








## Article

# Inoculation with *Bradyrhizobium elkanii* Reduces Nitrogen Fertilization Requirements for *Pseudalbizzia niopoides*, a Multipurpose Neotropical Legume Tree

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**Abstract:** This study investigated the effects of *Bradyrhizobium elkanii* inoculation and nitrogen (N) fertilization on the growth of *Pseudalbizzia niopoides* seedlings in a nursery and their subsequent performance in soil. *P. niopoides* is a legume tree native to Latin American tropical forests, known to nodulate but with no previously identified rhizobial partner. Seedlings were grown in a nursery under varying N fertilization rates (0, 250, 500, 1000, and 2000 mg L<sup>-1</sup>) with and without *B. elkanii* inoculation. Morphological traits, nodulation, and post-planting growth were assessed. Both inoculation and N fertilization significantly enhanced seedling growth in the nursery. However, high N rates suppressed nodulation and caused root toxicity. Inoculated seedlings exhibited improved growth after planting, particularly at lower N rates. Notably, inoculated seedlings without added N demonstrated vigorous new root proliferation after three months, highlighting the beneficial effects of the symbiosis. In terms of nitrogen fertilization in nurseries, a N rate up to 500 mg L<sup>-1</sup> produced satisfactory plant growth and no prejudicial effects on the symbiosis establishment. However, it is possible to raise seedlings even in the 0 mg L<sup>-1</sup> N rate, with a vigorous root emission during the post-planting growth. This study provides valuable insights into the interaction between a specific rhizobia strain and *P. niopoides*, with implications for nursery practices and sustainable agroforestry systems.

**Keywords:** *Albizzia niopoides*; nitrogen fixation; nodulation; rhizobia; soil bacteria; South America; urea



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## 1. Introduction

The symbiosis between legume plants and rhizobia, a diverse group of alpha and beta Proteobacteria [1], can significantly enhance plant growth. Nitrogen-fixing bacteria (NFB) are widely utilized in legume crop species, thereby reducing the reliance on nitrogen

fertilizers and consequently mitigating the environmental impacts and economic costs associated with their industrial production [2].

In recent decades, there has been a growing body of research investigating the role and ecology of NFB in legume trees, including the compatibility between rhizobia and tree species [3] and on the effects of the N fixation on both the host plant and the surrounding environment. The benefits of rhizobia and legume tree symbiosis extend from enhanced growth of the individual host tree to nitrogen enrichment of the soil and surrounding plant community [4], which is useful even for restoration projects [5]. However, these benefits are contingent upon several factors, including both environmental conditions and intrinsic characteristic of the symbiotic partners [6]. Soil nitrogen availability is believed to be inversely proportional to the establishment and functioning of the symbiosis [7]. This occurs through the activation of symbiosis regulation mechanisms within the plant host [8]. The degree of nodulation varies according to partner species, their physiology [9], and environmental conditions [10].

First isolated from U.S. soybean fields in the 1990s [11], *Bradyrhizobium elkanii* has since become a widely studied inoculant for legumes, with strains identified in diverse ecosystems [12,13]. Further studies have identified strains of *B. elkanii* in soils under diverse vegetation cover systems in South America [14,15]. In Brazil, the Normative Instruction n° 13 of the Secretary of Agricultural Defense (MAPA—Ministry of Agriculture and Livestock <https://www.gov.br/agricultura/pt-br/assuntos/insumos-agropecuarios/insumos-agricolas/fertilizantes/legislacao/in-sda-13-de-24-03-2011-inoculantes.pdf/view> (accessed on 25 July 2024))—authorizes diverse strains of *B. elkanii* as inoculant for native and exotic multipurpose legume species, including some mimosoid clade ones, like *Enterolobium timbouva* Mart. and *Leucaena leucocephala* (Lam.) de Wit.

*Pseudalbizzia niopoides* (Spruce ex Benth.) E.J.M.Koenen & Duno (*P. niopoides*, hereafter), a neotropical legume tree species in the mimosoid clade (subfamily Caesalpinoideae [16]), is native to seasonal forests from southern Mexico to northeastern Argentina (<https://www.tropicos.org/name/100532070> (accessed on 15 January 2025)). *Albizzia niopoides* is a synonym for this species. While its nodulation ability has been documented [17], no detailed reports exist on identified rhizobia species with established effects on this species. Identifying compatible symbiotic partners is crucial for understanding the natural history of legume–rhizobia interactions and holds promise for inoculant development [18]. Given its nitrogen-fixing capabilities and valuable traits for pastures [19], agroforestry systems [20], and urban forestry [21], *P. niopoides* is commonly used in reforestation projects, ecological restoration efforts [21], and is cultivated in commercial nurseries. Furthermore, a recent study suggests potential medicinal applications for the species, with its leaves demonstrating antitumor potential against cervical cancer cells [22]. Therefore, given its critical and strategic role in environmental and socioeconomic systems, further specific studies are warranted.

