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Contribution of Biological Nitrogen Fixation to the Biomass Productivity of Elephant Grass Grown in Low-Fertility Soil for Energy Purposes

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Abstract: Elephant grass has high biomass production potential and can benefit from biological nitrogen fixation (BNF) as its main external nitrogen source. This study evaluated the effect of BNF on biomass productivity and total nitrogen accumulation in different elephant grass genotypes. This experiment was conducted in a 120 m^2 concrete tank filled with soil labeled with ^{15}N to estimate the contribution of BNF. The experimental design was randomized blocks with four replications, and the evaluation was over three years of cultivation, with semiannual cuts. The productivity of fresh and dry mass of the shoot, Nitrogen (N) accumulation, and the contribution of BNF by the ^{15}N natural abundance technique were evaluated. The annual average of BNF was 38%. There was a statistical difference between the treatments, with the genotype P13G13 presenting fresh and dry mass productivity 50% higher than P6G4. The annual average of fresh mass, dry matter, total N, and N derived from BNF in the genotypes was approximately 70, 30, 100 Mg ha^{-1}, and 35 kg ha^{-1}, respectively. The results obtained by the P13G13 genotype allow us to recommend its use for biomass production aimed at bioenergy, favoring sustainability, reducing greenhouse gas emissions, and dependence on synthetic nitrogen fertilizers.

Keywords: nitrogen; dry matter; ^{15}N natural abundance; soil fertility; energy plants; genotype

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1. Introduction

Energy is fundamental to human activities and plays an important role in global development by providing goods and services. The growing global demand for energy, driven by population growth, raises concerns about the environmental impacts caused by fossil fuel combustion [1,2]. As a result, the search for renewable energy sources proves to be essential, with plant biomass being an excellent option [3]. Another concern in this scenario is the search for biofuels from energy crops. Recent studies with C_4 grasses, such as sorghum, sugarcane, and elephant grass, among others, indicate that it may be possible to produce biofuels in a sustainable way that could compete with petroleum-based fuels [4,5].

Elephant grass (*Pennisetum purpureum* Schum) is a crop highly efficient in atmospheric CO_2 fixation during the photosynthesis process, demonstrating the potential and quality to be used as an alternative energy source, as the burning of its biomass does not contribute to global warming [6]. Additionally, its high productive potential is complemented by the possibility of performing more than one cut throughout the year, and being a vegetatively propagated crop, it highlights the crop's potential for biomass productivity, alternative energy production, energy applications such as direct combustion, gasification, charcoal production, and bagasse hydrolysis [7–9].

It is a perennial crop belonging to the Poaceae family and originating from the African continent, which has adapted well in Brazil due to the edaphoclimatic characteristics present in the country. Elephant grass is cultivated practically throughout the Brazilian territory, presenting good biomass productivity rates per year [7,10]. Productivity success, however, can be highly influenced by the choice of cultivar, the characteristics of the production region, and the management adopted by producers [11]

Elephant grass bagasse is similar to sugarcane bagasse, consisting of 65% fiber and 35% non-fibrous material. Annual biomass production of elephant grass can reach 50 Mg ha^{-1}, provided efficient genotypes are used and the region's edaphoclimatic conditions, as well as crop management, are favorable [12]. The selection of an ideal plant species as a bioenergy source considers two criteria: a high capacity to accumulate biomass and good crop development in environments with nitrogen (N) deficiency or without the need for high nitrogen fertilization, as the use of nitrogen fertilizer increases production costs and poses environmental risks when applied under inappropriate conditions [6]. Additionally, it is crucial that the energy produced by biomass burning exceeds the fossil energy used in its production, as a positive energy balance is achieved when more biomass is produced without increasing the costs of inputs and agricultural operations.

The excessive use of synthetic nitrogen fertilizers compromises the soil and agricultural sustainability, with biological nitrogen fixation (BNF) being an alternative to overcome this problem [13]. BNF in crops increases with the reduction in nitrate concentration in the soil and plant, and in addition, good moisture, light, and adequate soil pH conditions favor the BNF process, leading to better crop development [6,14]. Several studies focused on BNF in poaceae have indicated the efficiency of this process and its contribution to productivity in other crops, in addition to elephant grass, such as sugarcane, maize, and wheat [15–20]. In these cases, BNF associated with crop development in low-fertility soils demonstrated a positive response to the fixation process.

The recognition of elephant grass as an alternative energy source began with studies on BNF associated with crop development in low-fertility soils. This is a crucial issue for both the economy and environmental preservation, as fossil energy sources are finite and contribute to environmental pollution, driving the increase in the greenhouse effect [21]. Elephant grass is an alternative due to its high photosynthetic efficiency, high dry matter accumulation, and BNF [22].

Although the use of elephant grass biomass as an energy source is already known [7–9,23,24], there is still a need for the development of specific genotypes for this purpose, which present characteristics different from those traditionally selected for animal feed. Evaluating new genotypes of elephant grass for energy purposes allows for the selection of those with high productivity characteristics combined with low production costs, resulting in a positive energy balance for the crop.

