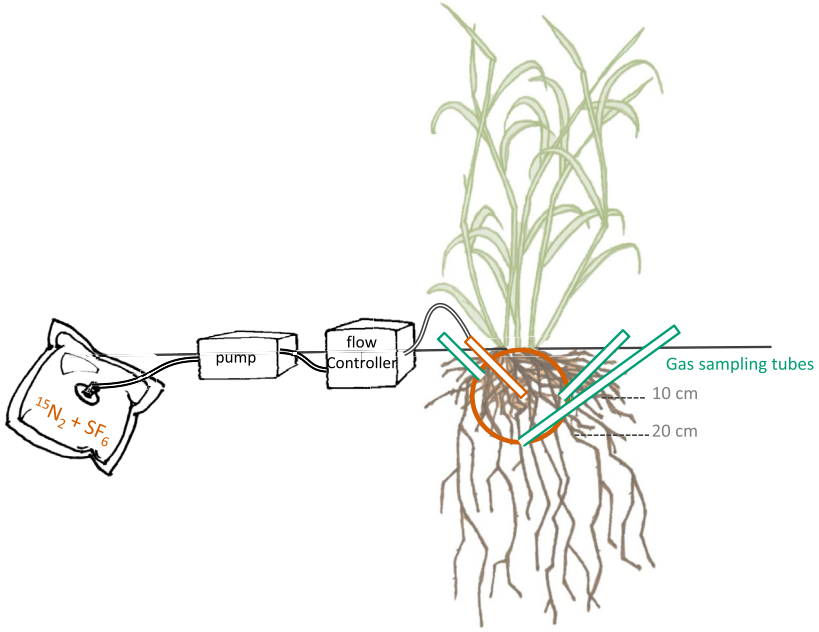


Fig. 2. Experimental design for *in situ* additions of $^{15}N_2$. The orange tube is the gas injection tube, which was perforated 2.5 cm from the end. The blue-green tubes are gas sampling tubes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Temperatures varied within and among incubations according to field conditions. On average, soil temperature was 19 °C at the start of the incubation and 21 °C at the end, while air temperature averaged 23 °C at the start and 24 °C at the end. Gravimetric soil moisture was relatively consistent, ranging from 14 to 17%, with a mean of 15%.

2.5. ^{15}N analyses

All soil and plant samples were dried for at least 1 week at 60 °C and ground. Small samples were pulverized in a SPEX Shatterbox (Metuchen, NJ, USA), while large samples (i.e., chamber samples) were first ground in a Christy Turner Mill (Christy Turner Ltd, Ipswich, Suffolk, England) with a 1-mm filter and then sub-samples were further pulverized in the Shatterbox. All solid samples were packed in tin capsules and analyzed at the UC Davis Stable Isotope Laboratory (Davis, CA, USA).

We analyzed the $^{15}N_2$ content with a HP 5890 gas chromatograph (Palo Alto, CA, USA) with a modified inlet system (Bergsma et al., 2001) connected to an Elementar Isoprime mass spectrometer (Mt. Laurel, NJ, USA). Using a gas-tight syringe, we injected 100 µL of sample into the modified inlet system.

For the greenhouse and *in situ* experiments, we estimated detection limits. First, we calculated the minimum $\delta^{15}N$ value for detection of N_2 fixation as the mean of the control $\delta^{15}N$ value plus two standard deviations (Boddey, 1987). Next, we calculated the equivalent N fixation rate corresponding to the detection limit, in µg N g^{-1} d^{-1}, based on average plant weights, $^{15}N_2$ concentration, and incubation time in each experiment. The field experiment detection limits are approximate, because the $^{15}N_2$ enrichment of the soil atmosphere throughout the root zone is indeterminate, but it allows us to compare among our field experiments where differences in incubation time and plant size changed the minimum detectable N fixation rate.

Commercially-available $^{15}N_2$ can be contaminated with other gaseous forms of reactive ^{15}N, including ^{15}NOx, which readily oxidizes to $^{15}NO_3^-$ when dissolved in water, and $^{15}NH_3$ (Dabundo et al., 2014). These contaminants may be taken up by plants and microbes, which can result in ^{15}N enrichment of plants and soils in the absence of fixation and thus inflate N fixation estimates or result in false positives. We addressed this issue in several ways. For experiments that occurred before we were aware of potential contamination, we calculated the maximum effect of potential contamination, based on levels reported by Dabundo et al. (2014), and adjusted rates accordingly. For other experiments, we tested our $^{15}N_2$ gas for contamination and purified it if necessary.

For the 2013 field experiment and the initial greenhouse experiment, we assumed contamination levels as high as those reported by Dabundo et al. (2014) for the same lot numbers as ours. If the gas used was not of the same lot numbers reported in that paper, then we applied the average contamination level for the gas source. To make our N fixation estimates conservative, we assumed that all potential $^{15}NH_3$ and ^{15}NOx contaminants were taken up by the plant tissues and soil and subtracted that from the measured values. If potential contamination was equal to or exceeded our measured rates, we excluded the results from this paper. In the *in vitro* experiment, we report the contamination-corrected values (Sigma-Aldrich lot #SZ1670V). For the field $^{15}N_2$ additions (Cambridge Isotope Labs, lot #I-17229), our calculations revealed that the potential effect on plant $\delta^{15}N$ content would be < 0.0001‰, even if all gas added to the rhizosphere remained there. Those levels are well below our detection limits.

For later experiments, we first scrubbed the gas of ^{15}NOx by pumping the $^{15}N_2$ through glass tubing packed with Purafil SP (Doraville, GA, USA). We then passed the scrubbed $^{15}N_2$ gas through ultrapure water prior to injecting it into the soil (2015 field experiments) or into greenhouse enclosures. The water was approximately the same pH as the water in the soil, and thus would capture NOx and NH_3 contaminants in the same way as would soil. We then measured the NH_3 and NO_3^- concentration in the water compared to water that was exposed to lab air. There was no difference between the water exposed to $^{15}N_2$ and lab air and so we conclude that contamination was not an issue in those experiments. Furthermore, in one of the greenhouse experiments, fixation was below detection. If our $^{15}N_2$ was contaminated with reactive ^{15}N, we would have observed enrichment in all samples.

2.6. Microbial analyses

For each experiment, we took subsamples of all fresh plant and soil material for analysis of the microbial community. In addition, to determine the effect of N availability on microbial communities, we obtained samples from plots fertilized at 196 kg N ha^{-1} yr^{-1} in the Nitrogen Rate Experiment. We collected these samples at the same time as the samples from the unfertilized switchgrass incubated *in situ* with $^{15}N_2$.

