The ^{15}N natural abundance technique to assess the potential of biological nitrogen fixation (BNF) in some important C4 grasses

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Abstract

Grasses such as sugarcane (*Saccharum* spp.), maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench) with C4 photosynthetic system are important crops that can host endophytic bacteria capable for biological nitrogen fixation (BNF). However, fixation measurements have yielded conflicting results. We determined BNF of five sugarcane and two sorghum varieties, maize and Guinea (*Panicum maximum* Jacq), elephant (*Pennisetum purpureum* Schum) and buffel (*Cenchrus ciliaris* L.) grasses in a pot experiment, using the ^{15}N natural abundance technique, with a soil naturally rich in ^{15}N (δ^{15}N > 16 ‰) and two reference species (castor bean, *Ricinus communis* L., and cotton, *Gossypium hirsutum* L.) that absorb only N from the soil (average δ^{15}N = 9.2 ‰). The initial results showed that no fixation occurred in elephant and buffel grasses. Sorghum, maize, Guinea grass and four sugarcane varieties had δ^{15}N signals significantly lower than both reference species, indicating that they absorbed N from the atmosphere (δ^{15}N = 0 ‰). The differences in signals from the reference species translates into proportions of plant N content originating from BNF varied from 12.8 to 19.4 % for sugarcane, 22.4% for maize, 20.9% for Guinea grass, and 24.7 to 31.2 % for sorghum. Nitrogen fixation by these species represents a potential of great fertilizer economy and high yields in low input agriculture.

Keywords: diazotrophic bacteria, energy crops, isotopes, tropical grasses, ^{13}C.
Abbreviations: BNF_biological nitrogen fixation, SC_sugarcane, masl_meters above sea level, MAR_mean annual rainfall.

Introduction

Grasses of C4 metabolism have high photosynthetic efficiency and high potential for biomass production (Burner et al., 2009; Morais et al., 2009; Pedroso et al., 2014; Gorlitsky et al., 2015). They provide human food and animal fodder products and are important energy crops (Lima et al., 2014). Ethanol production from sugarcane, especially in Brazil, and from maize, especially in the USA (Balat and Balat, 2009) has been increasing since the oil crisis of the 70´s of last century. Other grasses such as *Pennisetum purpureum* (Morais et al., 2009), sorghum (Davila-Gomez et al. 2011), *Panicum maximum* and *Brachiaria brizantha* (Lima et al., 2014) can be strategic energy crops for the cultivation in areas less suitable for more traditional cultures. The possibility of high biomass production with minimal use of nitrogen fertilizers, which are dependent on fossil energy for their manufacture, emphasizes the importance of biological nitrogen fixation (BNF) in tropical grasses. The pioneer isolation of N$_2$-fixing (or diazotrophic) bacteria associated with sugarcane (Döbereiner and Ruschel, 1958) indicated that the plant could benefit from this process and has brought a great interest for the isolation of new microorganisms. Several nitrogen-fixing species, especially genera *Azospirillum*, *Herbaspirillum*, *Gluconacetobacter* and *Burkholderia,* have already been identified and colonized

from the surface or the inner roots of sorghum (Coelho et al., 2009; Luna et al., 2010; Yoon et al., 2016), maize (Roesch et al., 2006; Montañez et al., 2009; Kifle and Laing 2016; Brusamarello-Santos et al., 2017; Norman et al. 2017), elephant grass (Videira et al., 2012) and other tropical grasses. Despite the vast literature on the topic, it remains unclear whether and to what extent, grasses benefit from N fixed by endophytic diazotrophs or if benefits are due to the production of indole-3-acetic acid (IAA) and other growth-promoting substances that are proven to cause morphological changes in roots (such as increased lateral roots and root hairs), increasing nutrient absorption (Steenhoudt and Vandereyden, 2000; Videira et al., 2012; Beneduzi et al., 2013; Alves et al., 2015).

