

Evidence of Downregulation in Atmospheric Nitrogen-Fixation Associated with Native Hawaiian Sugarcane (*Saccharum officinarum* L.) Cultivars

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Abstract

Aims

The study of nitrogen fixation in sugarcane has a long history that has demonstrated high potential but with substantial variation in results. This 32-month study sought to assess the response of nitrogen fixation associated with sugarcane (*Saccharum officinarum* L. cvs. 'Akoki, Honua'ula, and 'Ula) to available soil nitrogen.

Methods

Plants were grown in large pots of perlite along with a fixing and a non-fixing plant control, and administered liquid fertigation with varying amounts of isotopically enriched nitrogen. Assessment of nitrogen fixation utilized nitrogen isotope tracing and acetylene reduction assay in the target and control plants.

Results

Isotope enrichment and acetylene reduction assay both indicated that nitrogen fixation peaked under low nitrogen application, and declined with higher application rates, with agreement between the two methods. These results suggest that sugarcane engages in a downregulation of nitrogen fixation under high nitrogen availability, potentially explaining the high variation in published experimental results.

Conclusion

This suggests that nitrogen management and fertilization strategy can impact the atmospheric inputs of nitrogen in sugarcane cultivation, and the potential to improve nitrogen application efficiency in cropping systems utilizing sugarcane.

Introduction

Nitrogen (N) is often the limiting factor for primary production in terrestrial ecosystems due to its importance in fundamental biological processes and its high degree of mobility and losses in ecosystems (Vitousek et al. 2002). The vast reservoir of atmospheric nitrogen gas (N₂) is inert and is converted to ammonium (NH₃) through the process of nitrogen fixation, only after which N can be utilized in biological processes in various organic and inorganic forms (Pudi et al. 2018).

In natural systems, vast majority of N is fixed by bacteria utilizing the nitrogenase enzyme, with several types of N-fixation recognized by different groups of bacteria, such as "free-living" in soil or water (e.g., *Anabaena spp.*, *Azotobacter spp.*), "symbiotic" bacteria associated with leguminous plants (e.g.,

Rhizobium spp., *Bradyrhizobium* spp.), “associative” residing in the rhizosphere of plants (e.g., *Azospirillum* spp., *Glucenobacter* spp.), and “endophytic” bacteria that dwell within organisms (e.g., *Azotobacter* spp., *Gluconacetobacter* spp.), with many genera able to occupy different niches. Nitrogen fixation is affected by numerous parameters, including plant species and cultivar, bacterial symbionts, and environmental conditions (Zheng et al. 2019). In particular, nutrient availability has long been considered a key regulator of biological N-fixation, with nutrients such as N, phosphorus (P), and molybdenum (Mo) having strong effects on N-fixation rates. For biological N-fixation associated with plants (symbiotic, associative, and endophytic), the evidence suggests that low N-availability induces the plant-microbe association and enables this mechanism. General mechanisms include facultative or over-regulating N-fixers, which downregulate rates of N-fixation in response to increases in available soil N, or obligate fixers, which tend to demonstrate unaltered rates of fixation regardless of N availability (Menge et al. 2015, 2019).

Human activities fix massive quantities of N through various industrial processes. Although some are inadvertent, the vast majority of anthropogenic N is deliberately fixed and distributed as fertilizers to agroecosystems, primarily applying the energy-intensive Haber-Bosch method, which produced approximately 290 million tons of ammonium in 2018 (Fernández 2022). Humans now fix more nitrogen than all natural processes combined (Stevens 2019), and the alteration of global nitrogen cycles has multiple consequences on the health and function of ecosystems (De Vries et al. 2013; Gruber and Galloway 2008; Lehnert et al. 2018; Vitousek et al. 1997). Substantial environmental impacts such as the eutrophication of waterways, nitrate poisoning of groundwater sources, and nitric oxide emissions contributing to global warming have warranted the increasing attention to N management. This is especially true for agricultural ecologies, where harvest can remove and farming activities can mobilize substantial amounts of N.

Although industrial N-fixation has resulted in the substantial increase in global food production, the dependency on human supplied N can result in negative impacts in socio-economic and environmental consequences (Zhang et al. 2015). Efforts to curtail the substantial demand in N fertilizers have included improved nutrient application methods, improved crop nutrient-use efficiencies, and the inclusion of crop-associated N-fixation. Biological N-fixation is well known to positively influence plant growth and overall health through mutualistic associations, although these relationships often become overlooked in present-day agriculture due to the use of human-mediated nutrient inputs (Gosling et al. 2006; Sattelmacher et al. 1991). While the roles of different N-fixation strategies have been explored for various ecosystems (Menge et al. 2009, 2015), consideration of human-dominated agroecosystem has received less attention. The implications of these two strategies can play a deterministic role in plant growth and production (Van Der Heijden et al. 2006). From an agroecological framework, the behavior of facultative fixers can create a negative feedback loop in which the use of N fertilizers decreases N-fixation, thereby leading to an increased reliance on fertilizers.

