Nitrogen Pools in Tropical Plantations of N₂-Fixing and Non-N₂-Fixing Legume Trees under Different Tree Stand Densities

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Abstract: We investigated the nitrogen pools in monocultures of legume species widely used in reforestation in Brazil that have contrasting growth and nitrogen acquisition strategies. The plantations were established with the slow-growing and N₂-fixing tree *Anadenanthera peregrina* var. peregrina, and the fast-growing and non-fixing tree *Schizolobium parahyba* var. amazonicum. The measurements of N pools in the tree biomass and the soil followed standard methods and were carried out on 54 experimental plots. The N₂ fixation pools were evaluated by abundance natural of ¹⁵N and the N accretion methods. The soil N content was of similar magnitude between species and stand densities. The species showed similar amounts of N in the biomass, but divergent patterns of N accumulation, as well as the ¹⁵N signature on the leaves. *S. parahyba* accumulated most N in the stem, while *A. peregrina* accumulated N in the roots and leaves. However, the N accumulation in biomass of *A. peregrina* stand was less constrained by environment than in *S. parahyba* stands. The percentage of N derived from N₂ fixation in *A. peregrina* stands decreased with the increase of stand density. The biological N₂ fixation estimates depended on the method and the response of tree species to environment.

Keywords: forest plantation; forest restoration; N₂ biological fixation; functional traits; ¹⁵N natural abundance; N balance

1. Introduction

The expansion of forest restoration is expected for this decade in tropical regions as one important way to improve many ecosystem services [1,2]. The Atlantic Forest biome has experienced an increase in forest cover (forest transition), with the new land-cover map revealing the cover of 28% of native vegetation [3], 12–17% higher than the previously available estimates [4]. In addition to socioeconomic drivers, such as farm credit and economic development, part of this expansion was attributed to Atlantic Forest remnants, but also the vicinity of eucalyptus plantations [5].

The Brazilian forestry sector has improved planted areas and businesses [6]. However, the sustainability of plantations faces challenges related to tree growth and survival affected

by biotic and abiotic constraints [7,8]. Moreover, the silvicultural behavior of tree species native to the Brazilian flora is less known than for exotic commercial species of the genera *Eucalyptus* and *Pinus* [9–11]. The choice of the tree stand density is a relevant question that directly affects biomass and nutrient accumulation. Furthermore, the response to competition is highly variable as a function of the demands for growth resources [12]. As pointed out by some authors [13], the fast-slow plant economics spectrum predicts that faster-growing species has a large biomass accumulation rate per individual trees, and nutrient uptake and water consumption (high-rate acquisition resources), while the resistance to environmental stress is in general higher from slow-growing species (with higher tissue density, long tissue lifespan and lower rates of resource acquisition) [13].

The forest expansion in Brazil and other tropical countries have occurred on previous cropland and grassland, and these areas are often degraded [5,7]. Biomass production and tree survival are limited by N availability in the soil, which is intimately associated with soil organic matter pools. N is abundant in the atmosphere (78%) but under this chemical form (N_2) is unavailable for most plants [14]. Legume trees are most abundant in the tropics and several tree species are used for wood production and forest restoration. The use of legume tree species capable to form an efficient symbiosis with diazotrophic bacteria strains (N_2-fixing trees), is considered an important strategy to increase N transfer from the atmosphere to the soil [15–19] and for companion plants [20,21]. Thus, the use of these species can reduce costs with nitrogen fertilizers in forest restoration or commercial plantations while increasing organic matter accumulation in the soil [18,22,23].

The percentage of N derived from atmosphere (%Ndfa), i.e., biologically fixed, can be affected by soil N availability and plant competition [24,25], also being influenced by stand density [26], as well as by phosphorus [27], iron [28], and other nutrients [29]. Furthermore, the growth rate of the species and the age of the trees are other factors that influence the N_2 fixation rate [30,31]. The %Ndfa may oscillate from inexistent to more than 90% in tree species, including drought-adapted species [32,33]. Many methodologies were developed to estimate biological nitrogen fixation [34,35], each one with its potential and limitations [32,36,37]. The ^{15}N natural abundance method is the most used technique to estimate the %Ndfa in undisturbed stands and natural forests, being relatively easy to apply and less sensitive to the choice of reference plant than enriched methods [37], besides to show relatively good accuracy [33,38]. Another technique commonly applied for its versatility about the components used (forest floor litter, soil, and biomass) and its low estimation costs is the N accretion method, calculated based on the differences in N accumulated in fixing and non-N_2-fixing species [35,39]. Although both methods facilitate quantification, few studies employ both techniques simultaneously [19], in Brazilian conditions.