Among the tropical grasses, attempts to prove BNF through isotopic techniques are more abundant for sugarcane, but the results are variable, suggesting a strong influence of environmental factors and/or plant genotype. Although this is not a new discovery, it is relatively recent and much remains to be studied. For example, results have shown that some varieties cultivated in Brazil (Urquiaga et al., 2012; Baptista et al., 2014) and Uruguai (Taulé et al., 2012) may obtain significant amounts of atmospheric N, but no evidence of BNF was found in sugarcane grown in other places, such as

Australia (Biggs et al., 2002) and South Africa (Hoefsloot et al., 2005). For other grasses such as sorghum (Santos et al., 2017; Ferreira Neto et al., 2017), Guinea grass (Miranda et al., 1990), maize (Montañez et al., 2009) and elephant grass (Morais et al., 2012), reports are scarce.

The aim of this study was to estimate the contribution of BNF in six C4 cycle tropical grasses (sugarcane, sorghum, maize, guinea grass, elephant grass and buffel grass) by comparing their natural abundance of ^{15}N with two non-leguminous C3 species. This study included five major sugarcane varieties grown in Northeastern Brazil, with no available report on FBN estimates, and two sorghum varieties.

Results

Biomass production and N accumulation

In general, sugarcane varieties, especially variety RB962962, have the highest biomass productions. However, no significant difference was observed compared to elephant grass (Table 1). The other grasses did not produce more biomass than the two C3 reference species (castor bean and cotton), which had $\delta^{13}C$ values much more negative than the C4 plants. The N concentrations in the biomass were highest in the reference plants and lowest values in the plants that produced more biomass (the five sugarcane varieties and elephant grass) and in guinea grass (Table 1).

Among grasses, maize and elephant grass accumulated the highest N concentrations (Fig. 1). A low N concentration was observed in biomass of sugarcane varieties, showing that sugarcane accumulates less N than all other species, except guinea grass (Fig. 1).

Biological nitrogen fixation (BNF) assessment

There was no significant difference between the $\delta^{15}N$ signals of both reference species (castor bean and cotton). The average value of both was used for comparison with the signals shown by grasses. Sorghum, maize, Guinea grass and four sugarcane varieties had ^{15}N natural abundances significantly lower than the average value of reference plants and were able to gain part of their N content through BNF. Buffel grass, elephant grass and sugarcane variety RB92579 had $\delta^{15}N$ values with no statistical difference from values of reference species and; thus, the hypothesis that they exclusively absorb N from the soil cannot be ruled out.

Sorghum cv. IPA-1011 was the grass that most benefited from atmospheric N_2 absorption, showing Ndda% greater than twice the least fixative sugarcane variety (RB863129). There was no difference between the proportions of N fixed in sugarcane varieties RB863129, RB867515, RB931011, and RB962962. Even producing twice the biomass produced by sorghum, sugarcane variety RB962962 fixed less than half the N fixed by this short-cycle grass (Fig. 1). There was no difference in the proportions of N absorbed from the atmosphere between guinea grass and the other species (Table 2), but its low biomass production led to a small amount of fixed N, which was lower than that of maize and sorghum (Fig. 1).

In spite of the extra N absorbed from the atmosphere in addition to the N absorbed from the soil, there were no significant relationships between the ^{15}N signals of plants with evidence of fixation and their N contents.

Discussion

Maize, sorghum, guinea grass and four sugarcane varieties exhibited significantly lower ^{15}N natural abundance compared to the average value of reference plants (Table 2). This indicates that these species were able to absorb part of their N content from sources more dilute in ^{15}N than the sources available for non-leguminous C3 plants (castor bean and cotton), probably atmospheric N_2. These species are colonized by nitrogen-fixing bacteria (James and Olivares, 1998; Roesch et al., 2006; Videira et al., 2013; Antunes et al., 2017), but there are still doubts about the real benefit of BNF for their nitrogen nutrition.