Research into agricultural N-fixation has focused primarily on legume related crops and their root-associated symbiosis with bacteria such as *Rhizobium*. However, more recently, multiple lines of research

have demonstrated that non-legume crops can occasionally boast high levels of N-fixation, typically through associative or endophytic relationships with bacteria, (Boddey et al. 2003; Dalla Santa et al. 2004; Kennedy and Tchan 1992; Rilling et al. 2018, Santi et al. 2013; Venieraki et al. 2011). Achieving high levels of fixation in cereal crops that currently supply most of the world's calories has been a long-cherished goal, although in general N-fixation levels are limited. The generation of plant-available nitrogen through atmospheric nitrogen fixation has tremendous potential not only for the reduction of N inputs into large scale monocrop production, but also the utilization of such crops and their residues in diversified cropping systems to support the cultivation of other crops.

Sugarcane (*Saccharum officinarum* L.), has a substantial history of study for N fixation (Boddey et al. 2003). Numerous naturally occurring N fixing bacteria have been isolated in association with and endophytic to sugarcane (Boddey et al. 2003; Reis et al. 2007). In previous studies, N-fixation in sugarcane has ranged up to 60% (Taulé et al. 2012) but has been highly variable (Boddey et al. 2003). To our knowledge, the N-fixation strategy of sugarcane has not been investigated as to whether strategies are obligate (maintain a fixed level regardless of nitrogen resources available in the soil) or facultative (mediate the level of fixation based on available resources) fixers (Menge et al. 2015).

In Hawai'i, we previously hypothesized that sugarcane was a substantial source of nitrogen to large, ancient field systems utilized by Native Hawaiians for several centuries (Lincoln and Vitousek 2016). With no evidence of in oral or written traditions, in paleo- or archaeological reconstructions, or in contemporary observations for legumes or other N inputs playing an important role in the pre-European cropping systems of Hawai'i, other sources of biological N-fixation must have been incorporated into dryland Hawaiian agricultural systems, which were both vast and maintained intensively for multiple centuries (Lincoln and Vitousek, 2017). While initial investigations suggested relatively high potential for asymbiotic N-fixation during the decomposition of sugarcane litter, our initial look at sugarcane-associated N-fixation suggested negligible results (Lincoln and Vitousek 2016).

In the current study, we aimed to examine the N-fixation strategy of sugarcane as a response to nitrogen availability in the soil. We hypothesize that in response to lower levels of available soil nitrogen sugarcane will demonstrate higher levels of atmospheric nitrogen fixation. The fixation strategy of sugarcane should be of high interest given the agricultural importance of sugarcane, the potential for beneficial economic and environmental impacts and the potential relevance to other cereal crops.

Materials And Methods

Experimental Design

At the Magoon experimental greenhouse facility (21°18'24.6"N, 157°48'36.1"W), three Hawaiian cultivars of sugarcane (*Saccharum officinarum* L. cvs. 'Akoki, Honua'ula, and 'Ula; Lincoln 2020) were subjected to experimental treatments to examine N-fixation under varying N-availability. Seed pieces were collected from *in situ* conservation plantings, surface sterilized then transferred to a mist table for two weeks until rooting. One half the seed pieces were inoculated with *Gluconacetobacter diazotrophicus* PA1 5 (ATCC

49037) by lightly misting the roots with inoculum, then starts were weighed and planted in individual 15-gallon pots of fine perlite. Random seed pieces were destructively sampled for the initial dry weight conversion and nutrient concentrations. Each pot received two types of fertilizer, a N-free hydroponic mixture of macro- and micro-nutrients, and isotopically labeled ammonium nitrate. Non-N soil nutrients were added at the rate of $0.3 \text{ g P m}^{-2} \text{ y}^{-1}$. Eight levels of N were applied at the rate of 0.00, 0.38, 0.96, 1.54, 2.50, 3.84, 5.76, and $7.68 \text{ g N m}^{-2} \text{ y}^{-1}$, applied monthly. After 16 months tissue samples were collected, dried, and ground for nutrient and isotopic analysis. The cane stalks were harvested and weighed, and the canes were allowed to regrow. At 30 months each pot was planted with a N-fixing (*Crotalaria juncea*) and non-N-fixing (*Brassica rapa*) control plant. At 32 months, root and leaf samples from all canes and control plants were collected and immediately subjected to acetylene reduction assay. Additional tissue samples were collected, dried, and ground for nutrient and isotopic analyses.