This study aimed to quantify the N accumulated in the biomass and topsoil of monocultures of *Anadenanthera peregrina* (L.) Speg. var. peregrina and *Schizolobium parahyba* var. amazonicum (Huber ex Ducke) Barneby grown under three stand densities (625, 1111, and 1666 trees ha^{-1}), and to estimate the percentage of N derived from biological N_2 fixation for *A. peregrina* using the ^{15}N natural abundance and the N accretions methods. These two species are among the most planted in Brazil, for both commercial and restoration purposes, with stands of *S. parahyba* occupying an area of about 90 thousand ha in northern Brazil. This faster-growing species presents a low-density wood (0.30 g cm^{-3}), has natural distribution throughout the Amazonian region and Atlantic Forest [40], and belongs to Fabaceae family and Caesalpinioideae subfamily, being considered a non-nodulation species [41]. The slow-growing *A. peregrina* presents a wood of higher density (0.66 g cm^{-3}) [42] and distribution in all Brazil, and belongs to Mimosoideae subfamily, being capable of developing symbiosis with N_2-fixing bacteria [43]. The research hypotheses were: (1) The N_2-fixing legume tree species accumulates more N in the biomass and the topsoil layer than the non-N_2-fixing tree species, regardless of stand density. (2) The stand densities affect the N_2 fixation in the *A. peregrina*. (3) The ^{15}N natural abundance and the N accretions methods generate similar fixed N estimates regardless of stand density.

2. Results

2.1. N Pools in the Biomass and Soil

The mean values of N concentration were in general larger for all tissues of *A. peregrina* than *S. parahyba*, with noticeable differences for leaves (22.3 versus 13.3 g kg^{-1}), bark (4.0 versus 1.8 g kg^{-1}), and roots (9.0 versus 3.9), and smaller differences for branches (5.8 versus 3.6) and wood (1.8 versus 1.7). The N concentration and content in the biomass of each species were not affected by stand density (data not shown).

The species showed contrasting patterns of biomass allocation independently of stand density (Figure 1). Aboveground N pools were larger in roots and leaves and roots of *A. peregrina*, while it was larger in stem of *S. parahyba*. Furthermore, proportion of N allocation in biomass was more affect by stand density in *A. peregrina* than *S. parahyba*.
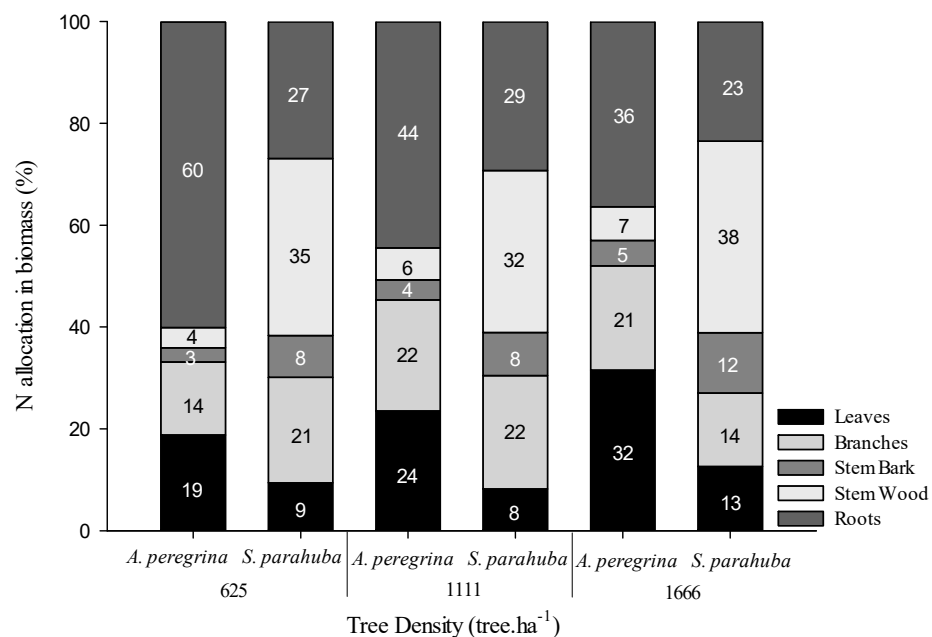
Figure 1. N pools in the leaves, branches, bark, and wood of *A. peregrina* and *S. parahyba* under different stand densities.