Techniques using ^{15}N isotope are the most accurate to estimate BNF in plants (Högberg, 1997). These techniques are based on the comparison between ^{15}N concentrations of a nitrogen-fixing species that obtains N from atmospheric N_2 in addition to the sources of soil nitrogen and the concentrations of one or more non-fixing reference species, which rely only upon soil-derived N. When natural concentrations of the heavy isotope are analyzed, the average $\delta^{15}N$ value of fixing plants must be significantly different from the average value of the reference plant and for greater accuracy, the difference between values must be greater than 2% (Högberg, 1997). This criterion is adopted because the methodology used is only appropriate when fixing and reference plants are growing under the same conditions, exploring the same soil volume and absorbing N in similar patterns, ensuring that there are no significant differences in ^{15}N signal in the N content of soil available to plants.

Mixing procedures and the small volume of soil in the pots used in this experiment minimized the N signal variation of soil available N to plants, as evidenced by the lack of significant difference in $\delta^{15}N$ between reference species (castor bean and cotton). Over the 180 days of experiment, no major differences in the soil exploitation by plant roots were observed, which was limited due to the pot volume. Furthermore, the high isotopic enrichment of the soil (Freitas et al., 2015) enables greater accuracy in BNF estimation (Boddey et al., 2000). Under the conditions of our experiment, the differences below 2 % among species should be carefully discussed for quantitative BNF estimates (Högberg, 1997). The smaller but significant differences are valid to separate fixing from non-fixing varieties or species.

For sugarcane, there is strong evidence that the different varieties planted in the Brazil are able to obtain part of their N from the atmosphere, but in quite different proportions (Urquiaga et al., 2012; Baptista et al., 2014), which would explain their production with amounts of nitrogen fertilizers much lower than those applied in other countries. However, available information does not cover much of the diversity of genotypes and environmental conditions of the different producing regions. The varieties evaluated in this study have been bred and recommended only few years ago and, in spite of being among the most planted in Northeastern Brazil, they have not been studied to be potentially used for atmospheric N_2. This study found indication of BNF ($\delta^{15}N$ value significant at p <0.05, compared to the average value of reference plants) in varieties RB863129, RB867515, RB931011 and RB962962. The Ndda% values did not exceed 20%, a proportion of fixed N below the values reported for other sugarcane varieties (Lima et al., 1987; Urquiaga et al., 1992, 2012; Baptista et al., 2014). The differences in $\delta^{15}N$ between reference species (castor bean and cotton) and sugarcane were small, ranging from 1.17 % for variety

Table 1. Biomass, N concentrations, and $\delta^{13}C$ signals of tropical grasses and reference species grown in pots, in greenhouse.

Species/varieties	Biomass (g)	N (mg g^{-1})	$\delta^{13}C$ (%)
Sacharum spp.			
RB86-3129	35.15 abc[1]	3.39 f	-13.56 bcd
RB96-2962	40.90 a	3.25 f	-13.79 d
RB92-579	35.65 abc	3.51 f	-13.67 cd
RB93-1011	38.31 ab	3.66 f	-13.85 d
RB86-7515	27.65 bcd	3.27 f	-13.63 cd
Zea mays	19.58 de	9.32 bc	-12.95 ab
Sorghum bicolor			
IPA-1011	20.06 de	7.37 cde	-13.03 abc
SF11	23.37 de	6.89 de	-12.71 a
Panicum maximum	13.31 e	4.09 f	-13.91 d
Cenchrus ciliaris	15.15 e	7.47 cd	-12.54 a
Pennisetum purpureum	35.40 abc	4.99 ef	-12.62 a
Ricinus communis	11.60 e	11.61 ab	-26.61 e
Gossypium hirsutum	17.01 de	12.95 ab	-27.26 e