All samples were refrigerated immediately after collection, and DNA was extracted within 3 days. To avoid contamination on plant surfaces, plant tissue samples were surface-sterilized following Miyamoto et al. (2004). Briefly, the whole plant was carefully washed with tap water and cut into leaf, stem, and root portions of ~2 cm length. Plant leaves were placed in 1% NaOCl for 0.5 min and stems and roots were placed in 2% NaOCl for 15 min, after which the plant parts were washed with sterilized water seven times and ground with liquid N_2 in mortar and pestle. Three grams of ground plant parts were used for DNA extraction using Mobio PowerMax soil DNA isolation kit.

NifH genes were amplified based on the PolF (TGCGAYCCSAARGCBGACTC) and PolR (ATSGCCATCATYTCRCCGGA) primer set with adaptors for Ion Torrent sequencing at the beginning followed by an 8 bp barcode to separate samples (Poly et al., 2001). Amplification and purification were conducted according to previously published protocols (Zhang et al., 2015). Sequence processing was conducted using FunGene Pipeline (Fish et al., 2013) (http://fungene.cme.msu.edu//index.spr). Sequences were barcode sorted and filtered to remove short and low quality reads. All sequences were passed through Framebot (Wang et al., 2013) to correct sequencing errors and translate reads into amino acid sequences. Taxonomy was assigned as the nearest neighbor (in GenBank) using Framebot. These sequences were aligned and clustered at 95% similarity. Raw abundances of clusters were normalized by Hellinger transformation (square root of relative abundance) and non-metric multidimensional scaling (NMDS) was performed to illustrate the beta-diversity between individual samples (Bray-Curtis distances between samples). Permutational multivariate analyses of variance (PERMANOVA) (Anderson, 2001) was performed to determine the significance of community composition differences between treatments. The OTU presence in each treatment was calculated when the OTU was present in at least three replicates of the treatment. All sequences were deposited in the NCBI Sequence Read Archive (SRA) database (Accession numbers: SRP137777).

3. Results

3.1. Experiment 1: location of fixation

In individual tissues exposed to $^{15}N_2$ *in vitro*, we found significant ^{15}N enrichment, relative to controls, in field-collected roots and switchgrass soil after exposure (Welch's one-tailed *t*-test, $t = -3.9$, $p = 0.015$; $t = -7.6$, $p = 0.002$, respectively), but not in stems, surface-sterilized roots, or leaves (Table 1). The mean potential fixation rates were 3.2 µg N g^{-1} plant d^{-1} ± 1.0 SE in washed roots and 0.81 µg N g^{-1} dry soil d^{-1} ± 0.2 SE in soils (Fig. 3). These values scale to 22.4 g N ha^{-1} d^{-1} ± 2.2 SE in roots (assuming root density of 700 g m^{-2} in the top 15 cm, as measured previously at the site) and 1.79 kg N ha^{-1} d^{-1} ± 0.1 SE potentially fixed in soils (assuming soil bulk density of 1.47 g cm^{-3}, as measured previously at the site).

Table 1
Nitrogen isotopic composition of switchgrass plant tissues and switchgrass soil exposed to $^{15}N_2$ in greenhouse experiments.

Timing	Tissue	$\delta^{15}N$ (‰)[a]	Minimum $\delta^{15}N$ (‰) for detection	# reps above detection	Minimum detectable rate (ug N g^{-1} d^{-1})	Mean rate (ug N g^{-1} d^{-1})[b]	Mean %N from fixation
2016 – tiller stage	Leaves	2.2 (0.3)	1.2	4/4	2.51	4.88 (0.73)	0.0004
	Roots	2.3 (0.1)	1.2	4/4	0.10	0.68 (0.06)	0.0002
	Stem	1.8 (0.1)	1.3	4/4	0.86	1.66 (0.14)	0.0002
	Soil	5.1 (0.3)	6.1	0/4	0.10	NA	NA
	Whole plant	2.2	1.1	4/4	0.22	0.99 (0.06)	0.0003
2016 – stem elongation phase	Leaves	0.5 (0.4)	1.9	0/4	1.20	NA	NA
	Roots	3.0 (0.6)	2.8	1/4	0.38	1.467	0.0006
	Stem	0.8 (0.2)	2.8	0/4	0.35	NA	NA
	Soil	5.2 (0.3)	6.3	0/4	0.10	NA	NA
	Whole plant	2.0	1.9	1/4	0.03	0.55	0.0002

[a] Mean (SE).
[b] Mean rate (SE) of samples that were above detection. No SE is reported if insufficient replicates above detection.

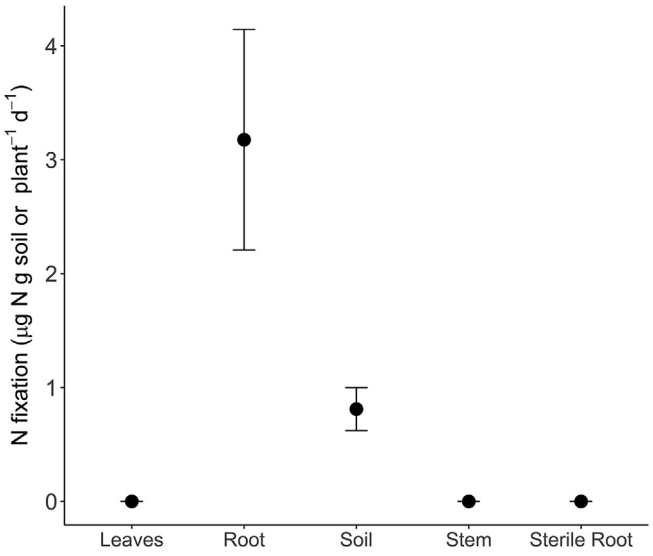


Fig. 3. Nitrogen fixation rates from *in vitro* experiments (mean ± SE), corrected for potential contamination.

3.2. Experiment 2: whole-plant incubations in greenhouse mesocosms

Incubation of whole plants showed that N fixation was above the detection limit for plants collected in the tiller stage, but not for those collected in the stem elongation stage (Table 1). In the tiller stage, all plant tissues (leaves, roots, and stem) became ^{15}N-enriched, but not the soils. These enrichments corresponded to an average of 0.0003% of total plant N coming from fixation during the incubation period. The average whole-plant fixation rate was 0.99 μg N g^{-1} d^{-1} ± 0.06 SE (Table 1), which is within the same range as the surface-sterilized roots and soils in the vial assays. In contrast to the vial assays, the highest fixation rate in the whole-plant assays was measured in the leaves, with a mean of 4.88 μg N g^{-1} d^{-1} ± 0.73 SE, although we note that the leaf ^{15}N content was potentially augmented with N fixed in and transported from the roots. For plants collected during the stem elongation stage, fixation was above detection in just one plant, where the roots had a $\delta^{15}N$ value of 4.89‰ compared to a detection limit of 2.8‰ (Table 1).