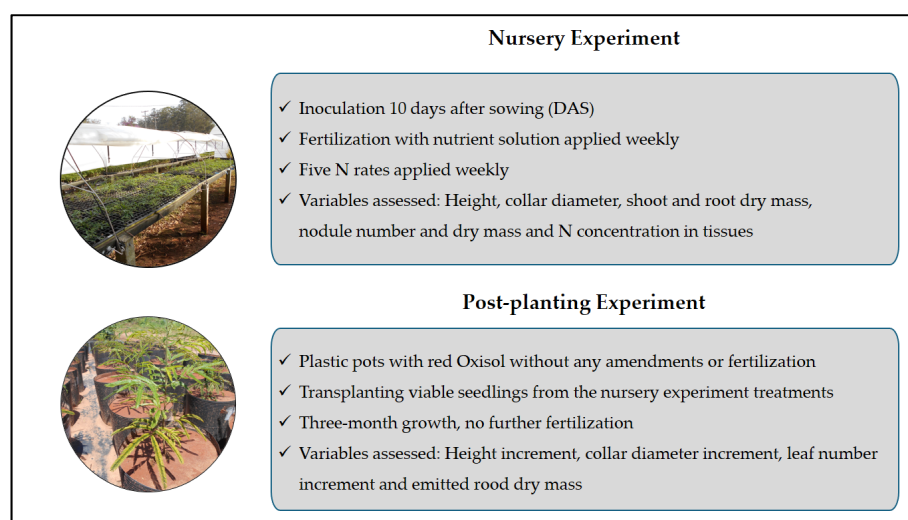
Nitrogen fertilization is a standard practice in nurseries to ensure adequate N supply for achieving the desired seedling quality [23], especially for native tree species, whose demands are growing due ecological restoration projects and landscaping [24,25]. Diverse N sources can be used as fertilizers, like ammonium sulfate and phosphate, calcium nitrate, and urea [26]. Since rhizobial inoculation does not always provide sufficient N for optimal growth in legume tree seedlings [27], there is a need to determine the optimal level of N fertilization that promotes suitable plant growth without compromising the N-fixing (NBF) symbiosis. This is particularly relevant for *P. niopoides*, given its incipient characterization of its symbiotic relationship with rhizobia. In addition, a deeper understanding of this legume–rhizobia symbiosis will provide valuable insights for the management of a species with a wide neotropical distribution and ecological ubiquity across natural and anthropized landscapes.

Building upon previous findings, this research seeks to (1) quantify the effects of a known rhizobia species on nodulation and growth of *P. niopoides* seedlings during nursery cultivation; (2) evaluate the influence of nitrogen fertilization on both plant growth and nodulation; and (3) examine the effects of rhizobia inoculation and N fertilization on initial growth in soil following nursery cultivation. We chose to use a strain recommended for inoculant production in legume tree cultivation, the *Bradyrhizobium elkanii* SEMIA 6432, which is tested for other mimosoid clade legume species.

## 2. Materials and Methods

### 2.1. Nursery Experiment

*P. niopoides* seedlings were grown in a nursery within the Forest Science Department of the School of Agriculture at the São Paulo State University (UNESP), Botucatu, Brazil (22°51'03" S, 48°25'37" W). The seeds originated from a healthy tree (14 m height, 45 cm diameter at breast height, unknown age), growing naturally in pasture. Seeds were sown in 115 cm<sup>3</sup> tubular-conical plastic containers using a peat-based substrate (Carolina Soil™, Pardinho, Brazil: composed of *Sphagnum* moss, vermiculite, rice hulls, limestone, agricultural gypsum, and trace amounts of NPK fertilizers; pH: 5.0 ± 0.5; EC: 0.7 ± 0.3 mS cm<sup>-1</sup>). One-week post-sowing, containers with emerged seedlings were selected for further treatments ( $n = 820$  seedlings) (Figure 1).



**Figure 1.** Overview of the experimental design of the study.

For rhizobial inoculation, we utilized strain SEMIA 6432 of *Bradyrhizobium elkanii* (GenBank accession # FJ025110), obtained from Embrapa Agrobiologia in Seropédica, Rio de Janeiro, Brazil. This strain is recommended for *Albizia lebbek* (L.) Benth. and *Falcataria falcataria* (L.) Greuter & R.Rankin (syn. *F. moluccana* (Miq.) Barneby & J.W. Grimes) [28].