[1]Averages followed by the same letter in the column are not significantly different by the Tukey test at 0.05 probability level.
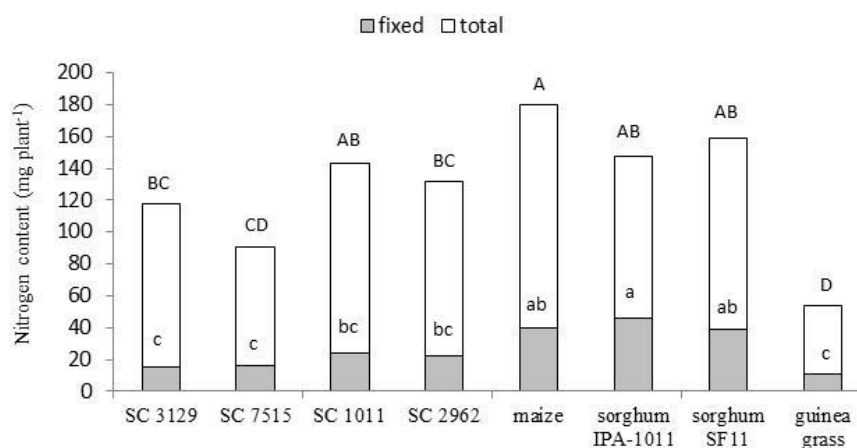

Fig 1. Total and fixed nitrogen in four sugar-cane varieties (RB863129, RB867515, RB931011 and RB962962), maize, two sorghum varieties (IPA-1011, forage, and SF11 grain producer) and Guinea grass grown in pots, in greenhouse. Averages with same capital letter (total) and small letter (fixed) are not significantly by the Tukey test at 0.05 probability   level.

Table 2. ^{15}N natural abundance and percentage of N derived from the atmosphere (% Ndda) in tropical grasses and reference species grown in pots in greenhouse.

Species/variety	$\delta^{15}N$ (‰)	Ndda (%)
References	9.18 ± 0.61	-
Sacharum spp.		
RB86-3129	8.01 ± 0.64 *[1]	12.80 b[2]
RB86-7515	7.40 ± 1.11 *	19.42 ab
RB92-579	8.34 ± 0.68 ns	-
RB93-1011	7.68 ± 0.31 *	16.33 ab
RB96-2962	7.64 ± 0.70 *	16.74 ab
Zea mays	7.12 ± 0.61 *	22.40 ab
Sorghum bicolor		
IPA-1011	6.32 ± 0.34 *	31.18 a
SF11	6.92 ± 0.81 *	24.67 ab
Panicum maximum	7.26 ± 0.80 *	20.92 ab
Cenchrus ciliares	8.12 ± 0.61 ns	-
Pennisetum purpureum	8.42 ± 0.36 ns	-

[1]Averages significantly different (*) or not (ns) from the average signal of reference species by the T test at the 0.05 probability level. [2]Averages followed by the same letter in the column are not significantly different by the Tukey test at 0.05 probability level.

RB863129 to 1.78 % for variety RB867515. These differences are smaller than the recommended limit for quantitative estimates, but show the potential of BNF in Brazilian varieties, in contrast to the lack of fixation observed in other countries, such as Australia (Biggs et al., 2002) and South Africa (Hoefsloot et al., 2005). This potential BNF can represent a large economy in nitrogen fertilizer costs.

Maize, sorghum and Guinea grass also demonstrated potential to obtain part of their N content by BNF, as they had natural ^{15}N abundance significantly lower than the average value of reference plants (Table 2). The differences in $\delta^{15}N$ mean values between these species and reference plants were greater than the differences presented by fixing sugarcane varieties, exceeding 2% for sorghum and maize.