Inoculum

Gluconacetobacter diazotrophicus PA1 5 (ATCC 49037) was grown following the recommendations of Tian et al (2009) with modifications. Briefly, *G. diazotrophicus* was grown in C2 medium for 48 hrs at 28 °C. Cells were harvested at 3500 rpm for 20 min and re-suspended in 0.8% NaCl solution to an OD600 = 0.2 for inoculation. The control solution was 0.8% NaCl in the absence of the bacterium.

Fertilizer Solution

A custom hydroponic mix was made using molecular and chelated forms of macro- and micro-nutrients. Concentrated stock solutions of each ingredient were mixed in the laboratory. A complete recipe of the fertilization solutions is reported (Supplemental Table 1). Stock solutions were added to 80 L of water and thoroughly mixed immediately prior to fertilization each month. An isotopically-enriched N fertilizer was made by mixing 98 atom % $^{15}\text{NH}_4^{15}\text{NO}_3$ (Cambridge Isotope Labs, Tewksbury, MA, USA) with natural abundance level ammonium nitrate for a final solution of 11.85 atom percent ^{15}N .

Acetylene Reduction

Acetylene reduction assay (ARA) was performed to assess nitrogenase activity (Hardy et al., 1968). Paired fresh samples were incubated in 10% acetylene gas or in air for 2 h. Gas samples were analyzed for ethylene concentrations on a Shimadzu 2A gas chromatograph. Correlation of ARA to N-fixation used a value of 3.3 as previously determined in Lincoln and Vitousek (2016) following the procedures in Crews et al. (2001). Samples for correlation were first incubated in 50% by volume ambient air, and 50% by volume 80% N₂ (99% $^{15}\text{N}_2$), 20% O₂ gas for 2 h, then subsequently aired out and incubated for ARA as above.

Nutrient and Isotope Analyses

Nitrogen levels and isotopes were assessed from the youngest mature leaf and a small section of stalk of each plant. All tissues samples were dried at 45°C for 48 hours until a constant weight was realized,

ground and analyzed for ^{15}N at the UC Davis Stable Isotope Laboratory using a PDZ Europa ANCA-GSL elemental analyzer coupled to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) following their submission protocols (stableisotopefacility.ucdavis.edu).

Calculation of N

Total N uptake was calculated as mass by N concentration. Isotopic mass balance was derived using a simple mixing model, using the difference in total N between starting and ending periods and applying a two end-member mixing model assuming an atmospheric $^{15}\text{N}/^{14}\text{N}$ ratio of 0.3663 (Dawson et al. 2002) and applying the calculated fertilizer $^{15}\text{N}/^{14}\text{N}$ ratio of 11.85. This approach does not account for fractionation rates of either atmospheric N-fixation nor uptake from the soil, and therefore we focus more on the patterns between treatments rather than absolute values of calculated fixation.

Results

The main treatment of N application level significantly impacted biomass, total N uptake, ^{15}N concentration, and ARA rates of sugarcane. The sub-treatments of sugarcane variety and inoculation with *Gluconacetobacter diazotrophicus* PA1 5 showed no significant differences between treatments. Therefore, all subsequent analysis ignored subtreatments and treated all samples as replicates to the main treatment of N level.

Sugarcane Growth

From initial seed pieces that were statistically identical, averaging 152.3 dry g, harvest dry weights in years 1 and 2 resulted in significant, increasing biomass with increasing nitrogen application, despite substantial variation in the biomass of individuals (Fig. 1, Table 1). Across all treatments, increases in biomass followed a log-linear relationship in response to increasing concentration of ammonium nitrate in the fertigation applications. Compared to the first year, second year growth demonstrated slightly higher biomass at lower N application and lower biomass at higher N applications, but followed very similar patterns of a log-linear growth response to increased nitrogen application.

Nitrogen Uptake and Isotopic Concentration

Nitrogen concentration demonstrated no significant differences between N treatments, however the plants demonstrated a significant difference in N concentration over time, from a mean of 1.00% in the leaves at installation, to 0.82% at year 1 sampling and 0.66% at year 2 sampling. Of interest, N concentration at installation differed significantly (ANOVA, r^2 0.41, $p < 0.0001$) and substantially (means of 1.04, 0.77, and 1.18% for 'Akoki', 'Honua'ula', and 'Ula' respectively) between varieties, but under experimental conditions no differences were observed at the year 1 and year 2 sampling.