Depending on the genetic characteristics of each material tested, the response of BNF on productivity is expected to be more or less efficient. The hypothesis raised in this study is that the BNF process is the main external nitrogen source in the cultivation of ten elephant grass genotypes, allowing for high productivity rates without the use of nitrogen fertilization. Therefore, this study aimed to evaluate the fresh mass and dry matter productivity of ten selected elephant grass genotypes for energy use, quantify the total N accumulation from the plant, and quantify the BNF contribution to the nitrogen nutrition of elephant grass using the ^{15}N natural abundance technique.

2. Materials and Methods

2.1. Plant Material and Experimental Conditions

This study was carried out in the experimental area of Embrapa Agrobiologia, in Seropédica- RJ, Brazil (22°44′38″ S, 43°42′28″ W and 26 m above sea level). The region is characterized by the Aw climate, according to the Köppen classification, with dry winters and hot and rainy summers, with temperatures that can vary between 16 and 34 °C and annual precipitation averaging 1224 mm. The elephant grass genotypes evaluated in this study come from the elephant grass breeding program of Embrapa Gado de Leite, Juiz de Fora, Minas Gerais (P3G1, P5G3, P6G4, P11G7, P14G10, P13G13, P21G15, P26G18, P27G19 and the cultivar BRS CAPIAÇU). Precipitation and temperature data during the experimental period are presented in Figure 1.

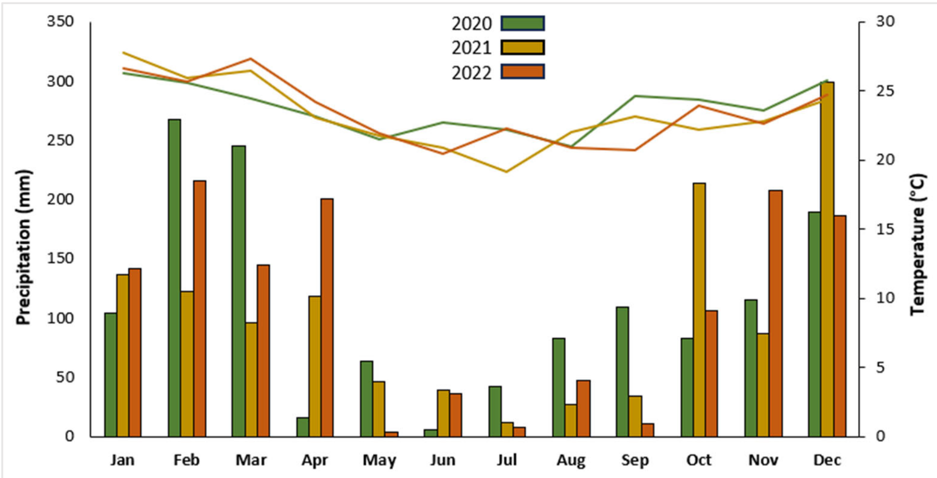

Figure 1. The average annual precipitation and temperature in the Seropédica, RJ (Brazil) from 2020 to 2023.

This experiment was carried out in a closed concrete tank with an area of 120 m^2 and 40 cm deep, located in the field (open air), filled with soil classified as Acrisol (WRB/FAO) or Typic Hapludult (USDA, Soil Taxonomy), labeled with ^{15}N [25] with the following properties: OM (organic matter), 21 g kg^{-1}; $pH_{(H20)}$, 5.7; total N, 1.1 g kg^{-1}; C:N ratio, 11.1; available P (Mehlich I), 11 mg L^{-1}; and exchangeable cations (cmol dm^3): Ca^{2+}, 3.3; Mg^{2+}, 0.6; K^+ 0.082 and Al^{3+}, 0,0. Sand, 800 g kg $^{-1}$; silt, 36 g kg^{-1}; and clay, 164 g kg^{-1}.

The experimental area was fertilized with 150 kg ha^{-1} of K_2O before planting and 100 kg ha^{-1} of K_2O, 200 kg ha^{-1} of P_2O_5, 200 kg ha^{-1} of fritted trace elements (FTE BR12) with a composition of 18 B, 8 Cu, 30 Fe, 20 Mn, 1 Mo, and 90 Zn (g kg FTE^{-1}) at planting. Topdressing fertilization was performed 77 days after planting, applying 100 kg ha^{-1} of K_2O, 15 kg ha^{-1} of $CuSO_4$, and 50 kg ha^{-1} of FTE BR12. After each elephant grass cut, 1 Mg ha^{-1} of limestone, 200 kg ha^{-1} of P_2O_5, and 180 kg ha^{-1} of K_2O were applied. No synthetic nitrogen fertilizer was used to encourage the biological nitrogen fixation (BNF) activity within the crop.

Planting was carried out using seed setts, each containing at least three viable buds. At the time of planting, the setts were placed in two rows within the furrow to ensure a uniform plant population. Planting was carried out in February 2020. The experimental design was randomized blocks with four replications. The plot consisted of a 3 m planting row with 1 m spacing between rows. Each plot had a treatment consisting of the nine genotypes evaluated and the control (BRS Capiaçu). This study was conducted for three years, and harvests occurred every 6 months of cultivation in the following periods: September/2020 (1st), March/2021 (2nd), September/2021 (3rd), March/2022 (4th), September/2022 (5th), and March/2023 (6th). The results obtained were presented by year, grouping two cuts per year. The variables evaluated were fresh mass productivity (FM); dry matter productivity (DM), in Mg ha^{-1} $year^{-1}$, of the whole plant; accumulated N content in dry matter, in kg ha^{-1} $year^{-1}$, contribution of BNF (%) and N derived from BNF.