This result means that BNF estimates in these species, ranging from 20 to 31% are even more robust than estimates for the four sugarcane varieties. The proportions found are within ranges reported by Miranda et al. (1990), for different guinea grass genotypes, and Montañez et al. (2009), for maize genotypes. Sorghum performance was lower than that reported by Ferreira Neto et al. (2017) and Santos et al. (2017) in the field, the only latest report of BNF in sorghum. Nevertheless, sorghum cultivar IPA-1011 was able to benefit from a higher proportion of atmospheric N_2 than sugarcane variety RB863129.

Despite producing less biomass (Table 1), the amount of N fixed in sorghum variety IPA 1011 was higher than the amounts fixed by all sugarcane varieties (Fig. 1). Taking into account the adaptation of sorghum for biomass production in semi-arid regions (Perazzo et al., 2014), this BNF capacity also means an important opportunity to combine productivity with relative economy of nitrogen fertilizers. Maize and sorghum variety SF 11 also outperformed two sugarcane varieties in the ability to accumulate N from the atmosphere. Despite relying on absorption of atmospheric N in addition to sources of soil N, no significant relationships were observed between ^{15}N signals of plants with evidence of fixation and their N contents. This intriguing result was also observed by Baptista et al. (2014).

Buffel grass and elephant grass had ^{15}N natural abundances without significant differences in relation to the average value of reference plants, indicating that they absorbed N exclusively from the soil. Even relying only upon the limited availability of soil N, elephant grass produced the same amount of biomass and accumulated more N than the sugarcane varieties. BNF estimates in elephant grass showed that there are considerable differences in the ability to accumulate fixed N among different elephant grass genotypes, but most of the tested genotypes absorbed over 30% of N from the atmosphere (Reis et al., 2001; Morais et al., 2012). The exclusive nutrition of soil N in elephant grass can be explained by the plant genotype or nutritional deficiencies that restricted the activity of associated diazotrophic microorganisms. In fact, both biomass accumulation and N concentrations were small, especially for sugarcane (Table 1), reflecting the reduced availability of this nutrient in the soil.

Materials and Methods

Experimental design and conduction of experiment

An experiment was conducted in greenhouse using a random block design, with four replicates and the following treatments: sugarcane (*Saccharum* spp., commercial interspecific hybrids RB867515, RB863129, RB92579, RB931011 and RB962962); sorghum (*Sorghum bicolor* L. Moench, varieties SF11, forage, and IPA-1011, grain producer); maize (*Zea mays* L., cv CMS 36.); Guinea grass (*Panicum maximum* Jacq, cv Mombaça); buffel grass (*Cenchrus ciliaris* L., cv Grass.); elephant grass (*Pennisetum purpureum* Schum cv Venezuela); castor bean (*Ricinus communis* L., cv Nordestina); and cotton (*Gossypium hirsutum* L. var. *latifolium* Hutch, cv. BRS 186). Plants were grown in pots containing 5 kg of soil.

Soil was collected in the 0-20 cm layer of a Latosol (Brazilian classification), Typic Acrortox (American classification) or Ferralsol (FAO classification) of loamy sandy texture in the municipality of Araripina (07° 33' S, 40° 34' W, 620 masl and 728 mm MAR) in an area covered with natural vegetation (savanna – deciduous forest transition).

This soil layer has a high isotopic enrichment ($\delta^{15}N > 16$ %) and low N (0.038%) and C (0.69%) contents (Freitas et al., 2015), which favors the determination of ^{15}N dilution due to BNF. Soil characteristics in the 0 to 20 cm superficial layer (analyzed following the procedures recommended by EMBRAPA, 1997) were: pH (water) 4.8; exchangeable Al^{3+}, Ca^{2+} and Mg^{2+}, 0.6, 0.2 and 0.3 $cmol_c$ dm^{-3}; respectively; extractable P and K (Mehlich I), 1.0 and 0.02 mg dm^{-3}, respectively.