3.3. Experiment 3: in-situ N fixation assays

We found sporadic evidence for fixation using *in situ* incubations with $^{15}N_2$. Plants sampled at every phenological stage had one replicate above detection, but a majority of replicates were always below detection limits (Table 2). Detection limits were generally higher for the *in situ* incubations than for the other experiments.

3.4. Microbial community

We successfully amplified *nifH* gene fragments in switchgrass soils, roots, leaves, and stems. Their sequences indicated a large diversity of N-fixing microbes in all four compartments. In general, there were more OTUs detected in soils (363 in the greenhouse experiment; 403 *in vitro*) compared to roots (251 in greenhouse; 69 *in vitro*), stems (144 in greenhouse; 73 *in vitro*), and leaves (111 in greenhouse; 104 *in vitro*). In both the greenhouse and *in vitro* experiments, all samples were generally grouped by tissue type, although the community difference between leaf and stem was not significant due to large variation among replicates (PERMANOVA, $p > 0.05$, Fig. 4). In comparing samples collected directly from fertilized and unfertilized field plots, the samples grouped first by tissue, but then fertilizer addition influenced microbial community composition in all four tissue types (Fig. 5). Shannon diversity was not different among tissues or by fertilizer addition. The nearest neighbors of the dominant sequences in the greenhouse, field, and *in vitro* experiments included *Hyphomicrobium* spp., *Bradyrhizobium* spp, *Geobacter uraniireducens*, and *G. bemidjiensis*. Additional common sequences were related to those of *Polaromonas naphthalenivorans* in the greenhouse and field, *Methylocella silvestris* in the greenhouse experiment, *Gluconacetobacter diazotrophicus* in the field, and *Dickeya dadantii* in roots incubated *in vitro* (Supplemental Figs. 1–3).

4. Discussion

Nitrogen fixation was detected consistently in tissue- and soil-specific *in vitro* (laboratory) incubations, under greenhouse conditions for whole plants collected at the tiller stage, and sporadically *in situ*. All switchgrass tissues and switchgrass soil contained nitrogenase genes whose sequences corresponded to a diverse array of diazotrophs. Measurable N fixation rates in whole-plant (greenhouse) incubations were within the range measured *in vitro* (Fig. 1, Table 1).

4.1. Timing of fixation

The episodic occurrence of fixation is likely a real characteristic of the system, but the alternative explanation is that the episodic observations are driven by Type I statistical error (false positives). False positives seem unlikely because other lines of evidence (mass balance and lab studies, Roley et al., 2018) show fixation is occurring at some level. When fixation was below detection, it's impossible to know if fixation was non-existent or occurring at a non-detectable rate. Regardless of the actual value of below-detection measurements, episodic is still an apt descriptor; these fixation episodes originate from either a baseline of 0 or a small positive number.

Many biogeochemical processes occur during highly episodic events, times during which a disproportionate amount of the

Table 2
Nitrogen isotopic composition of switchgrass plant tissues and switchgrass soil after field additions of $^{15}N_2$ gas.

Timing	Phenology	Tissue	$\delta^{15}N$ (‰)[a]	Minimum $\delta^{15}N$ (‰) for detection	# reps above detection	Minimum detectable rate (ug N g^{-1} d^{-1})
2013 June	Stem elongation	leaves	1.54	0.48	1/1	9.30
2013 July	boot	leaves	1.19	0.28	1/1	8.87
2013 July	boot	flowers	1.19	−0.01	1/1	20.31
2015 June	tiller	whole plant	0.48 (0.14)	0.76	1/3	3.27
2015 June	tiller	leaves	1.18 (0.36)	1.20	1/3	11.66
2015 June	tiller	roots	1.10 (0.43)	1.10	1/3	3.61
2015 July	Stem elongation	whole plant	0.38 (0.17)	0.55	1/4	2.38
2015 July	Stem elongation	leaves	0.11 (0.58)	0.90	1/4	5.91
2015 July	Stem elongation	roots	0.41 (0.27)	1.10	1/4	2.79
2015 July	Stem elongation	soil	4.01 (0.27)	4.39	1/4	NA
2015 Aug	flowering	flowers	0.77 (0.33)	0.75	1/3	5.08
2015 Aug	flowering	roots	0.47 (0.32)	1.20	0/3	1.05
2015 Aug	flowering	soil	4.66 (0.19)	4.39	1/3	NA
2015 Aug	flowering	whole plant	−0.17 (0.23)	0.02	1/3	0.89

[a] Mean (SE).

transformation occurs (McClain et al., 2003). These ecosystem control points are often characterized by a single missing component or trigger condition, and once that condition is met, transformations proceed rapidly (Bernhardt et al., 2017). Associative N fixation may be similarly episodic, such that microbes only fix N when, for example, they have sufficient access to energy and key nutrients (e.g., iron (Fe), molybdenum (Mo), phosphorus, or vanadium (V)) or when soil moisture is sufficient. In our *in situ* measurements, fixation was sporadic in both space and time (i.e., detectable during all time points, but in different plots), suggesting that optimal fixation conditions can occur during all phenological stages but that such conditions are not consistently present.

One potential driver of the observed temporal variation could be soil wetting and drying. Soil wetting events often stimulate biogeochemical processes, such as denitrification and methanotrophy, because of their effects on nutrient and oxygen availability and the reduction and oxidation of key substrates (Kim et al., 2012). Nitrogen fixation may also respond positively to wetting events because the resultant decrease in oxygen tension will make the nitrogenase enzyme less vulnerable to deactivation (Postgate, 1998). In addition, post-wetting leaching, re-mineralization, and redox reactions may make key nutrients more available.

Our closely situated plots experienced the same precipitation regime, so variation in wetting events likely do not explain the observed spatial variation. Spatial variation seems more likely due to small-scale differences in soil water-holding capacity or availability of key nutrients (e.g., Mo and V enzymatic co-factors). Alternatively or additionally, observed spatial variation may be due to an inability to detect all but the highest rates of fixation.