2.2. Determination of Fresh Mass and Dry Matter Productivity

The evaluation of the total fresh mass productivity (FM) of the aerial part of elephant grass was conducted at the end of each cut and carried out every six months. The aerial parts of the plants that make up the plots were cut and weighed in the field to obtain the total weight of each planting line and the FM yield of elephant grass. Then, four plants from the planting line were randomly selected and fractionated into straw, stem, and green leaf, and immediately after, subsamples were taken from these fractions, weighed to determine the fresh mass of the subsample, followed by drying in a forced circulation oven at 65 °C for 72 h, and after the drying process, the subsamples were weighed again to obtain the dry matter productivity (DM) of the subsample. From the fresh mass and dry matter values of the subsamples, it was possible to calculate the DM total of elephant grass.

2.3. Determination of Accumulated N in Plants

The oven-dried subsamples were pre-ground in a Willey mill (2 mm) to determine the total N content of the plant tissue using the CHNSO elemental analysis technique. Nitrogen (N) accumulation was quantified in the different fractions of the grass plants, identified as stem, green leaf, and straw. N accumulation was calculated from the product of the dry mass and the N concentration of each fraction, and the total accumulated N was represented by the sum of the three separate plant fractions of each plant (straw, green leaf, and stem).

2.4. Determination of the Contribution of BNF and BNF-Derived N

The quantification of BNF in this study was performed using the ^{15}N Natural Abundance technique [26]. In this case, it is necessary to know the ^{15}N natural abundance value of the available N in the soil and the ^{15}N natural abundance value of the fixing test plant (elephant grass). The elephant grass samples, previously ground in a Willey knife mill (2 mm) in the experiment, were subjected to a second grinding in a roller mill until they reached the appropriate particle size (samples with a talc appearance).

An additional study was conducted at two different times, in a greenhouse, with the aim of determining the natural abundance value of ^{15}N of the available N in the soil by cultivating non-N-fixing test plants Millet (*Panicum miliaceum*), Pearl millet (*Pennisetum glaucum*), and Sorghum (*Sorghum bicolor*). These non-N-fixing plants were chosen as references in this study because they have characteristics similar to those of elephant grass, especially with regard to high dry mass production, nutritional requirements, root system, hardiness, and adaptation to the same environmental conditions. Thus, the cultivation of the reference plants was carried out in a way that resembled the cultivation of elephant grass as much as possible. The plants were grown in 400 mL pots filled with the same soil as the concrete tank and grown until they reached soil N depletion, observed from signs of leaf chlorosis (approximately 40 days). The plants were cut and subjected to the drying process in an oven and grinding in a roller mill, following the methodology used in the elephant grass plant samples.

The samples were analyzed in a Delta V Advantage isotope ratio mass spectrometer coupled to a Flash 2000 elemental analyzer (Thermo Fisher Scientific, Waltham, MA, USA) to obtain the natural abundance values of ^{15}N of the samples. The calculation of the percentage contribution of BNF was performed by applying the formula:

$$\%\text{Ndfa} = 100\left(\delta^{15}N_{\text{ref}} - \delta^{15}N_{\text{fifixing\_plant}}\right) / \left(\delta^{15}N_{\text{ref}} - B\right) \quad (1)$$

where $\delta^{15}N_{ref}$ is the δ ^{15}N value of the available soil N obtained from the non-fixing control plants, used as a reference; $\delta^{15}N_{fifixing\ plant}$ is the δ ^{15}N value of the N_2-fixing test plant; B is the value of the ^{15}N isotopic discrimination during the BNF process. In this study, the B value will be considered as zero [15].

The values of accumulated N derived from BNF were calculated from the fixation rate and the total N accumulated by the plants.

2.5. Statistical Analyses

The experimental design used was a randomized block design, with 4 blocks and 10 treatments. This design was chosen to ensure repetition, randomization, and local control, as it is a field experiment where variations in the position of each treatment may occur. Examples of such variations include the spatial variability of organic matter, mineral nitrogen in the soil, soil fertility, and physical–hydric attributes of the soil. The use of this design minimizes the influence of these factors on crop development and BNF, allowing the effects of the treatments to become more evident.

Randomization meets one of the assumptions of analysis of variance (ANOVA), ensuring that the data were collected independently, without any influence from one experimental unit on another. Additionally, before performing the analysis of variance, we checked whether the data followed a normal distribution using the Shapiro–Wilk normality test with a significance level of 5%. The data dispersion within each group was also examined using Bartlett's test for homogeneity of variances, with a significance level of 5%.

After verifying the assumptions for ANOVA, the data were subjected to analysis of variance, and when a significant effect of the treatments was observed through the F-test, the means were compared using Tukey's test at a 5% significance level. This test was chosen because it offers a good balance between error control and detection power, in addition to being suitable for comparing all possible combinations of groups, as is the case in the present experiment with multiple genotypes. The data were analyzed using RStudio software (R Development Core Team, 2018), and SigmaPlot 11 (SystatSofware Inc., 2007) was used to generate the graphical elements.