The N pools at 0–20 cm soil depth were statistically equal between the stand density of the *A. peregrina* and *S. parahyba*, although soil N pools were smaller in the stand with 1666 trees ha^{-1} of both species (Table 1).

Table 1. Mean values (±standard deviation) of N pools (kg ha^{-1}) at 0–20 cm soil depth, and in aboveground and belowground biomass of *A. peregrina* and *S. parahyba* at the three stand densities. For a given species, different letters indicate statistical differences between stand density.

Species	Density	Soil N	Aboveground N	Belowground N	Total N
	(Trees ha^{-1})	——————————— (kg ha^{-1}) ——————————-			
A. peregrina	625	4902.8 ± 1088 a	74.7 ± 43.6 b	43.9 ± 33.2	5019.4
	1111	4997.5 ± 1093 a	143.1 ± 55.9 a	71.7 ± 38.3	5211.4
	1666	4464.7 ± 1113 a	174.7 ± 52.2 a	99.7 ± 26.3	4741.8
S. parahyba	625	4954.4 ± 1117 a	87.5 ± 53.4 b	32.1 ± 14.2	5074.8
	1111	4906.7 ± 1111 a	98.9 ± 55.1 b	40.9 ± 17.0	5043.5
	1666	4499.1 ± 1100 a	115.8 ± 53.4 a	35.5 ± 11.17	4650.3

The N pools in the aboveground biomass were larger in *S. parahyba* than in *A. peregrina* for the density of 625 trees ha^{-1} (Table 1). However, N accumulation tended to be larger in denser stands of *A. peregrina* than in *S. parahyba*, with gains of 44% and 49% in N for the

densities of 1111 and 1666 trees ha^{-1}, respectively. Although no significant difference was observed, the N accumulated in the belowground biomass of *A. peregrina* was 1.4, 1.8, and 2.8 times higher than in *S. parahyba* for the densities of 625, 1111, and 1666 trees ha^{-1}.

We observed soil N pools were affected by experimental blocks, with smaller values in one block to the other two for both species (Table 2). Moreover, the species showed contrasting responses related to N pools in the soil. The mean amount of N in the aboveground biomass of *S. parahyba* was lower in the block with a lower soil N amount, while the contrary was observed for *A. peregrina*.

Table 2. Effect of stand density (D, trees ha^{-1}), block (B), and interaction (D × B) on the soil N pools (Nsoil, kg ha^{-1}) and the N content of the aboveground biomass (Nbio, kg ha^{-1}) in monospecific stands of *A. peregrina* and *S. parahyba*. For a given species and N pool, different letters indicate statistical differences between blocks. Significant differences in the mean values are indicated by different letters.

Species		*p*-Value			Mean per Block (*n* = 9)		
		Density	**Block**	**D × B**	**1**	**2**	**3**
A. peregrina	Nsoil	0.507	0.01	0.963	5233.3 a	5247.5 a	3884.3 b
	Nbio	<0.0001	<0.0001	0.827	125.1 b	84.3 b	183.2 a
S. parahyba	Nsoil	0.347	<0.0001	0.392	3479.8 b	5298.6 a	5582.0 a
	Nbio	<0.0001	<0.0001	<0.0001	56.6 c	138.6 a	109.5 b

2.2. N Pools via Biological N$_2$ Fixation

The mean values of δ^{15}N were smaller in *A. peregrina* than in the non-fixing species *S. parahyba*, varying from 0.8 to 6.3, 1.9 to 5.6, and 2.8 to 5.4 for the respective densities of 625, 1111, and 1666 trees ha^{-1} (Figure 2A). Furthermore, the values of δ^{15}N in the species *S. parahyba* tended to decrease between the lowest and the highest stand density, while in the N$_2$-fixing species *A. peregrina* there was an upward trend in the values of δ^{15}N with the increase in stand density. Thus, the proportion of N$_2$ derived from fixation tended to decrease between the lowest and the highest density in the stands of *A. peregrina*, with mean values of 85%, 59%, and 36% for the densities of 625, 1111, and 1666 trees ha^{-1} (Figure 2B).