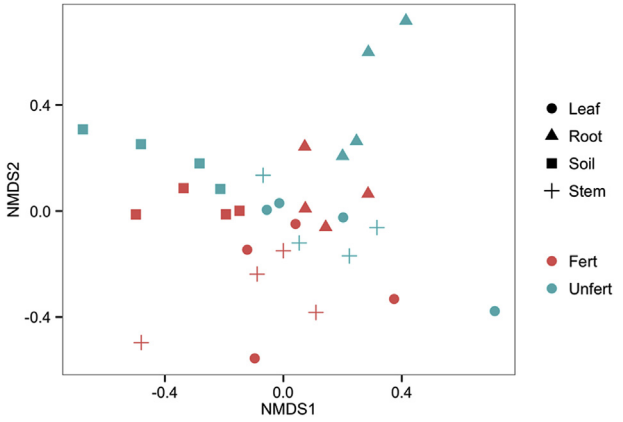


Fig. 5. Non-metric multidimensional scaling (NMDS) comparison of the microbial communities in switchgrass tissues and soils that were unfertilized (Unfert) or fertilized at 196 kg N ha^{-1} yr^{-1} (Fert).

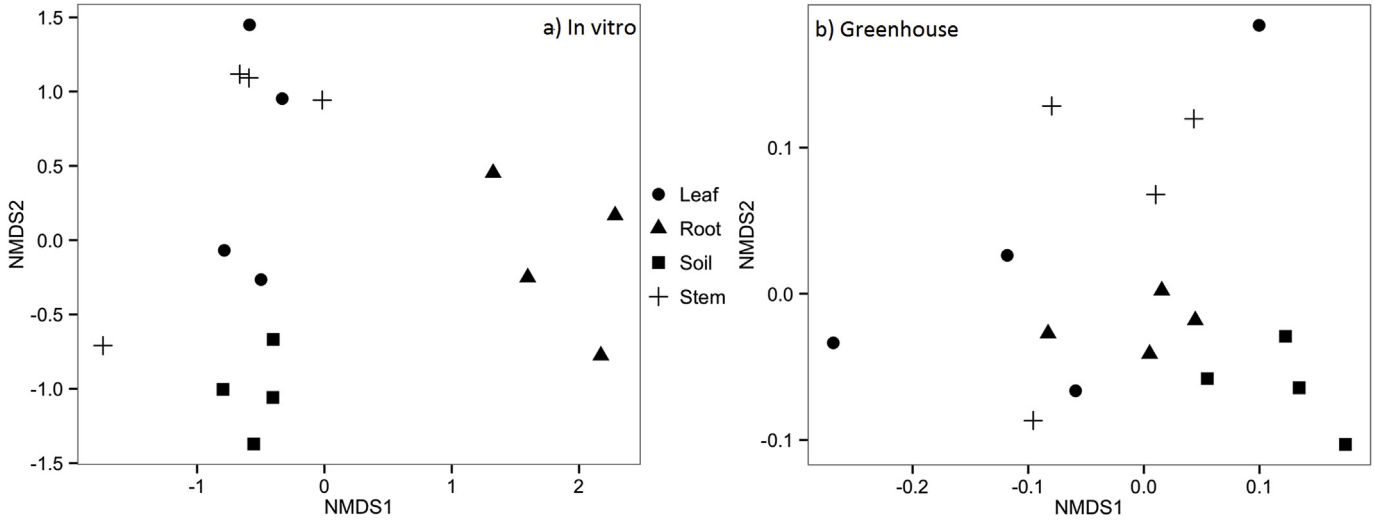


Fig. 4. Non-metric multidimensional scaling (NMDS) comparison of the microbial communities in switchgrass tissues and soils from a) the *in vitro* laboratory experiment and b) the whole-plant experiment in the greenhouse.

4.2. Microbial community

Switchgrass promotes microbial biomass compared to annual monocultures and also harbors a distinct community of N-fixers (Jesus et al., 2016; Liang et al., 2016). It could thus be fostering a microbial community that augments its N needs through ANF. A study in Oklahoma, USA found that few diazotrophs are transcribing *nifH* at any given time (Bahulikar et al., 2014), which is consistent with our finding of episodic fixation; en masse transcription of *nifH* is likely to occur only when conditions are appropriate for high rates of fixation. Addition of N fertilizer decreases microbial biomass in switchgrass and prairie plots (Oates et al., 2016), and our results suggest that it also alters diazotroph community composition.

Some of the most abundant N-fixing taxa from our unfertilized plots overlapped with the most abundant taxa in switchgrass plots in Oklahoma, while others were distinct. *Bradyrhizobium* was one of the most abundant taxa at both sites, although in Oklahoma it was not transcribing *nifH* at sampling time (Bahulikar et al., 2014). *Bradyrhizobium* is classically known as a symbiont of soybean but it is now recognized to be common feature of many soil microbial communities, especially in Midwest tallgrass prairies (c.f. Wang et al., 2013; Mackelprang et al., 2018). *Hyphomicrobium*, *Geobacter*, *Polaromonas*, and *Methylocella* relatives were among the most abundant taxa at our site but were not present in any of the sequences in Oklahoma. Overall, N-fixing communities appear to be influenced by a combination of plant species, site-specific soil and climate conditions, and N availability.

4.3. Ecology of ANF

The ecology of fixation in non-nodulating plant-soil systems is not well-characterized. The term “free-living” refers to diazotrophs that do not have a symbiotic relationship with a host plant, even though they may occur within rhizosphere soils or plant tissues (Postgate, 1998; Reed et al., 2011). Associative fixation is a mutualistic relationship whereby both partners can live satisfactorily without the other (Postgate, 1998). The distinction between free-living and associative fixation is somewhat ambiguous, because it is hard to determine if N fixation is fueled by plant exudates and if the N fixed is provided directly to the plant or if it becomes available after cycling through the soil food web (James, 2000).

In our *in vitro* assays, fixation was detectable in soils and on root surfaces but not in leaves or stems. The lack of detectable *in vitro* fixation in aboveground tissues may have been due to experimental conditions: leaves and stems separated from the plant lack a source of mineral nutrients and water, stressing the plant and its endophytes. We found diazotrophs in aboveground tissues and others have found detectable fixation in leaves or stems (e.g., Boddey et al. (2003) for sugarcane and Moyes et al. (2016) for conifers). Further, plants in our greenhouse experiment sometimes accumulated fixed N in leaves and stems despite undetectable fixation in soil (Table 1). We conclude that fixation in aboveground tissues may be happening is worthy of further investigation.

4.4. Magnitude of inputs from N fixation

Temporal variability in the detection and estimated rates of N fixation prevents us from confidently scaling our fixation measurements to annual rates. But mass balance evidence from this site suggests that N fixation is an important part of the switchgrass N economy: N removed from unfertilized plots as harvested biomass exceeds atmospheric deposition inputs by $> 50\,kg\,N\,ha^{-1}\,yr^{-1}$ in the post-establishment period (Roley et al., 2018). This phenomenon is not restricted to our site; switchgrass grown in New York, USA and Oklahoma, USA have also exhibited a net N deficit with no yield penalty (Fike et al., 2017). Here, we show that N fixation is sometimes detectable on a whole-plant scale, meaning that if N fixation is responsible for the N deficits, then it likely does so in patchy, sporadic bursts of activity, perhaps only as environmental conditions warrant.