3. Results

3.1. Fresh Mass Productivity and Dry Matter Productivity

The effect of BNF on elephant grass cultivation resulted in high FM and DM indices during the evaluation period, even under unconventional cultivation conditions. The average FM during the rainy season was 40 Mg ha^{-1} in the three years of this experiment, except in the period from March to September 2020, when, despite the dry season, productivity was exceptionally high, with an average FM above 45 Mg ha^{-1}. A variation in the averages of each genotype was observed from one cut to the next, with a pattern of higher and lower productivity depending on the season of the year, with the rainy season being the most productive for elephant grass FM (Figure 2).

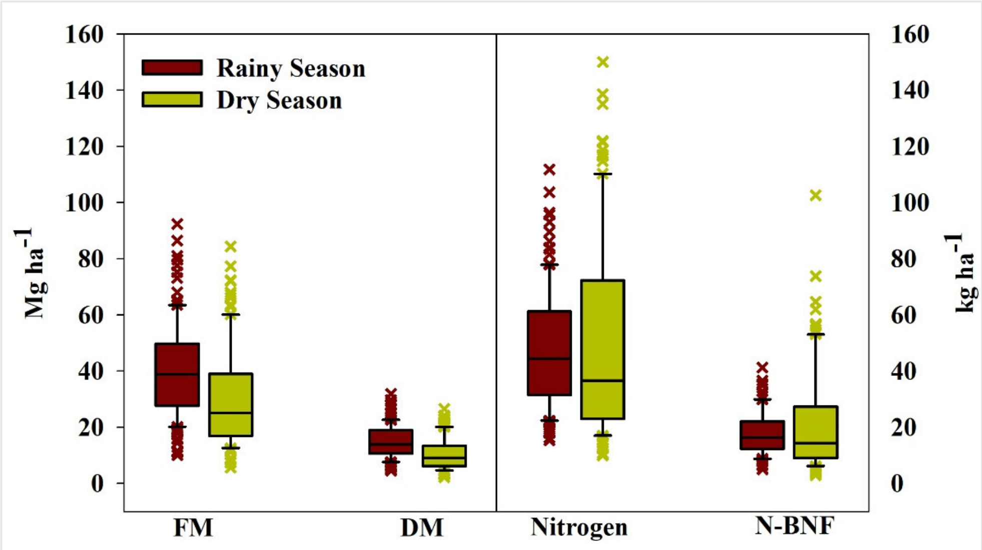

Figure 2. Distribution of fresh mass, dry mass, nitrogen, and N derived from BNF evaluated in two seasons of the year (dry and rainy seasons).

Considering the time of year with the best productivity response, it is observed that climate change directly interferes with elephant grass production, influenced by factors such as temperature, precipitation, and photoperiod. During the rainy season, the FM and DM response was considerably more positive, as elephant grass performs better at high temperatures, requires a large amount of water, and has more expressive growth during longer days.

The genotypes P13G13, BRS CAPIAÇU, P11G7, P21G15, and P27G19 showed the highest total productivity of fresh mass (Figure 3) compared to the other genotypes, with a significant effect of the treatments for this variable. The annual average of genotype P13G13 differed statistically from the others over three years, showing itself to be a promising genotype for production under cultivation conditions similar to those of the present study.

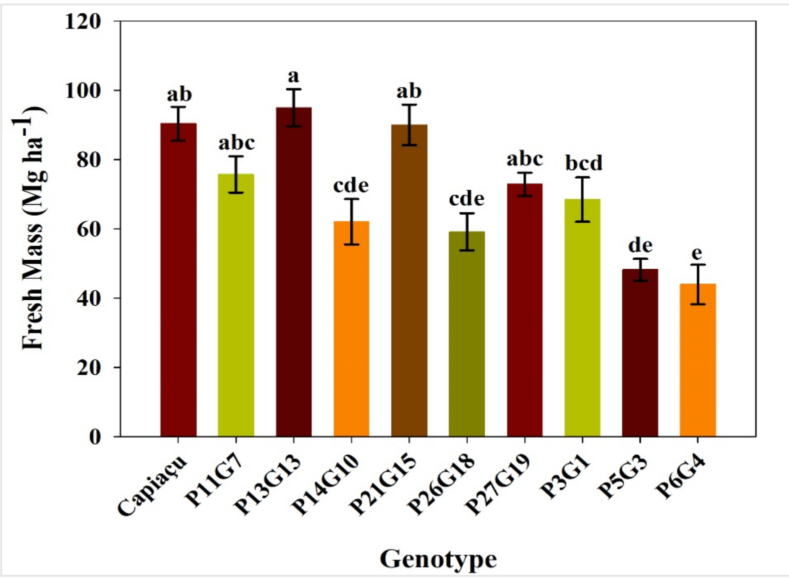

Figure 3. Fresh mass (FM) of elephant grass grown in ^{15}N-labelled soil for six crop cycles (Mg ha^{-1}). Means followed by the same letter or without a letter do not differ from each other at a 5% probability level ($p < 0.05$) using the Tukey test.