Nitrogen isotopic ratios, which were statistically identical across treatments at installation (mean $\delta^{15}\text{N}$ of 8.04‰), diverged significantly over the course of the experiment (Fig. 2). For sugarcane, the response of

foliar $\delta^{15}\text{N}$ to the amount of N fertilizer applied was well fit by a three-parameter exponential growth model in both year 1 (r^2 0.96, $p < 0.0001$) and year 2 (r^2 0.93, $p < 0.0001$). Year 2 samples produced consistently and significantly lower $\delta^{15}\text{N}$ values compared to year 1. The two control plants, grown only prior to year 2 sampling, displayed different response patterns compared to sugarcane and each other, with $\delta^{15}\text{N}$ values that bracketed the sugarcane values at each treatment. *C. juncea* produced lower $\delta^{15}\text{N}$ values that increased significantly over the two lowest N applications, followed by relatively stable values across the remaining treatments, while *B. rapa* produced higher $\delta^{15}\text{N}$ values that were stable across all treatments that included N fertilization, breaking only with the treatment that excluded N altogether.

Contributions of Nitrogen from Air

Patterns of N contribution from air were evaluated both through isotopic mass balance and through the measurement of N-fixation potential through ARA. Patterns between the two approaches aligned well.

Using total N uptake between each time period and a two-end mixing model, total N contribution derived from air was determined (Fig. 3). The N omission treatments demonstrated low accumulation of atmospheric N. Total inputs from atmospheric N peaked in the low N application treatments, with atmospheric N contributions declining with higher N application.

Estimation of atmospheric N-fixation by ARA suggested active N-fixation of sugarcane, primarily in the roots, and declining fixation rates with increasing N fertilizer applications (Fig. 4). The growing media, *B. rapa*, and sections of sugarcane stalk all demonstrated very low rates of acetylene fixation, translating to low atmospheric N-fixation potential, that did not differ significantly across N fertilization treatments. *C. juncea*, a known obligate N-fixer, demonstrated consistently high, statistically indistinguishable, levels of N-fixation across all N fertilization rates. Sugarcane roots demonstrated levels of fixation that were substantially higher than the non-N-fixer control and significantly lower than the known N-fixer control. Furthermore, the potential fixation associated with sugarcane roots declined significantly in the higher N fertilization treatments.

Discussion

Much of the initial work on N-fixation in sugarcane occurred in low-N environments, often as a response to observations of mass-balance discrepancies in no- or low-N input cultivation systems. Productive sugarcane cultivation in Brazil, for example, was clearly uptaking much more N than was available in the soil system alone (Boddey 1995; Dong et al. 1994; Reis et al. 2007). Despite the potential importance of N availability in determining N fixation levels, there have been minimal investigations examining sugarcane's N fixation potential across nitrogen gradients. Liu et al. (2014) demonstrated that nitrogenase activity in sugarcane increased significantly when cutting nitrogen application in half, suggesting that sugarcane may exhibit a facultative N fixation strategy.

The results from our well-replicated experiment suggest that both instantaneous (acetylene reduction) and long-term (isotopic dilution) N-fixation is strongly affected by soil N availability. These results could explain why numerous studies, including a previous study by the lead author, have resulted in results indicating an absence of N-fixation during sugarcane growth (e.g. Biggs et al. 2002; Lincoln and Vitousek 2016; Temorshuizen et al. 2005). If examinations of N-fixation in sugarcane were conducted under high soil-N conditions, N-fixation could decline to a level that is statistically indistinguishable from a non-N-fixing control, as was seen in both the ARA and the fertilizer isotope tracing in this study.

Facultative N-fixation in agricultural crops has substantial implications for sustainable agricultural practices. The crop and management practices produce a strong feedback mechanism, whereas the more N fertilizer that is applied the less atmospheric N is sourced, increasing the need for N fertilization (or conversely, the less N fertilizer that is applied the more atmospheric N is sources, reducing the need for N fertilization). The results of this study suggest that the cycle breaks down if there is no available soil N, as reduced biomass and production limits the amount of N that can be fixed. However, with relatively small additions of available N in the soil, N-fixation rates and total accumulation rapidly increase to a maximum level, before declining under high soil-N conditions. The interactions between N-fixation and fertilization were predicted as a possible explanation of previous results that demonstrate an initial peak followed by a trough in the biomass response to N-fertilization (Purchase 1980; Wood 1972)