The total N pools in both soil and biomass were lower in *A. peregrina* than *S. parahyba* only when stand density was 625 trees ha^{-1} (Table 1). This result prevented the quantification of the N fixed in the lower density by the accretion method (Figure 3). In contrast, the total N pools were higher in N$_2$-fixing than non-N$_2$-fixing trees for the remaining densities (Table 1). Moreover, according to with accretion method, the biological N$_2$ fixation may improve about 167.8 and 91.4 kg ha^{-1} for the densities of 1111 and 1666 trees ha^{-1} of A. peregrine, respectively (Table 1; Figure 3).
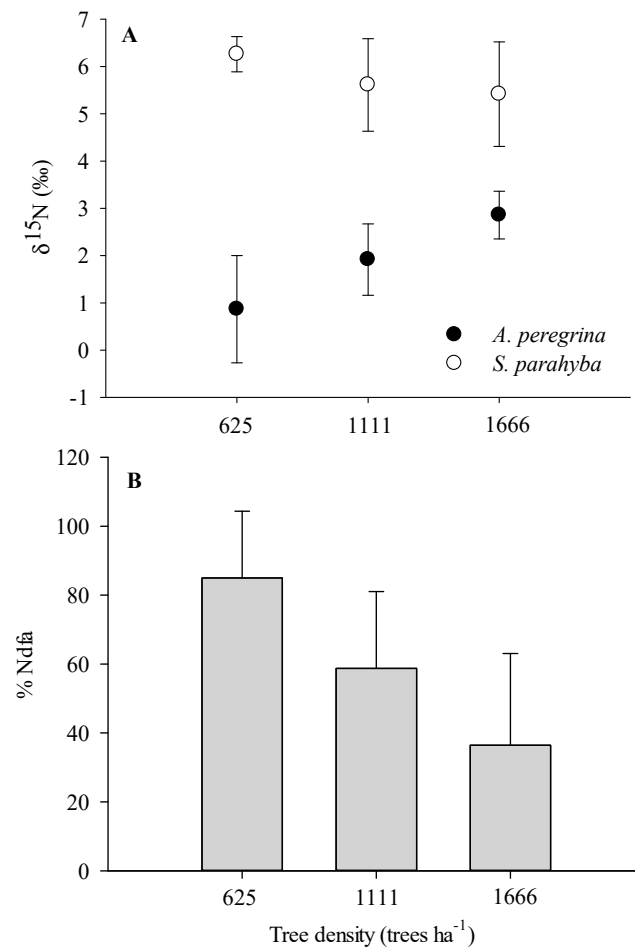
Figure 2. (**A**) δ^{15}N (‰) in leaves of *A. peregrina* and *S. parahyba* under three different stand densities, and (**B**) Percentage of N₂ derived from fixation (%Ndfa) by *A. peregrina*, under three different stand densities. Vertical bars indicate the standard deviation of the mean (N = 3).
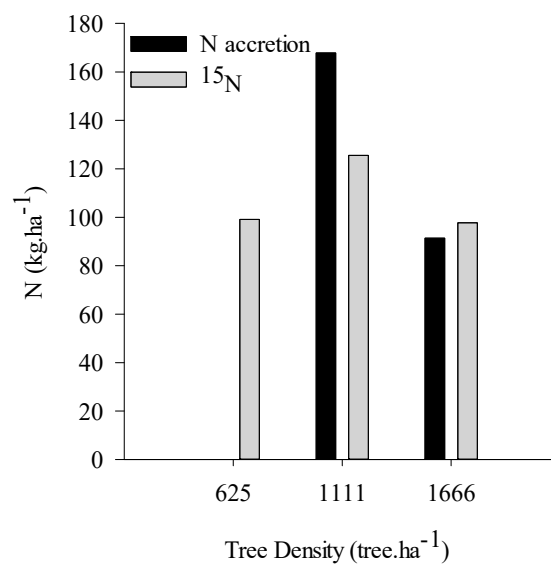
Figure 3. N fixed (kg ha^{-1}) by *A. peregrina* in three stand densities estimated by the N accretion (black columns) and by ^{15}N natural abundance (grey columns) methods.

The mean amounts of N biologically fixed in the aboveground and belowground biomass of *A. peregrina* were similar in the three stand densities by the ^{15}N natural abundance method, with values varying across 99, 125 and 101 kg ha^{-1}. Thus, the N$_2$ fixing rate in aboveground biomass of *A. peregrina* was remarkably similar for in the three stand densities ranging between 21 and 27 kg ha^{-1}year^{-1}. The accretion method estimates N$_2$ fixation values 45% higher than the natural abundance for the density of 1111 trees ha^{-1} and 6% lower for the density of 1666 trees ha^{-1}. Besides, the N$_2$ fixing rate was different for the density of 1111 (36 kg ha^{-1} year^{-1}) but similar for 1666 trees ha^{-1} (20 kg ha^{-1} year^{-1}).