The average annual accumulation of total dry matter (biomass) (Figure 4) was equivalent to 30% of the PMF. Total dry matter productivity followed the same trend observed for PMF, reflecting the direct influence of fresh mass productivity on DM. There was a significant effect of the treatments on biomass accumulation, with the genotypes P13G13 and BRS CAPIAÇU reaching the highest DM averages in the three years of cultivation, with values above 30 Mg ha^{-1} in the year.

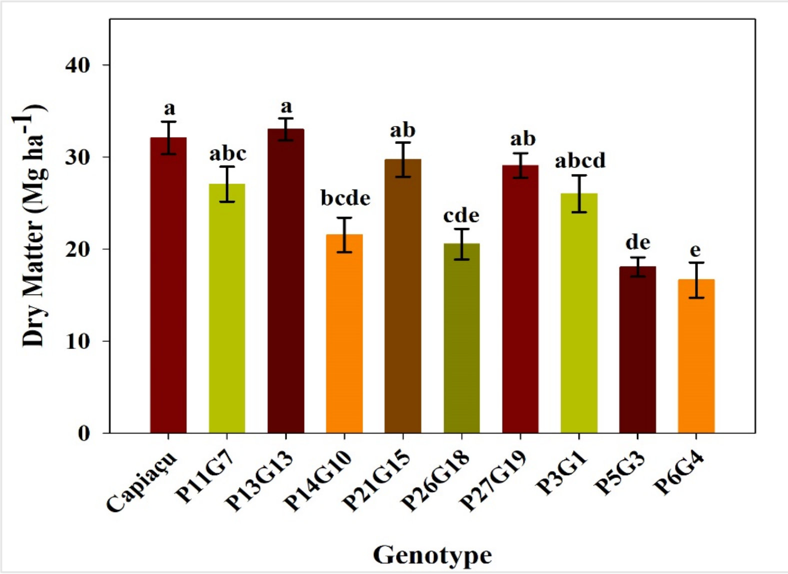

Figure 4. Dry matter accumulation of elephant grass grown in ^{15}N-labelled soil for six crop cycles (Mg ha^{-1}). Means followed by the same letter, or without a letter, in the same columns do not differ from each other at a 5% probability level ($p < 0.05$) using the Tukey test.

Plant genetics and BNF rate were considered determining factors for the productivity achieved in each cycle. An important factor related to DM is the amount of water present in the plant, which directly influences the accumulation of dry matter. Different genotypes may present high or low DM depending on their genetic characteristics. In the present study, a variation in DM of 15 to 30 Mg ha^{-1} in the year was observed.

The P13G13 genotype stands out compared to lower-performing treatments, such as the P6G4 genotype, which presented an annual average of 50% lower. In addition, the P5G3 and P26G18 genotypes also demonstrated lower efficiency in dry mass accumulation when compared to P13G13. This performance pattern among the genotypes was also observed in fresh mass productivity, as previously described.

3.2. Accumulated N Content, Contribution of BNF to Nitrogen Nutrition and N Derived from BNF

Nitrogen (N) accumulation in plants followed the same trend observed in the fresh mass (FM) and dry matter (DM) productivity variables among the ten genotypes evaluated, with fluctuations in the average N accumulation values throughout the cycles, depending on the season (Figure 2). N accumulation was determined based on the dry matter of elephant grass and the N concentration in the samples. Despite the annual variations in N accumulation, the total accumulated N (Figure 5) corresponded to the expected, aligning with the trend observed in dry matter accumulation. In the first year, the average N accumulation exceeded 130 kg ha^{-1}. In the following years, there was a reduction of approximately 35% in N accumulation, similar to what was observed in DM, which also showed a 30 to 35% decrease in productivity over the two subsequent years.

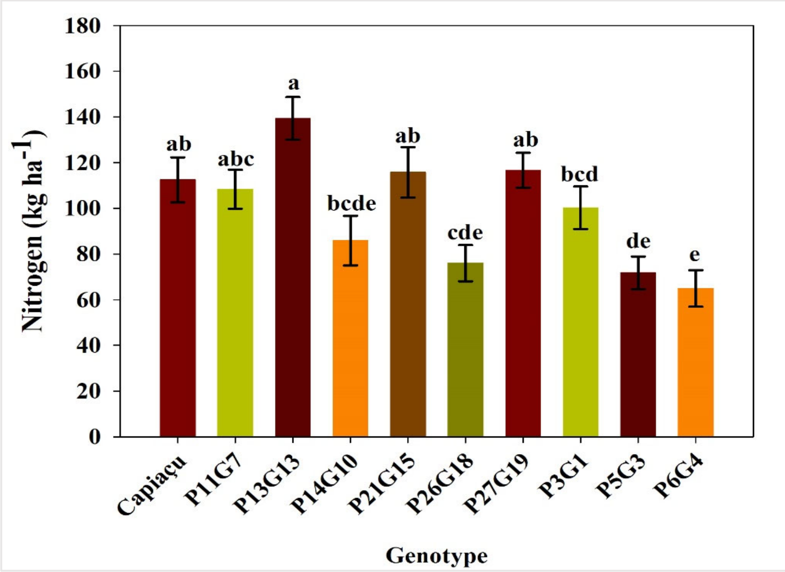

Figure 5. Accumulation of nitrogen by the elephant grass grown in ^{15}N-labelled soil for six crop cycles. Means followed by the same letter, or without a letter, in the same columns do not differ from each other at a 5% probability level ($p < 0.05$) using the Tukey test.