While the patterns of fixation appear clear in our results, several unexpected outcomes deserve some discussion. One is the lack of response to inoculation by *Gluconacetobacter diazotrophicus* PA1 5 (ATCC 49037). While this specific strain of bacteria was anticipated to increase N-fixation rates in our experiment, *G. diazotrophicus* is known to be widespread in Hawai'i and N-fixing bacteria have previous been isolated from sugarcane in Hawai'i (Boddey et al. 1991; Vega et al. 2005). It seems unlikely that the successful inoculation would not have yielded significant differences in the treatments. One potential explanation is that our source material was already inhabited by a N-fixing endophyte that resisted the inoculation of *G. diazotrophicus*, or that our attempt at inoculation was otherwise unsuccessful. Unfortunately, our controlled biological permit required we sterilize and destroy the experimental plants and we were not able to submit the plants for genetic assessment to determine what N-fixing bacteria might have been associated with our experiment.

Additionally, no significant difference between the sugarcane varieties were observed, although upon initiation of the experiment, the varieties demonstrated significantly different $\delta^{15}\text{N}$ values, suggesting that there were differences of N-fixation, or at least differences of N isotope fractionation, that were occurring in the field cultivation where the material was collected. These differences quickly disappeared under the experimental conditions, although we are unclear as to why. Although our expectation was that the cultivars would perform differently in their rates of N-fixation, these were not detected in the experiment. It may be that the varieties do differ subtly in the field conditions that, over time, resulted in the divergence of the immediate soil isotope signatures and the signatures of the plants in the field, but these small differences were not detectable in the controlled settings.

Differences between experimental Year 1 and 2 were also evident, with the high N treatments fixing more N from the atmosphere in Year 2 compared to Year 1. We believe that the second-year growth, in which the experimental plants were more root-bound in the pots, resulted in greater N losses from the pots and the behavior of the plants as if they were more N deficient than they actually were. This may also be a function of growing the control plants with the cane, which would have competed for N-uptake.

Conclusion

N-fixation in plants and crops is a critical component of ecosystem health and agricultural productivity, with increasing attention applied to understanding N-fixation potential in non-leguminous plants. N-fixation strategies can cause feedback, in which N-availability influences the rates of fixation. Here, we demonstrate two independent measurements of N-fixation in a controlled experiment that suggest sugarcane is engaging in a facultative N-fixation strategy, in which atmospheric N-fixation occurs only when soil-N availability is well below required levels. This research is in agreement with past research, and provides an explanation for some of the highly variable outcomes of previous research examining N-fixation in sugarcane.

Declarations

Acknowledgements

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Competing Interests

The authors declare no financial interests.

Author Contributions

Lead authors contributed the study conception and design, funding, analysis, writing and revising. The remaining authors contributed to the material preparation, experimental management, sample analysis, data collection, and editing. All authors read and approved the manuscript.

Data Availability

The datasets generated during the current study are available from the corresponding author upon request.

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Table 1

Table 1 is not available with this version

Supplemental Table 1

Supplemental Table 1: Stock solution recipes, and amount of stock solution utilized for each monthly fertilization application.

Nutrient	Stock Solution (per 1 Liter of deionized H ₂ O)	Stock solution per fertilizer application
Phosphoric Acid (P ₂ O ₅)	260 mL	2 drops
Boric Acid (H ₂ BO ₃)	25g	1 mL
Chelated Iron	100g	2 mL
Micronutrient solution (Mo, Mg, Zn, Cu)	175mL Mo; 140mL Mg; 270mL Mg; 65mL Cu	2 mL
Potassium Phosphate, monobasic (KH ₂ PO ₄)	200g	7 mL
Chelated Calcium	200g	30 mL
Chelated Magnesium	200g	30 mL
Potassium Sulfate (K ₂ SO ₄)	50g	65 mL