Perennial grasses can also obtain N from soil organic matter as it decomposes, and this could help account for the N imbalance. At our study site, however, soil N content has not declined over the course of the experiment (Ruan et al., 2016; Valdez et al., 2017), a pattern generally observed in non-tilled agricultural ecosystems (West and Post, 2002). We thus conclude that soil organic N is a minor N source to switchgrass at our site and does not fully explain the observed negative mass balances.

In temperate grasslands, annual ANF inputs have been estimated from scaled lab measurements and from mass balances. There is a wide discrepancy between the two techniques, with mass balance results estimating $34\,kg\,ha^{-1}\,yr^{-1}$ beneath native grasses in Texas, USA (Smith et al., 1954) and $54\,kg\,N\,ha^{-1}\,yr^{-1}$ in barley and Sudan grass grown in lysimeters (Chapman et al., 1949). Similarly, in sugar cane, a combination of mass balance and natural abundance isotope data estimated $> 40\,kg\,N\,ha^{-1}yr^{-1}$ (Urquiaga et al., 2011). In contrast, laboratory measurements scaled to annual inputs have reported $< 4\,kg\,N\,ha^{-1}\,yr^{-1}$ (Tjepkema and Burris, 1976; Morris et al., 1985). Both techniques have limitations, but our observations can perhaps reconcile these differences: we hypothesize that N fixation rates beneath switchgrass are low most of the time, but occasionally high enough to make up for N deficits on an annual scale.

5. Conclusion

We found support for both of our hypotheses, although not always in expected ways. Switchgrass augments its N requirements with fixed N, as we predicted, but it does so episodically, perhaps in response to transiently suitable environmental conditions coinciding with appropriate N-fixing populations. Consistent with our second hypothesis, we found that fixation occurs in switchgrass soils and on root surfaces, but we cannot rule out fixation in other tissues. A diverse N-fixing community inhabits switchgrass soils, roots, stems, and leaves, and the composition of that community varies with N availability and location (in or around the plant). The magnitude of inputs from ANF has long been a puzzle and we suggest that making numerous measurements throughout the growing season, in a range of moisture conditions, may help capture potentially episodic ANF inputs.

Acknowledgements

Support for this research was provided by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research (Awards DE-SC0018409 and DE-FC02-07ER64494) to the Great Lakes Bioenergy Research Center; by the National Science Foundation Long-Term Ecological Research Program (DEB 1637653); by the Chinese Scholarship Council stipend to CX; and by Michigan State University AgBioResearch. We thank K. Kahmark for help with methods development, N.E. Ostrom and H. Gandhi for help with isotope analyses, I. Schley for help in the field and lab, and B. McGill for drawing Figures 1 and 2. Thanks also to two anonymous reviewers who provided constructive comments that improved this manuscript.

Appendix A. Supplementary data

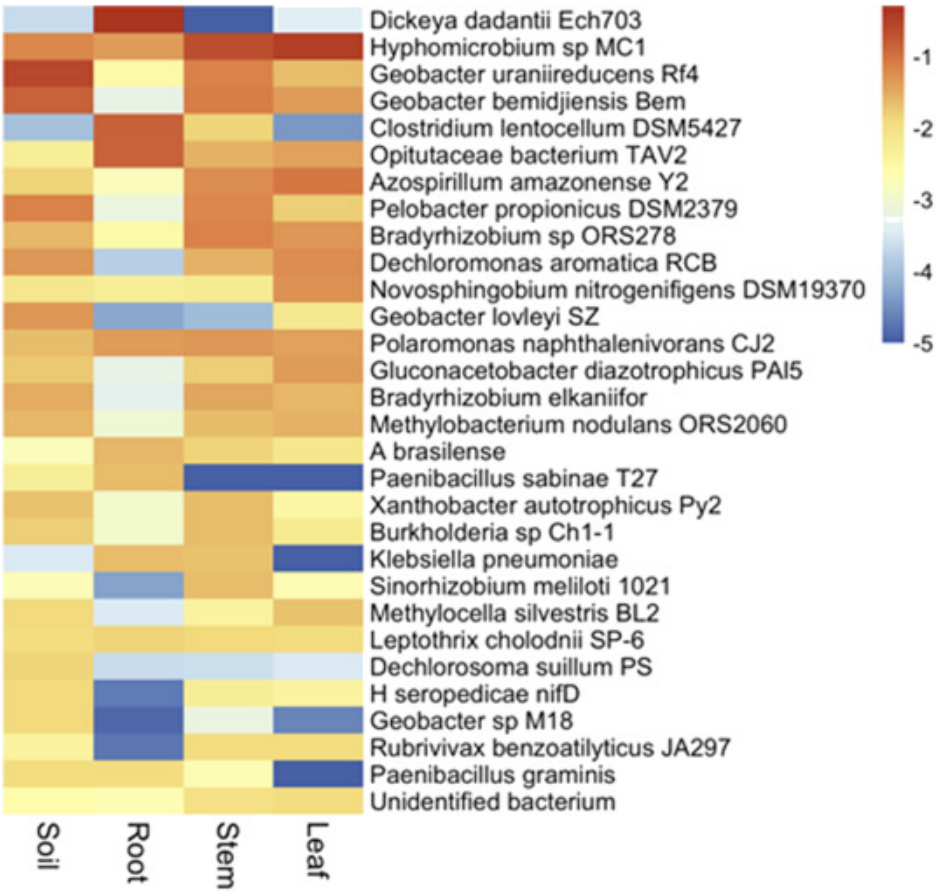
Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2018.11.006.