The %Ndfa in *A. peregrina* could be higher using only the two blocks with higher soil N pools (i.e., B1Ap; B2Ap and B2Sp; B3Sp). In this case, the %Ndfa was 100.0%, 73.4% and 53.2% for the stand density of 626, 1111, and 1666 trees ha^{-1}. In contrast, the %Ndfa could be lower using only the block with the lower soil N pools (i.e., B3Ap and B1Sp), with 55.3%, 54.5%, and 37.1%. The use of the accretion method was unfeasible for blocks with the higher soil N pools lead to a negative estimative of N fixation for all the stand densities. Moreover, the accretion method applied to block with the lower soil N pools leads to larger N$_2$ fixation, with 236; 1169 and 426 kg N ha^{-1} for densities 625, 1111, and 1666 trees ha^{-1}, respectively.

3. Discussion

3.1. N Pools in Stands with N$_2$ and Non-N$_2$ Fixing Species

Slow-growing species allocate a large proportion of biomass in leaves and branches, while fast-growing species allocate more biomass in stem wood [13,29]. The larger leaf biomass of *A. peregrina* may explain larger root production to uptake water and nutrients to sustain physiological processes in the canopy [44]. The lack of statistical significance for N pools in the biomass between species prevents us from accepting the first hypothesis in full. Moreover, different explanations can justify these patterns, as the natural variability of N pools in the study area and by the exclusion of the belowground N of the analysis. Moreover, the larger N pools in the canopy and roots biomass of *A. peregrina* are counterbalanced by smaller stem wood biomass than in *S. parahyba* [45,46] (unpublished data). Furthermore, studies with the species *S. parahyaba* have suggested that wider spacings, such as 4 × 4 m, provide better development and final production [47], which corroborates with our data. On the other hand, the *S. parahyba* trees are more sensitive to environmental stresses. A mortality of trees was subtly larger for *S. parahyba* than for *A. peregrina* in the study area, been related to wind-breaking, rainfall variability, and attack by pests and diseases [45,46] (unpublished data). *S. parahyaba* tree was also more sensitive regarding soil fertility and tree competition than *A. peregrina.* The soil proprieties were in general similar between species and experimental blocks. However, in B1 of *S. parahyba* showed the most depleted soil fertility parameters and lower N accumulation in biomass, and for B3 of *A. peregrina*, which showed less concentration of Ca and Mg, the biomass N accumulation was larger [48] (Unpublished data). This pattern suggests soil proprieties were likely more limiting to *S. parahyba* than *A. peregrina* growth independently of stand density. The response of trees for the availability of nutrients in the soil is usually greater in fast-growing than in slow-growing species [13,29]. Further studies are necessary to assess the environmental factors affecting the growth of *A. peregrina* and *S. parahyba* in our study area.

The amount about four times higher of N in the leaf biomass and roots of *A. peregrina* compared with *S. parahyba* should be an important input of N to the soil. However, we observed no statistical difference between species regarding soil N pools, although a significant effect of the block was verified on the N pools. These patterns indicate that soil N pools are affected mainly by soil attributes on a local scale, as clay mineralogy and soil granulometry [49]. Furthermore, the relatively young age of the stands could explain the lack of differences between species for the N accumulated in the topsoil. Although N$_2$-fixing species tend to provide greater N availability in the soil [16,19,23,30], the changes in the total soil N status could not see in the short-term, when trees are planted in pastures [50], and even in forestry stands [19]. Such processes not measured as leaching and denitrification may influence those patterns of N accumulation. We suspect that

nitrification may be favored in plots of *A. peregrina*, as observed in other sites with N_2-fixing species in Brazil [19,51]. This process could minimize differences between *S. parahyba* and *A. peregrina* plots. Moreover, we do not know the causes of the tendency to decrease soil N pools in the highest stand density in both species. An explanation may be the about three times higher inputs of N via fertilizer in stand density of 1666 than 625 trees ha^{-1} overplanting, that associated to inputs of litter could cause a priming effect [52]. These patterns suggest OM decay is affected by other environmental factors than only differences between leaves and roots biomass [53].