The strain was propagated for six days in liquid Medium 79 of Fred and Waksman [29] with shaking. The liquid culture was then used directly as the inoculant [30]. Inoculation was performed 10 days after sowing (DAS) by applying 4 mL directly to the plantlet collar using a syringe.

Seedlings were fertilized weekly using a capillarity-fed nutrient solution. The fertilization method consisted in submerging the base of the tubes in the nutrient solution, allowing capillarity action to transport moisture up to the substrate surface. The nutrient solution contained (in mg L<sup>-1</sup>): P 88, K 200, Ca 168, Mg 40, S 52, B 3, Mn 3.9, Zn 1.2, Cu 0.6, Mo 0.3, and Fe 48. N fertilization treatments were applied by adding the amount of urea to the standard solution: 0, 250, 500, 1000, and 2000 mg L<sup>-1</sup> of N. Urea was chosen as N

source because its composition does not contain other nutrients that could interfere with the plant nutrition. The applied urea concentrations were based on usual N levels used in nutrient solution for native seedling production research [31,32].

The seedling tubes were arranged in trays containing 27 tubes each. Of these, the 14 central tubes in each tray were selected for measurements and statistical analysis. Trays were placed on a stand of 1 m above the ground, covered with a transparent plastic film (150 micra), and irrigated daily with 3–8 mm of water, depending on temperature and air humidity.

The end of the nursery period was determined when the plastic pot volume appeared to be restricting seedling growth, which occurred at 350 days after sowing. To assess the effects of treatments on seedlings growth, height, collar diameter and shoot, and root dry mass were measured. The shoot-to-root ratio and the Dickson Quality Index [33] were calculated, as follows:

$$\text{Shoot to root ratio} = \frac{\text{shoot dry mass (g)}}{\text{root dry mass (g)}} \quad (1)$$

$$\text{Dickson Quality Index} = \frac{\text{Total dry mass (g)}}{\frac{\text{height (cm)}}{\text{diameter (cm)}} + \frac{\text{shoot dry mass (g)}}{\text{root dry mass (g)}}} \quad (2)$$

To assess the effects of treatments on the establishment of legume–rhizobia symbiosis, the number and dry mass of nodules per seedling were counted. In addition, the nitrogen concentration of root and shoot was analyzed by titration after sulfur digestion, using the Kjeldahl method [34].

## 2.2. Post-Planting Experiment

Eight-liter plastic pots were filled with red-yellow Oxisol (without any amendments or fertilization, Table 1). Six seedlings were selected based on their height and collar diameter standard deviation and then transplanted into eight-liter plastic pots filled with red Oxisol without any amendments or fertilization (Figure 1). Height and diameter measurements were taken at this time.

**Table 1.** Soil physical–chemical features.

Clay	Silt	Sand	pH (CaCl <sub>2</sub> )	Organic matter	P (resin)
----- % -----				---- g dm <sup>-3</sup> ----	--- mg dm <sup>-3</sup> ---
16	4	80	3.9	5	4
H + Al	K	Ca	Mg	SB	CEC
----- mmol <sub>c</sub> dm <sup>-3</sup> -----					
31	0.9	4.0	1.0	5	36

Pots were placed in an open area and irrigated daily. After three months of growth, seedling height and collar diameter were measured, and the increment was calculated by subtracting the initial measurements taken at planting. The shoot and the emitted roots of each seedling were weighed for dry mass.

## 2.3. Statistical Analysis

Analysis were performed by the SAS Statistical Software v9.4 (SAS University Edition ([http://www.sas.com/en\\_us/software/university-edition.html](http://www.sas.com/en_us/software/university-edition.html), accessed on 5 September 2023)).

For nursery experiment data, nonlinear regression models [35] were fitted to predict height, collar diameter, shoot, root, and total dry mass as a function of nitrogen rates, with and without rhizobial inoculation:

$$Y_i = \alpha + \beta X_i e^{-\frac{X_i}{\gamma}} + u_i \quad (3)$$

where  $Y_i$ : variable estimated result;  $X_i$ : nitrogen rates ( $\text{mg L}^{-1}$ );  $\alpha$ : parameter related to horizontal shift;  $\beta$ : parameter related to the growth stage of the curve;  $\gamma$ : parameter matching the nitrogen rate at which the curve reaches its maximum point;  $u_i$ : random component with normal distribution.