References

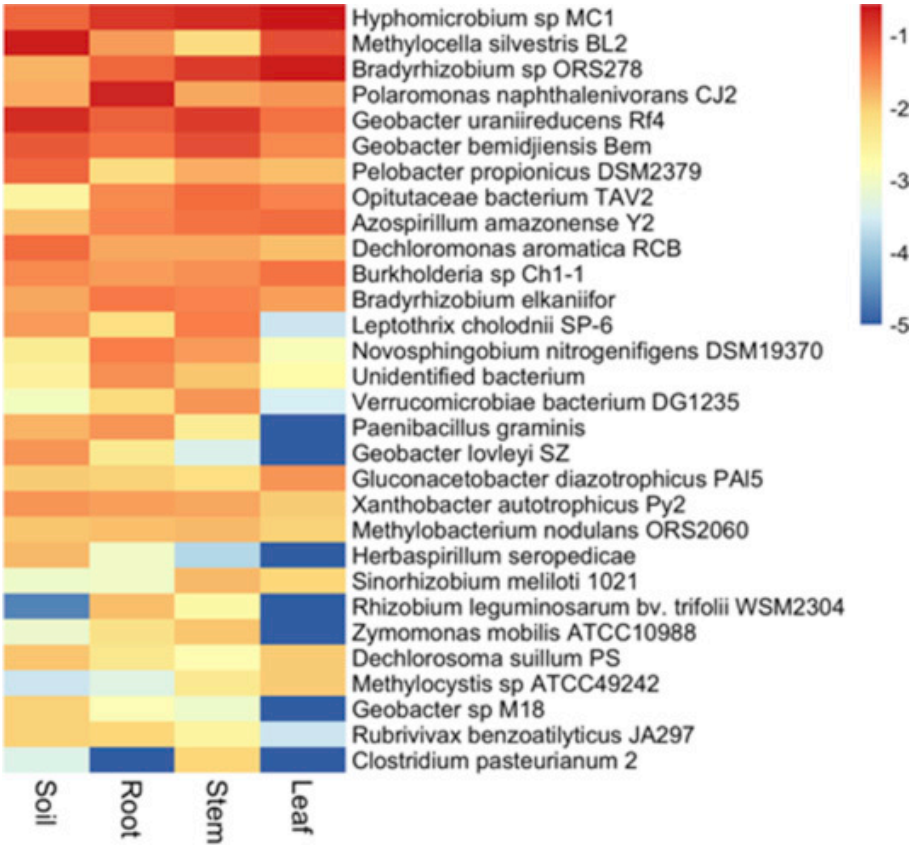
Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26, 32–46.

Bahulikar, R.A., Torres-Jerez, I., Worley, E., Craven, K., Udvardi, M.K., 2014. Diversity of nitrogen-fixing bacteria associated with switchgrass in the native tallgrass prairie of northern Oklahoma. Applied and Environmental Microbiology 80, 5636–5643.