Figures

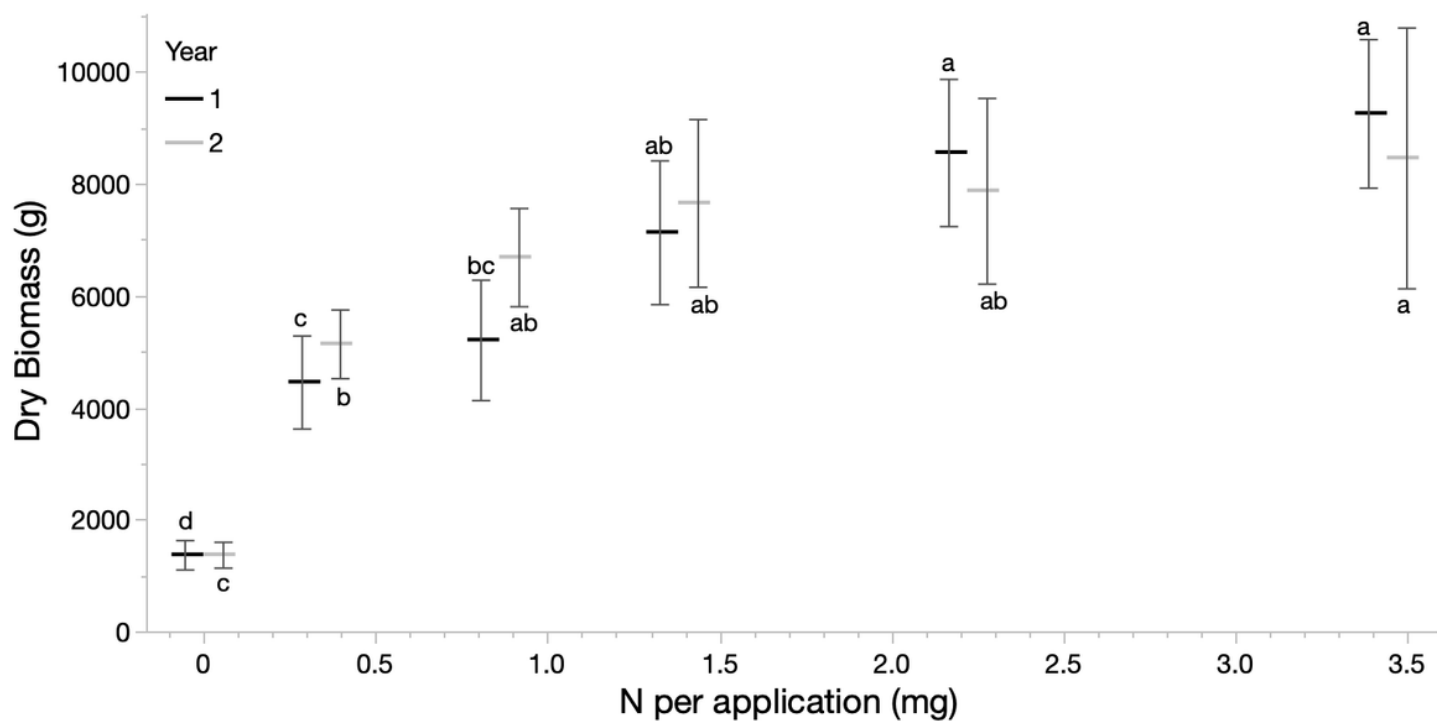


Figure 1

Mean dry biomass and 95% confidence intervals of sugarcane harvested in years 1 and 2 of the experiment in response to nitrogen application.