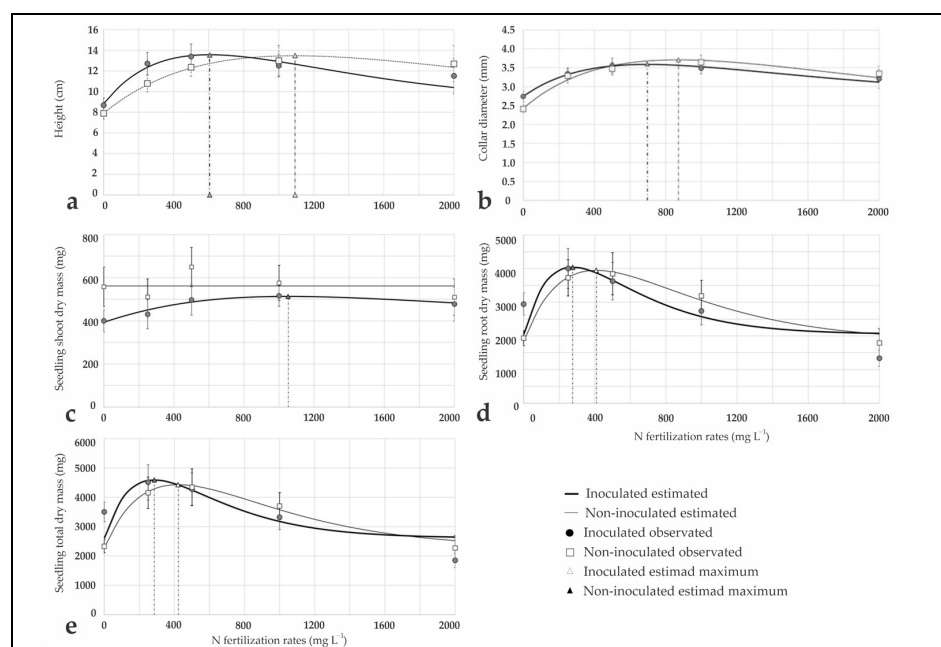
The parameter estimators and their confidence intervals were used as criteria for comparing the effect of rhizobia inoculation (yes or no).

Generalized Linear Models (GLMs) were used to analyze data on nodulation, plant tissue N concentration, and all variables from the post-planting experiment. We employed a gamma distribution with a log link function [36,37]. Model fitting was performed using maximum likelihood estimation. Deviance analysis was used to assess the goodness of fit. For comparisons between means, the Tukey–Kramer test was applied [38], with all comparisons reported at  $\alpha = 0.05$ .

### 3. Results

#### 3.1. Nonlinear Regression Models for Nursery Experiment

Nonlinear regression curves for height and diameter differed between inoculated and non-inoculated treatments (Table 2). Nitrogen fertilization increased seedling height and collar diameter, but the highest rates led to a reduction in these variables (Figure 2a,b). Inoculated seedlings required a smaller nitrogen amount ( $605 \text{ mg L}^{-1}$ ) to reach maximum height compared to non-inoculated seedlings ( $1093 \text{ mg L}^{-1}$ ).



**Figure 2.** Fitted nonlinear regression models for the variables in seedlings of *P. niopoides*, under nitrogen rates by rhizobia inoculation: (a) height; (b) collar diameter; (c) shoot dry mass; (d) root dry mass; and (e) total dry mass. The model fitted is the one described in Equation (1), except for the inoculated treatment in shoot dry mass, where the mean itself fitted the observed data ( $\hat{Y} = \alpha = \text{mean}$ ). The vertical bars indicate the standard error of the mean.

**Table 2.** Estimated parameters (with confidence intervals), maximum estimated  $\hat{Y}$ , adjusted  $R^2$  and  $p$ -value of regression, according to treatments and variables height, collar diameter and shoot, root and total dry mass measured in *P. niopoides* seedlings, after nursery growth. The model fitted is the one described in Equation (1). Details of goodness of fit are available as Supplementary Materials (Tables S1–S5).