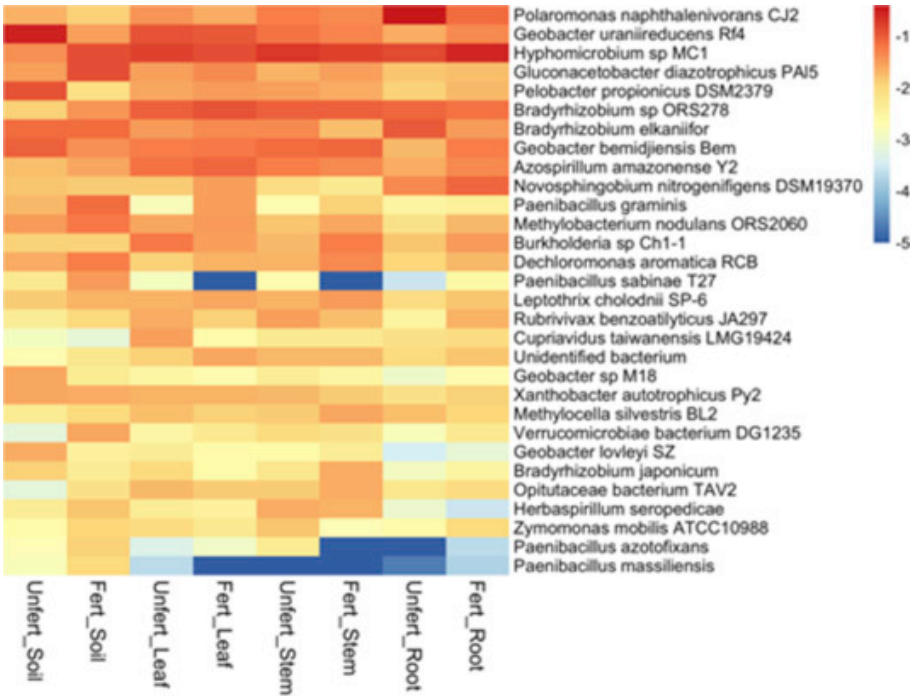
Baldani, J.I., Caruso, L., Baldani, V.L.D., Goi, S.R., Dobereiner, J., 1997. Recent advances in BNF with non-legume plants. Soil Biology and Biochemistry 29, 911–922.
Baptista, R.B., de Morais, R.F., Leite, J.M., Schultz, N., Alves, B.J.R., Boddey, R.M., Urquiaga, S., 2014. Variations in the N-15 natural abundance of plant-available N with soil depth: their influence on estimates of contributions of biological N-2 fixation to sugar cane. Applied Soil Ecology 73, 124–129.
Bergsma, T.T., Ostrom, N.E., Emmons, M., Robertson, G.P., 2001. Measuring simultaneous fluxes from soil of N2O and N2 in the field using the 15N-gas "nonequilibrium" technique. Environmental Science & Technology 35, 4307–4312.
Bernhardt, E.S., Blaszczak, J.R., Ficken, C.R., Fork, M.L., Kaiser, K.E., Seybold, E.C., 2017. Control points in ecosystems: moving beyond the hot spot hot moment concept. Ecosystems 20, 665–682.
Binkley, D., Son, Y., Valentine, D.W., 2000. Do forests receive occult inputs of nitrogen? Ecosystems 3, 321–331.
Boddey, R.M., 1987. Methods for quantification of nitrogen fixation associated with Graminae. CRC Critical Reviews In Plant Sciences 6, 209–266.
Boddey, R.M., Urquiaga, S., Alves, B.J.R., Reis, V., 2003. Endophytic nitrogen fixation in sugarcane: present knowledge and future applications. Plant and Soil 252, 139–149.
Bormann, B.T., Bormann, F.H., Bowden, W.B., Pierce, R.S., Hamburg, S.P., Wang, D., Snyder, M.C., Li, C.Y., Ingersoll, R.C., 1993. Rapid N2 fixation in pines, alder, and locust - evidence from the sandbox ecosystem study. Ecology 74, 583–598.
Bottomley, P.J., Myrold, D.D., 2015. Biological N inputs. In: Paul, E.A. (Ed.), Soil Microbiology, Ecology, and Biochemistry. Elsevier.
Brejda, J.J., Yocom, D.H., Moser, L.E., Waller, S.S., 1993. Dependence of 3 Nebraska sandhills warm-season grasses on vesicular-arbuscular mycorrhizae. Journal of Range Management 46, 14–20.
Casler, M., 2012. Switchgrass breeding, genetics, and genomics. In: Monti, A. (Ed.), Switchgrass: a Valuable Biomass Crop for Energy. Springer-Verlag, London, pp. 29–53.
Chapman, H.D., Liebig, G.F., Rayner, D.S., 1949. A lysimeter investigation of nitrogen gains and losses under various systems of covercropping and fertilization, and a discussion of error sources. Hilgardia 19, 57–128.
Dabundo, R., Lehmann, M.F., Treibergs, L., Tobias, C.R., Altabet, M.A., Moisander, P.H., Granger, J., 2014. The contamination of commercial N-15(2) gas stocks with N-15-labeled nitrate and ammonium and consequences for nitrogen fixation measurements. PLoS One 9.
Dalton, D.A., Kramer, S., Azios, N., Fusaro, S., Cahill, E., Kennedy, C., 2004. Endophytic nitrogen fixation in dune grasses (Ammophila arenaria and Elymus mollis) from Oregon. FEMS Microbiology Ecology 49, 469–479.
Davis, S.C., Parton, W.J., Dohleman, F.G., Smith, C.M., Del Grosso, S., Kent, A.D., DeLucia, E.H., 2010. Comparative biogeochemical cycles of bioenergy crops reveal nitrogen-fixation and low greenhouse gas emissions in a *Miscanthus x giganteus* Agro-Ecosystem. Ecosystems 13, 144–156.
Depolli, H., Matsui, E., Dobereiner, J., Salati, E., 1977. Confirmation of nitrogen fixation in 2 tropical grasses by (N-15)2 incorporation. Soil Biology and Biochemistry 9, 119–123.
Dobereiner, J., 1961. Nitrogen-fixing bacteria of the genus *Beijerinckia Derx* in the rhizosphere of sugar cane. Plant and Soil 15, 211–216.
Eskew, D.L., Eaglesham, A.R.J., App, A.A., 1981. Heterotrophic (N-15)2 fixation and distribution of newly fixed nitrogen in a rice-flooded soil system. Plant Physiology 68, 48–52.
Fike, J.H., Pease, J.W., Owens, V.N., Farris, R.L., Hansen, J.L., Heaton, E.A., Hong, C.O., Mayton, H.S., Mitchell, R.B., Viands, D.R., 2017. Switchgrass nitrogen response and estimated production costs on diverse sites. Global Change Biology Bioenergy 9, 1526–1542.
Fish, J.A., Chai, B., Wang, Q., Sun, Y., Brown, C.T., Tiedje, J.M., Cole, J.R., 2013. Functional gene pipeline and repository: tools for functional gene analysis. Frontiers in Microbiology 4, 291.
Gupta, V.V.S.R., Kroker, S.J., Hicks, M., Davoren, C.W., Descheemaeker, K., Llewellyn, R., 2014. Nitrogen cycling in summer active perennial grasses in South Australia: non-symbiotic nitrogen fixation. Crop & Pasture Science 65, 1044–1056.
Hamilton, S.K., Ostrom, N.E., 2007. Measurement of the stable isotope ratio of dissolved N_2 in ^{15}N tracer experiments. Limnology and Oceanography: Methods 5, 233–240.
Herridge, D.F., Peoples, M.B., Boddey, R.M., 2008. Global inputs of biological nitrogen fixation in agricultural systems. Plant and Soil 311, 1–18.
James, E.K., 2000. Nitrogen fixation in endophytic and associative symbiosis. Field Crops Research 65, 197–209.
Jesus, E.D., Liang, C., Quensen, J.F., Susilawati, E., Jackson, R.D., Balser, T.C., Tiedje, J.M., 2016. Influence of corn, switchgrass, and prairie cropping systems on soil microbial communities in the upper Midwest of the United States. Global Change Biology Bioenergy 8, 481–494.
Ker, K., Seguin, P., Driscoll, B.T., Fyles, J.W., Smith, D.L., 2014. Evidence for enhanced N availability during switchgrass establishment and seeding year production following inoculation with rhizosphere endophytes. Archives of Agronomy and Soil Science 60, 1553–1563.
Kim, D.G., Vargas, R., Bond-Lamberty, B., Turetsky, M.R., 2012. Effects of soil rewetting and thawing on soil gas fluxes: a review of current literature and suggestions for future research. Biogeosciences 9, 2459–2483.
Liang, C., Jesus, E.D., Duncan, D.S., Quensen, J.F., Jackson, R.D., Balser, T.C., Tiedje, J.M., 2016. Switchgrass rhizospheres stimulate microbial biomass but deplete microbial necromass in agricultural soils of the upper Midwest, USA. Soil Biology and Biochemistry 94, 173–180.
Mackelprang, R., Grube, A.B., Lamendella, R., Jesus, E.D., Copeland, A., Liang, C., Jackson, R.D., Rice, C.W., Kapucija, S., Parsa, B., Tringe, S.G., Tiedje, J.M., Jansson, J.K., 2018. Microbial community structure and functional potential in cultivated and native tallgrass prairie soils of the Midwestern United States. Frontiers in Microbiology 9, 1775. https://doi.org/10.3389/fmicb.2018.01775.
McClain, M.E., Boyer, E.W., Dent, C.L., Gergel, S.E., Grimm, N.B., Groffman, P.M., Hart, S.C., Harvey, J.W., Johnston, C.A., Mayorga, E., McDowell, W.H., Pinay, G., 2003. Biogeochemical hot spots and hot moments at the interface of terrestrial and aquatic ecosystems. Ecosystems 6, 301–312.
Mitchell, R., Vogel, K.P., Uden, D.R., 2012. The feasibility of switchgrass for biofuel production. Biofuels 3, 47–59.