The BNF rate was calculated using the equation described in the methodology, which compared the abundance of ^{15}N in elephant grass plants (Table 1) with the abundance of ^{15}N non-N_2-fixing plants grown in a greenhouse, thus determining the contribution of biologically fixed N. The natural abundance values of ^{15}N of the three non-N_2-fixing plant species grown in a greenhouse were used to determine the abundance of ^{15}N of available soil N. For each crop cycle, an average value of $\delta^{15}N$ was calculated among the three reference plants, and this average was used to quantify the BNF. The ^{15}N abundance in the reference plants, used to calculate the BNF (%), was 46.0825 for the first, second, and third cycles and 43.43104 for the fourth, fifth, and sixth cycles.

Table 1. Values of ^{15}N abundance (‰) of elephant grass grown in ^{15}N-labelled soil for three years.

GENOTYPE	2020/21	2021/22	2022/23
BRS CAPIAÇU	29.71481	26.150755	27.67077
P11G7	29.64103	27.40141	31.52896
P13G13	29.547055	24.891255	27.99227
P14G10	30.388415	26.943885	27.427755
P21G15	28.244895	25.22509	28.67502
P26G18	29.77144	23.40055	26.509755
P27G19	28.97286	25.914805	29.194425
P3G1	28.12682	23.76335	26.551095
P5G3	28.753235	24.959655	26.08045
P6G4	29.31908	23.72163	24.44017
MEAN	29.247964	25.2372385	27.607067
SD	0.72	1.37	1.94
CV	2.51	5.43	7.03

Values of table represent the ^{15}N abundance (‰) means of elephant grass grown in ^{15}N-labeled soil over 3 years of cultivation. The coefficient of variation (CV) is expressed as a percentage, and the standard deviation (SD) is expressed in ‰.

Regarding N derived from BNF (Figure 6), a significant effect was observed among treatments for the ten elephant grass genotypes over the three years of cultivation. The average contribution of BNF reached 43% among genotypes during the studied period. BNF proved to be efficient for the growth and nutrition of elephant grass, with variations in the BNF rate over the years possibly related to environmental conditions. The observed BNF rates were sufficient to contribute significantly to elephant grass productivity under the experimental conditions.

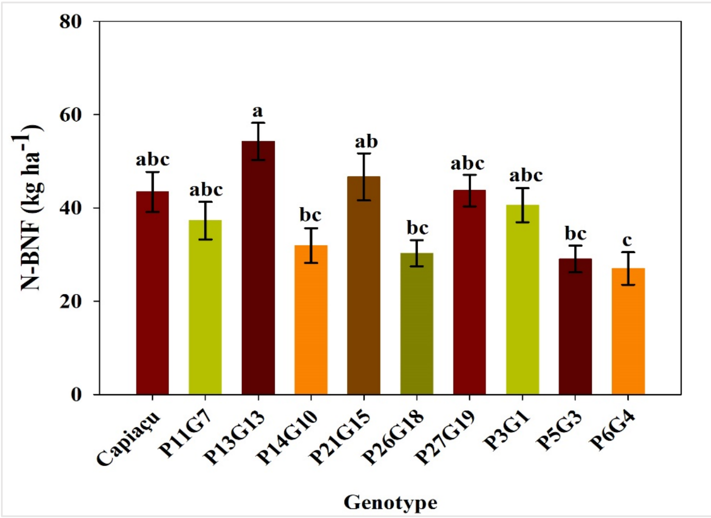

Figure 6. Total N derived from BNF elephant grass grown in ^{15}N-labeled soil for six crop cycles. Means followed by the same letter, or without a letter, in the same columns do not differ from each other at a 5% probability level ($p < 0.05$) using the Tukey test.

The correlation analysis (Figure 7) revealed a positive relationship between total N and mass fresh and dry mass variables. This suggests that increased absorption and concentration of total N, as well as N derived from BNF, contribute to higher elephant grass productivity. However, no correlation was observed between the BNF rate (%) and total N, fresh mass, or dry mass. This implies that the BNF percentage, on its own, does not directly influence the productivity of fresh mass, dry mass, and total N accumulation.