Variables		Inoculated	Non-Inoculated	
Height	$\hat{\alpha}$	8.83 (8.20–9.47)	7.93 (7.42–8.44)	ns
	$\hat{\beta}$	0.021 (0.014–0.028)	0.014 (0.010–0.0175)	ns
	$\hat{\gamma}$	605 (604–606)	1093 (803–1382)	*
	$\hat{Y}$	13.56 cm	13.49 cm	
	Adjusted $R^2$	40%	52%	
	$p$ -value	1.0	1.0	
Collar diameter	$\hat{\alpha}$	2.75 (2.65–2.85)	2.435 (2.34–2.53)	*
	$\hat{\beta}$	0.0033 (0.0023–0.0043)	0.0040 (0.0032–0.0047)	ns
	$\hat{\gamma}$	695 (532–861)	872 (711–1033)	ns
	$\hat{Y}$	3.59 mm	3.70 mm	
	Adjusted $R^2$	45%	66%	
	$p$ -value	0.2318	0.0608	
Shoot dry mass	$\hat{\alpha}$	$\hat{Y} = \max.$	5.92 (5.82–6.02)	
	$\hat{\beta}$	$\hat{Y} = \alpha = \text{mean}$	0.0070 (0.0012–0.0013)	
	$\hat{\gamma}$		1078 (374–1781)	
	$\hat{Y}$	560.7 mg	512.5 mg	
	Adjusted $R^2$		9.14%	
	$p$ -value		1.000	
Root dry mass	$\hat{\alpha}$	2050 (1763–2336)	1842 (1573–2110)	ns
	$\hat{\beta}$	19.53 (10.1–30.0)	14 (9.6–18.4)	ns
	$\hat{\gamma}$	276 (182–370)	409 (314–504)	ns
	$\hat{Y}$	4033 mg	3945 mg	
	Adjusted $R^2$	30.43%	41.34%	
	$p$ -value			
Total dry mass	$\hat{\alpha}$	2608 (2288–2928)	2282 (2058–2505)	ns
	$\hat{\beta}$	18.84 (9.5–28.2)	13.80 (9.0–18.6)	ns
	$\hat{\gamma}$	285 (182–387)	422 (320–524)	ns
	$\hat{Y}$	4587 mg	4425 mg	
	Adjusted $R^2$	27.44%	40.05%	
	$p$ -value	0.647	0.999	

\* Confidence intervals of estimated parameters did not overlap between inoculated and non-inoculated groups, meaning that the regression curves are different. ns: the confidence intervals of estimated parameters overlaps between inoculated and non-inoculated groups.

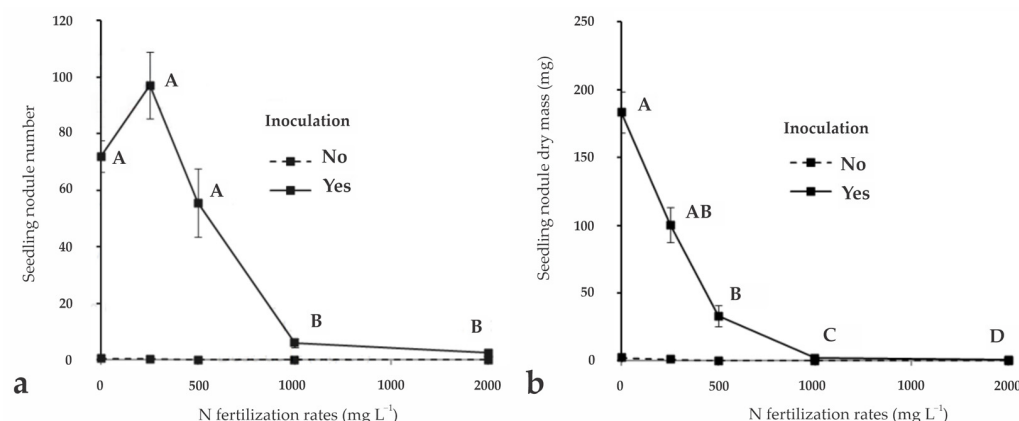
Regression curves for root and total dry mass did not differ between inoculated and non-inoculated seedlings. For both variables, a more pronounced reduction in plant dry mass occurred starting from 400 mg L<sup>-1</sup> N (Figure 2d,e).

A single model could not be fitted for inoculated and non-inoculated treatments on shoot dry mass. Therefore, the model fitted for non-inoculated seedlings was the same as for the other variables, with its maximum shoot dry mass (512 mg per plant) achieved at 1077 mg L<sup>-1</sup> N. The mean itself fit the inoculated treatments ( $\hat{Y} = \hat{\alpha} = \bar{Y} = \text{mean}$ ), meaning that at any nitrogen rate, the predicted shoot dry mass was 560 mg per plant (Figure 2c).

### 3.2. Generalized Linear Models for Nursery Experiment

Nodulation was affected by the interaction of treatments, both in relation to nodule number ( $p = 0.017$ ) and nodule dry mass ( $p = 0.0428$ ). Inoculation with *Bradyrhizobium elkanii* strain SEMIA 6432 induced the formation of indeterminate-shaped nodules in seedlings (Supplementary Figure S1). The highest amounts occurred between 0 and 500 mg L<sup>-1</sup> N

(Figure 3). Above this threshold, both nodule number and dry mass were significantly reduced. In the non-inoculated group, few small nodules were counted in some seedlings.



**Figure 3.** Nodulation of *P. niopoides* seedlings, after nursery growth under rhizobia inoculation and N fertilization treatments. (a) Nodule number per plant and (b) nodule dry mass per plant (mg). Uppercase letters mean differences by Tukey–Kramer test ( $p < 0.05$ ). The vertical bars indicate the standard error of the mean.