3.2. Biological N Fixation as a Function of Tree Densities and Methods

The stand density affected the %Ndfa in the *A. peregrina* confirming our second hypothesis. The intraspecific competition could increase $\delta^{15}N$ abundance in the leaves due to the probably greater uptake of N from the soil. The soil N availability may be higher in the largest stand density due to microclimate and input of N via litterfall and root, and this may contribute to ^{15}N fractionation in the leaves [26]. The larger roots biomass in denser stands improves soil N uptake to sustain the larger canopy biomass. Studies have indicated that biological fixation is more expensive in terms of ATP than the uptake of mineral N from the soil [52]. However, the metabolic cost explains partially the %Ndfa since larger %Ndfa was observed in the smallest stand density. Moreover, when we look at the fixation within the blocks with the highest amounts of N in the soil, the %Ndfa values were increased in all stand densities in a proportion like that observed for the general average showed in Figure 2. In contrast, the %Ndfa values were reduced and less affected by intraspecific competition when we used only the two blocks with the lowest soil N stock. The regulation trend of biological N_2 fixation with stand density is reasonable since biogeochemistry cycling and competition were likely intensive. However, an important bias of this discussion is related to the number of repetitions to compare the $\delta^{15}N$ and the %Ndfa simultaneously between stand density and blocks. Besides, the effect of rhizobium and mycorrhizal status on N_2 fixing were not evaluated here [35]. We did not find articles to subside any conclusion related to ^{15}N natural abundance values found in leaves of native species as a function of stand density and symbionts status. The possibility of them accessing different N sources may cause fractionation as stressed by some authors [19,35,54]. Although some authors argue that reference plant may differ in $\delta^{15}N$ (lower) than 5‰ in the leaves of fixing [54], the difference between the means obtained in o our study was 3.88‰, similar to other studies that reported the N fixation estimative [31,39]. Other explanations for small differences in $\delta^{15}N$ are the absence of inoculation with specific strains, and the natural microbiota of the soil, which is different from the original region [16].

The N accretion method indicated gains by biological fixation at the density of 1111 trees ha^{-1} and an equivalent value to the ^{15}N natural abundance method at the density of 1666 trees ha^{-1}. This method requires accurate quantification of N pools because the input and output of N should be similar between fixing and non-fixing species [54]. Furthermore, the method could involve measurements of the litter accumulated on the soil and deeper soil [39], which was not evaluated in the present study. In contrast, the ^{15}N natural abundance was not applicable in several studies that showed noticeable inputs of N by N_2 fixing legumes trees using the N accretion method [19,35]. The use of the accretion method was unfeasible for blocks with the higher soil N pools lead to a negative estimative of N fixation for all the stand densities. These patterns may be explained by the positive response of fast-growing and non-N_2-fixing *S. parahyba* to soil conditions. Moreover, the very larger amounts of N_2 fixation estimative by accretion method applied to block with the lower soil N pools and other soil fertility parameter, reinforce the divergent patterns of species response to soil proprieties and stand densities, as well as the positive impact of N_2-fixing to improve N for the legume trees.

4. Materials and Methods

4.1. Characterization of the Study Area

The study was conducted in monospecific stands of *A. peregrina* and *S. parahyba* located within the area of the Federal Institute of Education, Science, and Technology of Espírito Santo (IFES) (20°46′15.46″ S and 41°27′13.04″ W), municipality of Alegre, state of Espírito Santo, Brazil. The elevation of the region varies from 130 to 205 m.a.s.l., and the climate type is Aw, according to the classification by Köppen. The mean annual temperature and rainfall are 23 °C and 1200 mm, respectively [55].

This study used data from experimental plots established according to three stand densities, corresponding to 625, 1111, and 1666 trees, ha^{-1} (4 × 4 m^2, 3 × 3 m^2, and 3 × 2 m^2, respectively) for each species. For each stand density and species, nine plots with an area equal to 1500 m^2 each were equally distributed in three blocks for the control of slopes, which ranged between <15% and 45% [45,46]. The stands were established in June 2011 in an area previously occupied by unmanaged pasture, with the predominance of *Brachiaria* sp. for more than 40 years. For the implantation of the experiment, the cattle were removed, and the pasture area was desiccated by applying glyphosate. The seedlings were planted in 30 × 30 × 30 cm planting holes. Despite seedlings of *A. peregrina* were not inoculated with any N$_2$-fixing bacteria, the subfamily Mimosoideae easily establishes beta-rhizobial symbioses in the field [56,57]. Each plant received 220 g of the NPK 06-30-06 commercial fertilizer and micronutrients (0.2% Cu; 0.2% Zn) per planting hole at planting. Maintenance occurred for 12 months after planting through re-planting, weed control, and ant control.