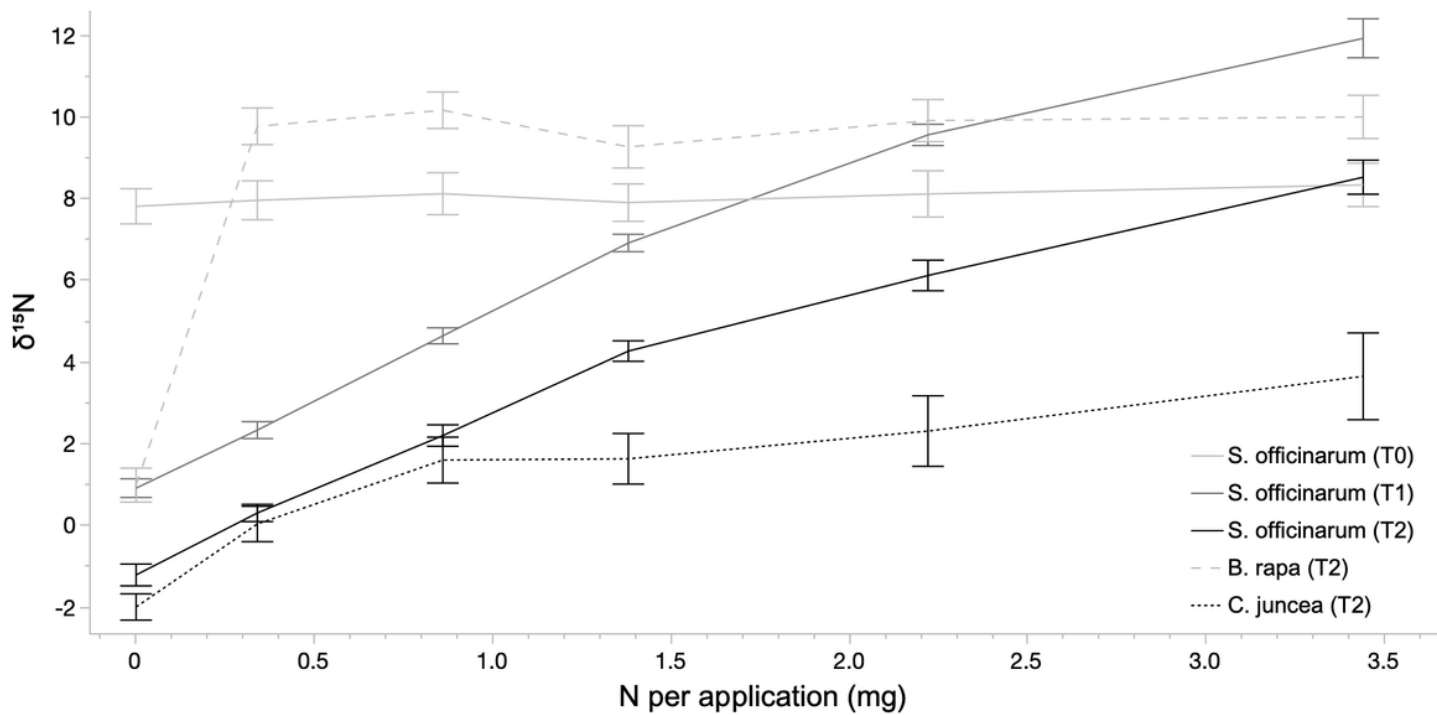


Figure 2

Values of $\delta^{15}\text{N}$ across nitrogen application rates for experimental and reference plants.

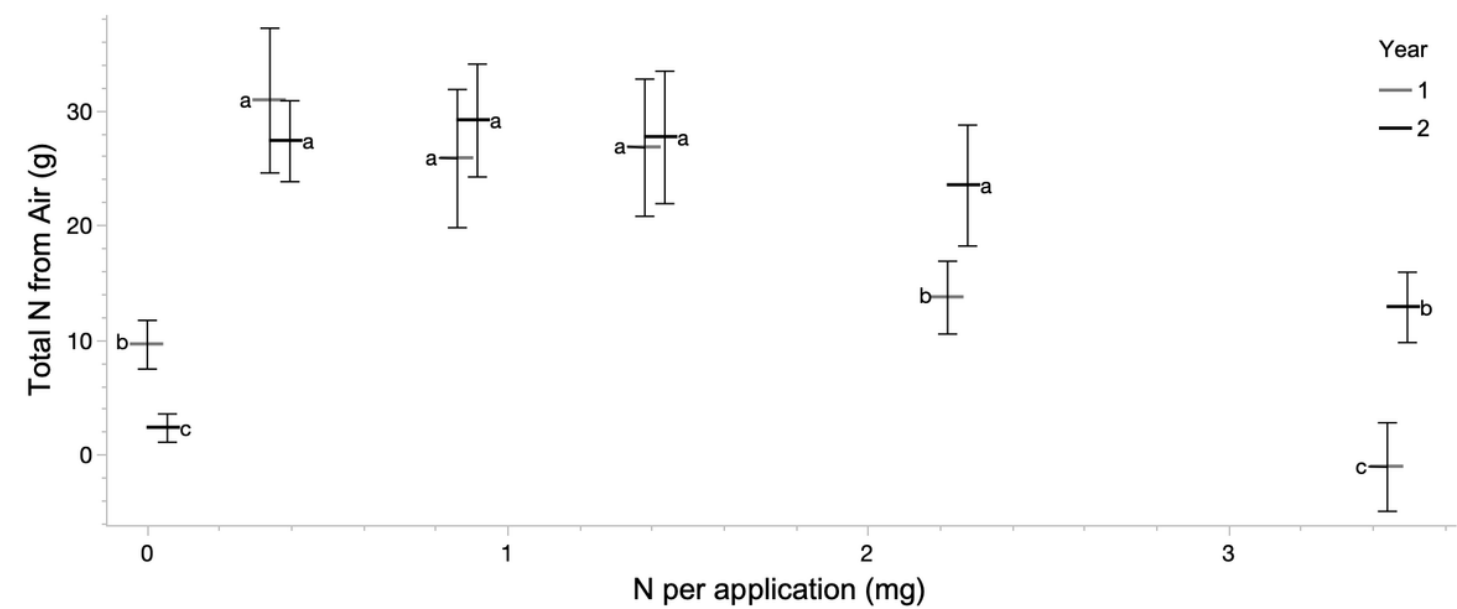


Figure 3

$\Delta^{15}\text{N}$ values across nitrogen application rates for experimental and reference plants.