Soil pH was corrected with 1.8 g kg^{-1} soil (equivalent to 1 ton ha^{-1}) of dolomite lime ($CaCO_3.MgCO_3$), applied 17 days before planting. Soil fertility was corrected with 1.6 g kg^{-1} soil of P_2O_5 (120 kg ha^{-1}), applied as triple superphosphate, and 0.5g kg^{-1} soil of K_2O (80 kg ha^{-1}), applied as KCl. Each pot received 1.74 mg kg^{-1} soil (400 g ha^{-1}) of Na_2MoO_4, three days after planting. No N fertilizer was applied and the pots received water daily to maintain the water potential close to field capacity.

The planting of sugarcane and elephant grass was performed using stem pieces (sets), measuring about 10 cm, containing one gem. For sugarcane, the sets were cut between leaves +3 and +6, with ¼ of upper and lower internodes. The other species were planted from seeds. Pruning was performed to leave one plant per pot.

Traits measured

Plants were harvested 180 days after planting and their shoots were oven dried at 65 °C for 72 hours to a constant weight, weighed and ground. Sub-samples were reduced into a fine powder using a roller mill for analysis. Aliquots were placed in a capsule and loaded into a ThermoQuest-Finnigan Delta Plus isotope ratio mass spectrometer (Finnigan-MAT; CA, USA) interfaced with an Elemental Analyzer (CarloErba model 1110; Milan, Italy) at the Laboratory of Isotope Ecology (CENA-USP, Brazil) to obtain the nitrogen and carbon isotope ratio and the total nitrogen content. Natural abundances of ^{15}N and ^{13}C were expressed using 'delta' notation (‰):

$$\delta = (R_{sample}/R_{standard} - 1) \times 1000$$

Where; R_{sample} and $R_{standard}$ are the $^{15}N:^{14}N$ and $^{13}C:^{12}C$ ratios of samples and of the standards, which are atmospheric N_2, for N, and Pee Dee Belemnite, for C.

Estimates of BNF

Estimates of the percentage of plant nitrogen derived from the atmosphere (Ndfa%) were made using the ^{15}N natural abundance technique. When the $\delta^{15}N$ value of a grass species (or variety) was significantly different from the average $\delta^{15}N$ of the reference plants, the proportion of fixed N in the plant was calculated using the following formula: (Shearer and Kohl, 1986):

$$\% Ndda = [(\delta^{15}N_{(reference)} - \delta^{15}N_{(fixing)}) / \delta^{15}N_{(reference)} - B] \times 100$$

Where; $\delta^{15}N_{(reference)}$ is the average value of $\delta^{15}N$ signals of reference plants (castor bean and cotton), $\delta^{15}N_{(fixing)}$ is the average value of $\delta^{15}N$ signals of each species (or grass variety) and B is the $\delta^{15}N$ value for fixing plants grown in the absence of N. As growth has not been entirely achieved on BNF of grasses species, the value was assumed to be zero (the same abundance of ^{15}N as air), as described by Morais et al. (2012), Baptista et al. (2014) and Alves et al. (2015).

The amount of nitrogen accumulated in plants was calculated by multiplying the total nitrogen by their respective biomass. The fixed N was calculated by multiplying the %Ndda by the total N accumulated by the plant.

Statistical analysis

Data normality was tested using the Shapiro-Wilk (W) test and, since this required condition was met in all cases, an analysis of variance was then performed with the application of the F-test. The average $\delta^{15}N$ value of each grass was compared with the average values of reference plants (castor bean and cotton), using the T-test at $p \leq 0:05$. The mean %Ndda values and amounts of accumulated and fixed N were compared by the Tukey test at 5% probability.

Conclusion

The absorption of atmospheric N through fixation contributes to the nitrogen nutrition of sorghum, sugarcane, maize and guinea grass with small variations in proportions among species. There was no evidence of biological fixation in elephant grass, buffel grass and sugarcane variety RB92579. The greatest proportion of N in nitrogen-fixing plants (>30%) was found in sorghum (variety IPA 1011), a species with a single previous fixation report.

Acknowledgements

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