The soils in the study area are Acrisols, Ferralsols, and Cambisols (FAO classification). The chemical attributes of the surface soil layer (0–20 cm) were determined before planting of species (Table S1, Supplementary Materials) [45,46]. In brief, the mean values for experimental plots were 5.7 for pH, 2.1 mg dm^{-3} for available phosphorus, 73.1 mg dm^{-3} for potassium, been statistically equal between species and blocks. The mean value of cation exchange capacity was slightly higher in *A. peregrina* than in *S. parahyba* blocks, with mean values of 7.9 and 6.2 cmol$_c$ dm^{-3}, respectively. The organic matter (OM) was also slightly higher in *A. peregrina* than in *S. parahyba* plots, with mean values of 22.3 and 16.2 g kg^{-1}, respectively.

4.2. N Pools in Tree Biomass

The N pools in aboveground biomass at stand level were estimated using inventory data, biomass equations, and N concentration in tree tissues. The forest inventory (e.g., height, diameter, and survival) was carried out at 44 months in all experimental plots; and biomass equations for leaves, branches, stembark, and stem wood were adjusted after selection and harvest of 45 trees by species at 56 months of age at the time of the study conducted by [45,46] (unpublished data). These measures were performed over the rain season (January–March). The biomass was measured in one medium-diameter tree ± one standard deviation per plot. The trees felled in the tested densities of 625, 1111, and 1666 trees ha^{-1} showed mean values of diameter at breast height equivalent to 7.4, 7.1, and 6.6 cm for *A. peregrina* and 14.3, 12.0, and 11.3 cm for *S. parahyba*, for the respective densities. The total tree height was 5.9, 6.3, and 6.2 m for *A. peregrina* and 11.4, 10.4, and 13.3 m for *S. parahyba* (details in [45,46]). The leaves, live branches, and trunk of all felled trees were separated and weighed in the field. One sample per biomass component was retrieved for moisture determination. Discs with bark were sampled, and the bark proportion in the discs was used to estimate the bark biomass. This study used samples collected from nine trees per species and stand density for N analysis (27 trees per species).

The roots of three trees per species and stand density (18 trees in all) were sampled with a backhoe loader in one of the blocks, and there was no separation of roots by size class. The excavation occurred in every projection of the crown and the maximum volume until the total pivoting root was obtained. The roots were separated from the soil using a 1.0 × 1.0 cm^2 mesh sieve and weighed in the field. The roots were cleaned with a damp

cloth, and a composite sample containing roots with different diameters was retrieved species and plot.

All samples were previously dried at 65 °C until constant weight and ground in a Willey mill (1.0 mm sieve). The N concentration was determined using the Kjeldahl method in the samples of leaves, branches, bark, wood, and roots of the trees felled in the three stand densities (243 samples in total).

N accumulation (kg ha^{-1}) in the aboveground biomass was calculated using the data of biomass accumulated in each plot, estimated using the previous equations and data from the inventory [45,46] (unpublished data) multiplied by the mean N concentration in the biomass components of each stand density (N = 9). Thus, only trees present in the plot were accounted for N accumulation. The trees survival was subtly larger in *A. peregrina* (73%–82%) than *S. parahyba* (65%–70%). The mean values of root biomass observed in each stand density and species were converted to hectare using an estimative of the excavated area. The N accumulated in the roots (kg ha^{-1}) was estimated for each stand density and species using the roots biomass multiplied by the mean N content (N = 3).

4.3. N Pools in the Soil

The N pool into 0–5, 5–10, and 10–20 cm soil layers was estimated to 67 months after planting of species. In each plot, six individual soil samples were collected in six different positions and distances to the trees and mixed, according to the methodology adapted from [19]. One sample per plot and soil layer was retrieved for N analysis. Soil density was determined in each soil layer using a 5-cm diameter metallic ring with a soil sample collected from the central region of each experimental plot. The N concentration was determined in dried, ground, and sieved soil samples according to the Kjeldahl method described by [58]. The samples used for density determination were dried at 105 °C for 72 h and weighed on a precision balance.

We calculated N pools for topsoil (0–20 cm soil depth) using the weighted value of N concentration in each plot (N = 54) and the corrected values of soil mass determined in each block per species (N = 9). The soil density was statistically equal between the stand density of the *A. peregrina* and *S. parahyba* in our study area [48].