Rhizobia inoculation ( $p \leq 0.0012$ ) and nitrogen fertilization ( $p \leq 0.0001$ ) had independent effects on root nitrogen (N) concentration, with no interaction between factors. Rhizobia inoculation increased root N concentration by 14% compared to non-inoculated seedlings. Nitrogen fertilization also increased root N concentration, ranging from 1.4% in the 250 mg L<sup>-1</sup> treatment to more than 4% in the highest N rate treatment (Table 3).

**Table 3.** Means of shoot and root nitrogen concentrations. Interaction between factors in shoot N ( $p < 0.0001$ ), not for root N ( $p = 0.1014$ ). Standard error of means in the brackets. Differences between inoculation treatments are described in text.

N Rates	Shoot N (%)		Root N (%)
	Inoculation		Both Inoculation Treatments (No Interaction)
	–	+	
0	1.78 E b (0.02)	3.18 C a (0.016)	1.83 C (0.167)
250	2.15 D b (0.073)	3.05 C a (0.056)	1.43 D (0.043)
500	2.84 C b (0.56)	3.09 C a (0.070)	1.84 C (0.021)
1000	3.67 B a (0.074)	3.78 B a (0.033)	2.71 B (0.104)
2000	4.49 A a (0.048)	4.43 A a (0.054)	4.23 A (0.105)

+ and – indicates the inoculated and non-inoculated groups, respectively. Uppercase letters compare differences across N rates (in the vertical), while lowercase letters compare inoculation (in the horizontal).

An interaction between treatments was observed for shoot N concentration ( $p \leq 0.0001$ ). Nitrogen fertilization increased shoot N concentration in seedlings. However, under low nitrogen rates, inoculated seedlings already exhibited higher N concentrations (Table 3).

### 3.3. Generalized Linear Models for Pot Experiment

Interaction between inoculation and N fertilization treatments was found in most variables, with exception of collar diameter increment (Table 4).

**Table 4.** Summary of deviance analysis for the assayed variables in *P. niopoides* seedlings after pot experiment (3 months).

	N Fertilization		Inoculation		Interaction	
	Chi <sup>2</sup>	Pr > Chi <sup>2</sup>	Chi <sup>2</sup>	Pr > Chi <sup>2</sup>	Chi <sup>2</sup>	Pr > Chi <sup>2</sup>
HI	11.14	<0.025 *	6.85	0.009 **	9.63	0.0471 *
CDI	14.89	0.0049 **	14.94	0.0001 **	5.91	0.2061
LNI	17.98	<0.0012 **	14.45	0.0001 **	9.89	0.0422 *
ERDM	31.40	<0.0001 **	50.82	<0.0001 **	21.94	0.0003 **

HI: height increment; CDI: collar diameter increment; LNI: leaf number increment; ERDM: emitted root dry mass. \* means significance at a level < 5%, while values signalized with \*\* means significance at level < 1%.

Height increment values of the seedlings ranged from 3.46 to 11 cm, with no significant differences detected by the Tukey–Kramer test (Table 5). The collar diameter increment ranged from 0 to 1.69 mm. Rhizobia inoculation doubled the diameter increment compared to non-inoculated plants ( $0.84 \pm 0.41$  to  $0.43 \pm 0.37$  mm). Regarding nitrogen fertilizing treatments, only the seedlings fertilized with 250 milligrams per liter showed an increment inferior to those fertilized with 1000 and 2000 milligrams per liter of nitrogen.

**Table 5.** Means of height and leaf number increment and root emission, according to N rates and rhizobia inoculation. Standard error of means in the brackets.

N	HI		LNI		REM	
	–	+	–	+	–	+
0	3.46 A a (0.78)	6.67 A a (0.70)	4.33 ab A (1.14)	5.00 A a (1.09)	0.195 B b (0.03)	1.383 A a (0.29)
250	3.90 A a (1.07)	6.71 A a (2.47)	1.50 B b (0.56)	5.67 A a (1.11)	0.708 A a (0.16)	1.298 A a (0.15)
500	9.25 A a (2.97)	5.80 A a (1.62)	4.67 AB a (1.56)	7.00 A a (1.71)	0.805 A a (0.13)	1.427 A a (0.15)
1000	4.43 A a (1.37)	11.20 a A (3.48)	4.17 AB a (1.56)	5.83 A a (1.40)	1.040 A a (0.19)	2.073 A a (0.32)
2000	8.68 A a (1.72)	11.67 a A (3.42)	6.33 A a (1.71)	7.00 A a (1.71)	0.750 A b (0.14)	1.580 A a (0.29)

HI: height increment (cm); LNI: leaf number increment; REM: root emission increment (g per plant); + or – means seedlings inoculated or non-inoculated, respectively. Uppercase letters compare differences across N rates (in the vertical), while lowercase letters compare inoculation (in the horizontal).