Miyamoto, T., Kawahara, M., Minamisawa, K., 2004. Novel endophytic nitrogen-fixing clostridia from the grass Miscanthus sinensis as revealed by terminal restriction fragment length polymorphism analysis. Applied and Environmental Microbiology 70, 6580–6586.
Morris, D.R., Zuberer, D.A., Weaver, R.W., 1985. Nitrogen fixation by intact grass soil cores using (N-2)-N-15 and acetylene reduction. Soil Biology and Biochemistry 17, 87–91.
Moyes, A.B., Kueppers, L.M., Pett-Ridge, J., Carper, D.L., Vandehey, N., O'Neil, J., Frank, A.C., 2016. Evidence for foliar endophytic nitrogen fixation in a widely distributed subalpine conifer. New Phytologist 210, 657–668.
Oates, L.G., Duncan, D.S., Sanford, G.R., Liang, C., Jackson, R.D., 2016. Bioenergy cropping systems that incorporate native grasses stimulate growth of plant-associated soil microbes in the absence of nitrogen fertilization. Agriculture, Ecosystems & Environment 233, 396–403.
Parrish, D.J., Fike, J.H., 2005. The biology and agronomy of switchgrass for biofuels. Critical Reviews in Plant Sciences 24, 423–459.
Poly, F., Monrozier, L.J., Bally, R., 2001. Improvement in the RFLP procedure for studying the diversity of nifH genes in communities of nitrogen fixers in soil. Research in Microbiology 152, 95–103.
Postgate, J.R., 1998. Nitrogen Fixation, third ed. University Press, Cambridge, UK.
Reed, S.C., Cleveland, C.C., Townsend, A.R., 2011. Functional ecology of free-living nitrogen fixation: a contemporary perspective. In: In: Futuyma, D.J., Shaffer, H.B., Simberloff, D. (Eds.), Annual Review of Ecology, Evolution, and Systematics, vol. 42. pp. 489–512.
Robertson, G.P., Hamilton, S.K., 2015. Long-term ecological research in agricultural landscapes at the Kellogg Biological Station LTER site: conceptual and experimental framework. In: Hamilton, S.K., Doll, J.E., Robertson, G.P. (Eds.), The Ecology of Agricultural Landscapes: Long-term Research on the Path to Sustainability. Oxford University Press, New York, New York, pp. 1–32.
Robertson, G.P., Hamilton, S.K., Barham, B.L., Dale, B.E., Izaurralde, R.C., Jackson, R.D., Landis, D.A., Swinton, S.M., Thelen, K.D., Tiedje, J.M., 2017. Cellulosic biofuel contributions to a sustainable energy future: choices and outcomes. Science 356, 1349.
Roley, S.S., Duncan, D.S., Liang, D., Garoutte, A., Jackson, R.D., Tiedje, J.M., Robertson, G.P., 2018. Associative nitrogen fixation (ANF) in switchgrass (*Panicum virgatum*) across a nitrogen input gradient. PLoS One 13 (6), e0197320. https://doi.org/10.1371/journal.pone.0197320.
Roper, M.M., Gupta, V.V.S.R., 2016. Enhancing non-symbiotic N2 fixation in agriculture. The Open Agriculture Journal 10, 7–27. https://doi.org/10.2174/1874331501610010007.
Ruan, L.L., Bhardwaj, A.K., Hamilton, S.K., Robertson, G.P., 2016. Nitrogen fertilization challenges the climate benefit of cellulosic biofuels. Environmental Research Letters 11, 064007.
Sanderson, M.A., 1992. Morphological development of switchgrass and kleingrass. Agronomy Journal 84, 415–419.
Sanford, G.R., Oates, L.G., Jasrotia, P., Thelen, K.D., Robertson, G.P., Jackson, R.D., 2016. Comparative productivity of alternative cellulosic bioenergy cropping systems in the North Central USA. Agriculture, Ecosystems & Environment 216, 344–355.
Santi, C., Bogusz, D., Franche, C., 2013. Biological nitrogen fixation in non-legume plants. Annals of Botany 111, 743–767.
Smith, R.M., Thompson, O., Collier, J.W., Hervey, R.J., 1954. Soil organic matter, crop yields, and land use in the Texas blackland. Soil Science 77, 377–388.
Tjepkema, J.D., Burris, R.H., 1976. Nitrogen fixation associated with some Wisconsin prairie grasses. Plant and Soil 45, 81–94.
Urquiaga, S., Xavier, R.P., Morais, R.F., Batista, R.B., Schultz, N., Leite, J.M., Maia e Sá, J., Barbosa, K.P., Resende, A.S., Alves, B.J.R., Boddey, R.M., 2011. Evidence from field nitrogen balance and 15N natural abundance data for the contribution of biological N2 fixation to Brazilian sugarcane varieties. Plant and Soil 356, 5–21.
Van Dommelen, A., Vanderleyden, J., 2007. Associative Nitrogen Fixation. Elsevier, New York.
Valdez, Z.P., Hockaday, W.C., Masiello, C.A., Gallagher, M.E., Robertson, G.P., 2017. Soil carbon and nitrogen responses to nitrogen fertilizer and harvesting rates in switchgrass cropping systems. BioEnergy Research 10 (2), 456–464.
Wang, Q., Quensen, J.F., Fish, J.A., Lee, T.K., Sun, Y., Tiedje, J.M., Cole, J.R., 2013. Ecological patterns of nifH genes in four terrestrial climatic zones explored with targeted metagenomics using FrameBot, a new informatics tool. mBio 4 (5). https://doi.org/10.1128/mBio.00592-13.
Warembourg, F.R., 1993. N_2 fixation in soil and plant systems. In: Knowles, R., Blackburn, T.H. (Eds.), Nitrogen Isotope Techniques. Academic Press, San Diego, CA.
West, T.O., Post, W.M., 2002. Soil organic carbon sequestration rates by tillage and crop rotation: a global data analysis. Soil Science Society of America Journal 66, 1930–1946.
Xu, J., Kloepper, J.W., Huang, P., McInroy, J.A., Hu, C.H., 2018. Isolation and characterization of N_2-fixing bacteria from giant reed and switchgrass for plant growth promotion and nutrient uptake. Journal of Basic Microbiology 58, 459–471.
Zhang, B.Z., Penton, C.R., Xue, C., Wang, Q., Zheng, T.L., Tiedje, J.M., 2015. Evaluation of the Ion Torrent Personal Genome Machine for gene-targeted studies using amplicons of the nitrogenase gene nifH. Applied and Environmental Microbiology 81, 4536–4545.



Supplemental Fig. 1. Heat map showing log-transformed relative abundances (mean of 4 replicates) of microbial taxa with the nearest nifH sequence in the in vitro laboratory experiment. For clarity, only the 30 most abundant taxa are shown.



Supplemental Fig. 2. Heat map showing log-transformed relative abundances (mean of 4 replicates) of microbial taxa with the nearest nifH sequence in the greenhouse whole-plant experiment. For clarity, only the 30 most abundant taxa are shown. The populations in the in vitro and greenhouse experiments would not necessarily be expected to be the same because the soil and plants were sampled at different times. In addition, the scale, incubation, and growth conditions were very different.



Supplemental Fig. 3. Heat map showing log-transformed relative abundances (mean of 4 replicates) of microbial taxa with the nearest nifH sequence. The leaf, stem, and root samples are from plants grown in fertilized (Fert) and unfertilized (Unfert) soil. For clarity, only the 30 most abundant taxa are shown.