4.4. N Pools via Biological N$_2$ Fixation

4.4.1. ^{15}N Natural Abundance Method

The natural abundance of ^{15}N was determined in samples of the leaves collected for biomass evaluation at 56 months. For each tree species and stand density, we mixed leaf samples of three trees in the three plots of each block. Thus, the number of repetitions per species was nine, been three repetitions per stand density. The δ^{15}N values [59] were determined with a Hydra 20–20 isotope ratio mass spectrometer connected to an ANCA-GSL automatic analyzer (Sercon Co., Krewe, UK), and approximately 10 mg of leaves were used in each analysis. The precision of isotopic measurements was 0.0001% × (^{15}N) [54]. The percentage of N derived from fixation (%Ndfa) was estimated using the values of δ^{15}N in the leaves of *A. peregrina* and *S. parahyba* in each stand density (Equation (1)) [60]:

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N } S. \text{ parahyba} - \delta^{15}\text{N } A. \text{ peregrina}}{\delta^{15}\text{N } S. \text{ parahyba} - \text{B}} \times 100 \qquad (1)$$

where δ^{15}N *S. parahyba* (reference plant) is the mean value of $10^3 \delta^{15}$N in the leaf samples of each stand density with *S. parahyba* (N = 3); δ^{15}N *A. peregrina* is the mean value of $10^3 \delta^{15}$N in the leaf samples of each stand density with *A. peregrina* (N = 3). Only the leaves were used for the estimates of the %Ndfa, as suggested in other studies [61]. It was assumed that the species assessed soil N with a similar abundance of δ^{15}N [38]. In the study, the value B was assumed to be zero. The B value represents the isotopic fractionation that occurs during the biological N fixation, and it is unknowledgeable for *A. peregrine*.

4.4.2. N Accretion Method

The estimative of biological N_2 fixation in *A. peregrina* stands was also performed using the N accretion method [62], by comparing the N pool in topsoil and the biomass of the N_2-fixing species with the non-N_2-fixing species (Equation (2)).

$$\text{N2 fixed} = (\text{N bio } A. peregrina - \text{N bio } S. parahyba) + (\text{N soil } A. peregrina - \text{N soil } S. parahyba) \tag{2}$$

where N bio and N soil refer to the mean values of N pools (kg ha^{-1}) in the aboveground and belowground biomass and the topsoil, in each stand density with *A. peregrina* and *S. parahyba*. The N pools in the forest floor, in understory vegetation and deep soil layer is not examined here. These N pools were not easy to assess since the presence of understory vegetation difficult sampling of forest floor and the understory was unequal among species and stand density ([48], unpublished data).

4.5. Statistical Analysis of the Data

We initially tested the effect of stand density and blocks on the N accumulated in the soil and biomass of each species using a two-way ANOVA. We disregarded the effect of the block in the comparisons between species for the N accumulated in the total biomass, as well as the 10^3 δ^{15}N. Therefore, for each stand density, the differences between species for the N accumulated in the aboveground biomass and the 10^3 δ^{15}N values were tested using a paired sample T-test. No statistical analysis was applied for the N content in the roots' biomass, Total N of species and %Ndfa due to the reduced number of replications. The data were subjected to the test of homogeneity of variances and the Shapiro-Wilk normality test. The statistical analysis was performed with the software SigmaPlot® 13.0 (Systat Software Inc., San Jose, CA, USA, 2014), with a statistical threshold value of $p = 0.05$.

5. Conclusions

The species showed divergent patterns of the N pools in the biomass despite no significant differences in aboveground N. *S. parahyba* accumulated most N mainly in the stem, while *A. peregrina* accumulated N in the canopy and roots. *A. peregrina* was less affected by intraspecific competition, accumulating larger N in biomass in stand density of 1111 and 1666 trees ha^{-1} than *S. parahyba*. Soil N pools were statistically equal between the studied species and their respective densities. However, differences of soil N among experimental blocks highlighted contrasting patterns of response between N_2-fixing and non-N_2-fixing. On the other hand, the N pools in biomass of *A. peregrina* were not depleted by soil N or other soil fertility indicators, while the opposite behavior was observed for *S. parahyba*. Furthermore, differences in δ^{15}N suggested more soil N dependence and lower %Ndfa by *A. peregrina* in higher stand density than in lower stand density. The methods used showed a similar magnitude of biological N_2 fixation only in the largest stand density with 1666 trees ha^{-1}. The biological N_2 fixation estimates depended on the method and the response of tree species to environment.

Supplementary Materials: The following are available online at https://www.mdpi.com/2504-3129/2/1/6/s1, Table S1: Mean values of soil attributes (0–20 cm) before the establishment of the monospecific stands of *A. peregrina* and *S. parahyba*.

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