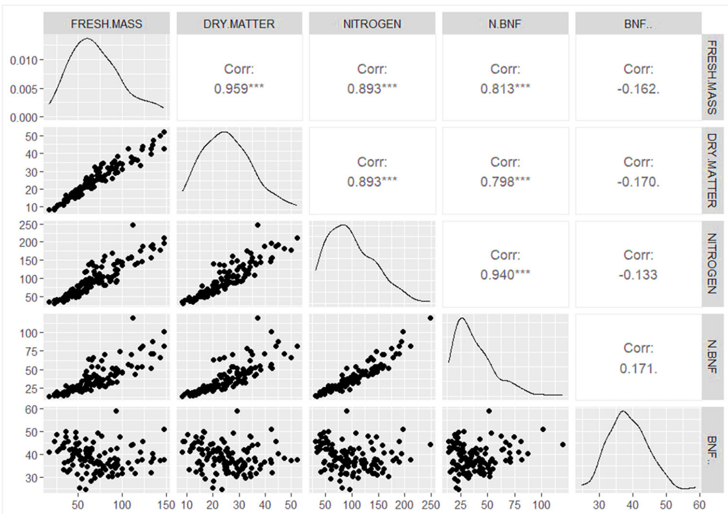

Figure 7. Correlogram between the variables evaluated (Fresh mass, dry mass, nitrogen, BNF (%), and N derived from BNF) in different elephant grass genotypes during three production cycles. Results statistically significant at the 5% level ($p \leq 0.05$) are marked with (***).

4. Discussion

4.1. Fresh Mass and Dry Matter Productivity

The good performance of elephant grass crops grown in N-poor soils associated with BNF is related to the environmental and management conditions to which it is subjected [6,14]. Although the crop shows positive growth and development responses from increasing nitrogen doses [27], there are studies that aim to understand the functioning and evaluate the productivity of poaceaes from BNF as the main source of N for crop nutrition [15,28,29]. Elephant grass is highly efficient in accumulating dry matter when grown under adequate management conditions, in addition to being highly responsive to increasing nitrogen doses, depending on the genotype [15,28].

As a rustic crop, elephant grass stands out among the poaceae due to its great potential for dry matter production in tropical regions [28]. Its ability to produce biomass, even under conditions of low soil N availability, is associated with interactions between microorganisms present in the soil that promote plant growth from BNF [21,24]. A study carried out in the experimental field of Embrapa Agrobiologia between 2009 and 2010 demonstrated the behavior of elephant grass when inoculated with diazotrophic bacteria. Endophytes developed for sugarcane, in this case, the inoculant, did not present a significant effect on the nitrogen fixation rate for elephant grass plants, which reinforces the idea that the interaction between the crop and soil diazotrophic microorganisms occurs with a certain specificity and independently [24].

In the present study, the biomass productivity values of elephant grass achieved were attributed to two main factors: the nutritional demand of the plants in relation to nitrogen and the genotypes used in this study. In an experiment conducted by Zanetti et al. [21], average biomass productivity values of 50 Mg ha^{-1} year^{-1} were found when two different genotypes of elephant grass were cultivated in Seropédica-RJ (Brazil) in 2009 and 2010. Morais et al. [28] obtained averages of 25 Mg ha^{-1} in an experiment with seven genotypes. It is noted that some elephant grass genotypes may be more adapted to certain soil and climate conditions than others, and therefore, the inadequate choice of genotypes may influence the high and/or low productivity of PMF and total PB of the aerial part.

4.2. Accumulated N Content

As observed in other crops, such as sugarcane, the amount of N that can be accumulated by plants throughout the crop cycles must take into account the availability of nutrients in the system, the type and texture of the soil, the climatic conditions, the availability of water, the genetics of the plant, among other factors [30,31]. Therefore, it is clear that there is a need to combine these factors so that the final response is positive in relation to crop productivity under low fertility conditions, as is the case of the experiment carried out in this work.

Elephant grass can benefit from BNF in its nitrogen nutrition, increasing and/or maintaining its productivity index even when grown in low-fertility soil [6]. It is known that nitrogen is a determining nutrient for good plant development [32] and that the inadequate use of fertilizers can generate environmental damage in the long term, making it necessary to find ways to meet the demand for N with more sustainable alternatives [21,24]. BNF, as the main source of N, can generate good gains in crop productivity for energy purposes, unlike when its use is oriented toward animal nutrition, without additional cost due to the use of fertilizers in production and reducing environmental risks arising from the inadequate use of synthetic fertilizer, as observed in this study.

Nitrogen has a fundamental role in crop productivity, and for this reason, higher fresh and dry matter production may be associated with greater N absorption [33]. Therefore, the amount of N increases total biomass productivity [34]. When nitrogen is obtained through

BNF, its contribution to increased productivity is determined by the BNF rate achieved. However, an increase in the BNF rate does not necessarily raise the total N concentration in the plant but rather the proportion of N derived from BNF, thereby reducing dependence on synthetic nitrogen fertilizers and making cultivation more sustainable [13,33].

4.3. ^{15}N Natural Abundance and Contribution of BNF to Nitrogen Nutrition

Using the ^{15}N natural abundance technique, it is possible to accurately evaluate the BNF rate in the field [26,27,35]. Applying this technique, Morais et al. [28] evaluated four elephant grass genotypes (Gramafante, Camarões, Bag 02, and Roxo) and obtained an accumulation of N between 36 and 132 kg N ha^{-1} year^{-1} from BNF, evidencing the high potential of the crop to benefit from BNF.

One of the criteria used in the methodology of the ^{15}N natural abundance technique is based on the requirement that the N-fixing test plants were grown under similar conditions to the non-fixing reference plants [27,29]. The present study was carried out in compliance with the methodological requirements, adapting the cultivation conditions, especially the soil characteristics, to ensure that the results were consistent with those found in previous studies. In this study, it was considered that the ^{15}N natural abundance value of the available N in the soil is equal to that of the non-fixing reference plants.