Significant differences were observed in leaf number increment between inoculated and non-inoculated seedlings under 250 mg L<sup>−1</sup> N fertilization in the nursery. Regarding seedling root emission, values ranged from 0.195 to 2 g, with significant differences observed only between inoculated and non-inoculated seedlings under 0 and 2000 mg L<sup>−1</sup> N (Table 5).

All inoculated seedling pots exhibited a noticeable number of nodules on the emitted new roots (Figure 4b). Nodules were also found in some pots with non-inoculated seedlings, but in significantly smaller numbers and were rare.



**Figure 4.** Details of the roots of *P. niopoides* seedlings after 3-month growth in soil pots, according to nursery treatments. (a) Comparison between plants without nitrogen fertilization ( $0 \text{ mg L}^{-1}$ ): non-inoculated (left) and inoculated with *B. elkanii* (right). (b) Example of root nodules in an inoculated plant, highlighted by the yellow arrow.

#### 4. Discussion

This study provides the first detailed investigation of the interaction between an identified rhizobia strain and *Pseudalbizzia niopoides*. The strain SEMIA 6432 of *Bradyrhizobium elkanii* was selected for *Albizia lebbek* and *Falcataria moluccana* [15,28]. The genus *Bradyrhizobium* is an important group of NFB for a wide variety of legume species of all habits [39] and environments [40–42], and was even recognized as the ancestor of all rhizobia species [43]. Nevertheless, other rhizobia genus are known to be compatible with legume tree species in South America, such as *Burkholderia*, which is a very common in other mimosoid clade species [44]. *Pseudalbizzia niopoides* has a wide range of natural occurrence in Central and South America, in both natural and human-altered landscapes.

This study did not use sterile environments, neither in the nursery production or the soil pots for post-growth experiment. Particularly in the post-planting experiment, we do not know the identity of rhizobia colonizing the new nodules in the new roots emitted during the three-month growth (Figure 4b). Given the fact that the soil can harbor resident populations of rhizobia symbionts, these strains can affect the efficiency of the symbiosis [45]. Therefore, further studies of how the species interacts with rhizobacteria species can help to understand the role of the legume–rhizobia symbiosis in its ecology [46] and physiology.

*P. niopoides* is also capable of establishing symbiosis with arbuscular mycorrhizal fungi [47]. Since the mycorrhizal fungal symbiosis has the potential to enhance biological nitrogen fixation (BNF) in legume species [48], this interaction should also be investigated. Other legumes within the mimosoid clade exhibit a legume–rhizobia symbiosis that is favored by mycorrhizal fungi, such as *Piptadenia gonoacantha* (Mart.) J.F.Macbr. [49]. As a matter of fact, increased phosphorus absorption is a main benefit to the plant mycorrhizal association, and this nutrient has effects on nitrogen metabolism in root nodules and other tissues in nitrogen-fixing legumes [50].

Positive effects of rhizobia inoculation were clearly observed on biomass accumulation, particularly in roots. Although regression curves between inoculated and non-inoculated treatments were not significantly different, deviance analysis and the Tukey–Kramer test support the beneficial effects of rhizobia on seedlings, especially in the absence of nitrogen fertilization. Beneficial influences of rhizobia inoculation on seedling traits of tropical legume trees are documented in several other species, including *Calliandra* [51], *Inga* [52], *Millettia*, and other mimosoid clade species [53]. Nodulation in inoculated seedlings and elevated nitrogen concentrations in tissues provide evidence that nitrogen fixation had occurred.

Establishment and functioning of the legume–rhizobia symbiosis were clearly influenced by urea fertilization in the nursery. At low N rates, seedlings exhibited high nodule number and dry mass. In fact, a minimal N amount is beneficial for the establishment of the symbiosis [7], which may have occurred even in the  $0 \text{ mg L}^{-1}$  N treatment due to the presence of monoammonium phosphate as the P source, which contains 12% N. Ferguson et al. [8] suggested that low external N sources promote nodulation by supporting plant health, but without reaching a level that renders biological nitrogen fixation (BNF) redundant.