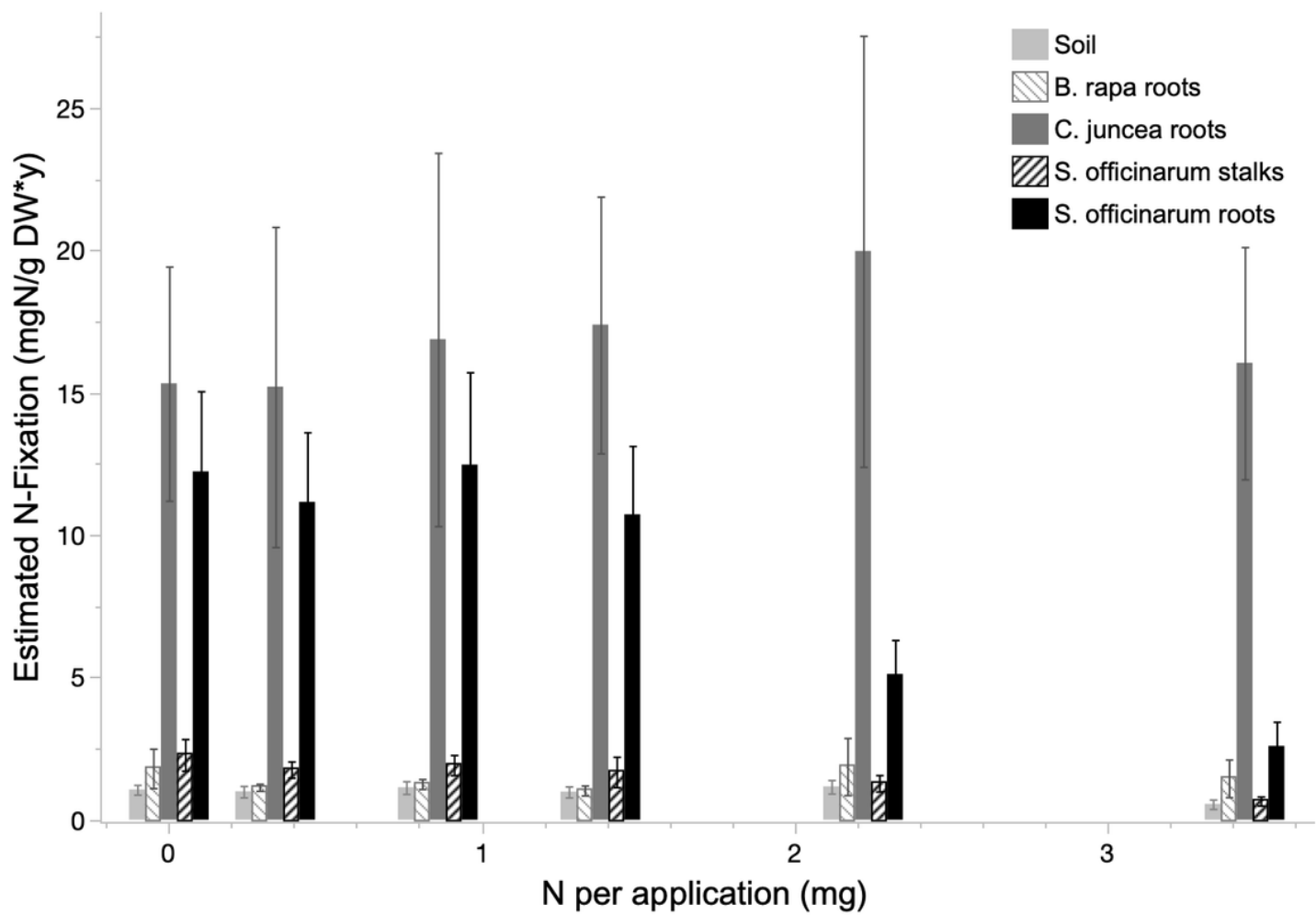


Figure 4

Estimated annual N fixation from acetylene reduction assay for sugarcane, the soilless media, and the N-fixing and non-N-fixing controls.