In a study on the contribution of BNF in elephant grass, it was stated that the evidence of BNF can be attributed to the fact that elephant grass plants have natural abundance values of ^{15}N lower than that of the invasive plants used in the experiment as a reference [28]. The natural abundance of ^{15} N was also described for varieties of sugarcane, corn, sorghum, and guinea grass in a study that aimed to measure the efficiency of BNF in these plants. In this specific study, the abundance of ^{15}N remained higher than the reference plants for all plants, evidencing that the N absorbed by them came from atmospheric N_2 [29]. These findings corroborate the data of the present study and demonstrate the importance of BNF as a source of nitrogen for the ten elephant grass genotypes evaluated since the natural abundance values of ^{15}N of elephant grass plants were significantly lower than those of the reference plants, evidencing a contribution of approximately 38% of BNF in the nitrogen nutrition of ten elephant grass genotypes cultivated throughout this study.

4.4. Influence of BNF on Elephant Grass Productivity

BNF performance is associated with the selection of diazotrophic bacteria and efficient genotypes [36], but there is a need to better understand the behavior and interaction between diazotrophic bacteria and elephant grass plants and how the association between them occurs [28]. Elephant grass depends on associations with diazotrophic bacteria to fix atmospheric nitrogen [33], with the genus Azospirillum being the most abundant among the group of associative bacteria [22,37,38]. These associations can occur in the rhizosphere, on the surface of roots, and inside plant tissues. Several studies have identified species responsible for BNF in Poaceae, including *Gluconacetobacter diazotropicus*, *Herbaspirillum seropedicae*, and *Azospirillum amazonense* [33,39–42]. In other poaceae plants, such as sugarcane and rice, the association of diazotrophic bacteria was observed and shown to have influenced the production of dry mass and nitrogen accumulation of plants due to high rates of BNF [19,25].

Among the genetic factors influencing elephant grass productivity through BNF, photosynthetic efficiency and root morphology stand out. The first is related to the plant's ability to capture light energy and convert it into carbon during photosynthesis, directly impacting its growth and development [43,44]. The second refers to the plant's interaction with soil microorganisms, especially the diazotrophic bacteria responsible for BNF. A more robust and deeper root system improves water and nutrient absorption and, most importantly, strengthens the relationship between roots and bacteria, making BNF more efficient [44].

Several studies on BNF quantification have shown that elephant grass was influenced by the biological process in question. The BNF rates found by some authors ranged from 30 to 35% [36], 20 to 50% [24], and 30 to 42% [28], where different genotypes were evaluated under cultivation conditions without the use of nitrogen fertilization, highlighting the biological nitrogen fixation capacity of elephant grass in low fertility soil.

The dry mass and nitrogen accumulation derived from BNF of the genotypes were higher when compared to previous studies or conventional crops, and the BNF rate remained similar to what has been found in the literature for this crop. Therefore, at least four of the nine genotypes tested, in addition to the control (BRS Capiaçu), responded positively with high BNF rates, as being a very important source of N, resulting in satisfactory FM, DM, and nitrogen accumulation for their development.

In the long term, BNF can enhance soil fertility, reduce reliance on synthetic nitrogen fertilizers, and consequently lower production costs [45]. However, the success of BNF depends on proper soil management and an adequate supply of nutrients such as phosphorus, potassium, and micronutrients. Additionally, sustainability is best achieved when production systems adopt agricultural practices that help maintain soil fertility, such as crop rotation or intercropping with legumes [46]. Compared to synthetic nitrogen fertilization, BNF presents itself as a more sustainable and cost-effective strategy. However, for highly demanding crops, nitrogen fertilization may still be necessary [47]. In such cases, it is essential to combine both agricultural production approaches to optimize cost reduction and minimize environmental impact.

5. Conclusions

The contribution of Biological Nitrogen Fixation (BNF) in the evaluated genotypes was 38%, which positively influenced biomass production and nitrogen accumulation in elephant grass grown in soil with low nitrogen availability and without the application of synthetic nitrogen fertilizers. In this study, average yields of 30 Mg ha^{-1} $year^{-1}$ of total dry matter from the aerial part and 100 kg ha^{-1} $year^{-1}$ of accumulated nitrogen were recorded. Considering the results of fresh and dry mass productivity, total N accumulation, and N derived from BNF obtained under the experimental conditions of this study, the genotype P13G13 has its use recommended for the production of biomass for bioenergy, especially when BNF is the main external source of N in production. These findings support the hypothesis that BNF can serve as a significant nitrogen source for elephant grass cultivation, promoting high productivity and reducing the need for synthetic nitrogen fertilizers in bioenergy production systems. Future studies involving other elephant grass genotypes, grown in different soil types, under different climatic conditions, and subjected to different production systems, may provide a better understanding of the efficiency of BNF in meeting the crop's nitrogen demand and how BNF behaves in different scenarios.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy15030605/s1, Table S1: Mean values of fresh mass, dry matter, total nitrogen and BNF-derived nitrogen over three years.

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