It is known that nitrogen fertilization can impair the development of the legume–rhizobia symbiosis [54]. In the nursery experiment, N rates above  $250 \text{ mg L}^{-1}$  already reduced the seedling's nodule dry mass, although only rates greater than  $500 \text{ mg L}^{-1}$  suppressed the nodule number. These findings show that the lowest N rate affected the symbiosis in some way, likely due to inhibition mechanisms that prevent the induction of nodulation genes to conserve plant resources [8,55]. These mechanisms include the autoregulation of nodulation, involving the production and reception of signaling peptides in plant roots and aerial tissues [56] in studies focusing primarily on legume model crops [57]. The nodulation suppression under high N availability has also been observed in other legume tree species, such as *Sesbania sesban* L. Merr., which showed no nodules under high  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations in a study by Dan and Brix [58], and *Faidherbia albida* (Delile) A.Chev., whose nodulation with *Bradyrhizobium* strains was very sensitive to mineral nitrogen availability [10]. Therefore, the relationship of *P. niopoides* and NFB should be further investigated regarding rhizobia species and strains and other factors like the substrate, nitrogen source, and environmental conditions.

Nitrogen fertilization had a positive effect on seedling growth up to  $500 \text{ mg L}^{-1}$  N. The decrease in plant dry mass (primarily roots) at the highest urea rates ( $1000$  and  $2000 \text{ mg N L}^{-1}$ ) indicates some toxicity effects of nitrogen fertilization on plant metabolism, such as ammonia excess in tissues. Dan and Brix [58] also reported short and stunted roots in *Sesbania sesban* exposed to high  $\text{NH}_4^+$  concentration. Urea application in peat-based substrates can alter the medium pH, consequently implying other effects on plant growth, such as nitrite accumulation and decreased micronutrient availability. Other sources of nitrogen, such as nitrate-based fertilizers (like calcium nitrate, also used in tree seedling nurseries), may yield different results in *P. niopoides* seedlings, as there are differences between  $\text{NH}_4^+$  and  $\text{NO}_3^-$  in plant metabolism [59].

When N fertilization was not applied in the nursery, the inoculated seedlings exhibited seven times greater new root emission after planting in the soil. This trait is an advantage over weed competition and potential droughts [60]. Considering that invasive species often exhibit rapid root growth [61], the increased root emissions resulting from rhizobia inoculation can enhance the competitiveness of *P. niopoides* seedlings in ecological restoration of natural ecosystems.

Overall, in both nursery growth and post-planting soil growth, *Bradyrhizobium* inoculation resulted in good seedling growth in the absence of urea fertilization. Under the conditions of these experiments, urea appears to be a suitable nitrogen (N) fertilizer for *P. niopoides* seedlings, in the application rate of  $250 \text{ mg L}^{-1}$  N or up to a maximum rate of  $500 \text{ mg N L}^{-1}$  (in nutrient solution, weekly), without significantly compromising the growth benefits of biological nitrogen fixation (BNF) symbiosis. Depending on the goals and management of seedling production, it might even be possible to obtain *P. niopoides* seedlings suitable for planting without the use of nitrogen fertilizers, as post-planting performance, particularly root emission, was already good in plants without urea fertilization. However, adopting this approach requires evaluating the economic viability of inoculation, given that multiple factors can influence symbiosis efficiency [62], including the produc-

tion scale of a given nursery. Further research using direct isolation of rhizobia from *P. niopoides* nodules would significantly advance the practical application of this symbiosis in seedling production.

## 5. Conclusions

*Pseudalbizzia niopoides* benefited from both *Bradyrhizobium elkanii* SEMIA 6432 inoculation and urea fertilization in terms of seedling production and post-planting performance in soil pots. Nitrogen fertilization also affected symbiosis establishment in the seedlings. At higher rates, nodulation was strongly decreased, and the root system exhibited nitrogen toxicity symptoms. Even without urea fertilization, inoculated seedlings showed good new root proliferation after three months of growth in soil, similar to nursery N-fertilized seedlings. If the need for N fertilization is considered, a rate of 250 mg N L<sup>-1</sup> in nutrient solution provided satisfactory plant growth without harming the rhizobia symbiosis. Further steps in this field could investigate natural rhizobia partners and the interaction of legume–rhizobia and other symbiosis for this legume species.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/nitrogen6020026/s1>. Table S1: Fitness of nonlinear regression models for seedling height; Table S2: Fitness of nonlinear regression models for seedling collar diameter; Table S3: Fitness of nonlinear regression models for seedling shoot dry mass; Table S4: Fitness of nonlinear regression models for seedling root dry mass; Table S5: Fitness of nonlinear regression models for seedling total dry mass; Figure S1: Nodulation in *Pseudalbizzia niopoides* seedlings: (a) Root nodules (yellow arrows) observed in inoculated plants after nursery production; (b) Detailed morphology of nodules, showing indeterminate shape or rounded shape in juvenile ones.

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