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Sewage Sludge Compared with Other Substrates in the Inoculation, Growth, and Tolerance to Water Stress of *Samanea saman*

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Abstract: This study evaluated the initial growth and tolerance to water stress after planting *Samanea saman* seedlings produced with different substrates and inoculation patterns. The experiment used a factorial design (3 × 3), with three substrates: standard (67% subsoil + 33% cattle manure), a commercial substrate (composed mainly of peat), and treated sewage sludge; and three inoculation patterns: control (no inoculation), fertilized (no inoculation + chemical fertilization), and inoculation with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi. The seedlings were planted in plastic pots inside a greenhouse. They received irrigation after planting and were submitted to water deficit for 35 days, followed by rehydration for 31 days. The inoculation promoted higher height and biomass for seedlings produced in the standard substrate. In the sludge, the roots biomass decreased when fertilized or inoculated. Seedlings grown in sludge showed higher height and biomass before planting and at the end of the experiment. Although, after rehydration, the height increment was similar for the sludge and the standard substrate. Seedlings grown with the commercial substrate are not recommended for planting sites subjected to water deficit. The standard substrate with inoculation and the sludge without inoculation or fertilization produced seedlings that showed better recovery and growth after water deficit.

Keywords: biosolids; degraded lands restoration; rhizobium; mycorrhizae; leaf water potential

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1. Introduction

The reuse of agricultural, forestry, and urban wastes as substrates for seedling production is increasing, which results in environmental and economic benefits [1]. The residues used in the substrate composition contain nutrients that will be recycled and can reduce chemical fertilization during seedlings production [2]. This practice generates benefits for the seedlings' production and waste management since reusing is a more sustainable alternative than landfill disposal and other similar destinations [3,4].

Sewage sludge is a residue with great potential for use as a substrate to produce tree seedlings since this material can benefit the seedlings' growth and quality [5–7]. The use of this material as a substrate is well explored for other tree species seedlings, but for *Samanea saman* only two studies were found. In their experiments, [8] observed that substrates containing 50 and 100% of sewage sludge benefited the growth of seedlings, and [9] verified acceptable growth for *Samanea saman* seedlings in a substrate composed of 100% sludge. These results suggest that sludge can be considered a suitable substrate for *Samanea saman*, even in high proportions (50 to 100% of the substrate's volume).

The use of sludge in the substrate can also promote savings in nursery production costs, reducing the acquisition of commercial substrates and chemical fertilizers [2,10]. In the chemical composition, the sludge generally presents high contents of nitrogen (N), phosphorus (P), and other macro and micronutrients, except potassium (K) [7]. As physical properties, the sewage sludge has a high water retention capacity, which results from its microporosity and contents of organic matter [11,12]. Adding sludge to the substrate improves water delivery to plants and promotes better rehydration after water restriction events [13].

Studies with nitrogen-fixing bacteria (NFB) and arbuscular mycorrhizal fungi (AMF) inoculation for tree seedlings using sewage sludge as substrate are scarce. For seedlings of *Acacia mangium* and *Acacia auriculiformis*, [5] evaluated the NFB inoculation in substrates with 33 and 100% of sewage sludge. Higher growth was observed for the 100% sludge substrate combined with inoculation. The AMF inoculation was studied by [14] for *Eucalyptus globulus* and [15] for *Caesalpinia ebano*. The first study observed synergic effects between small doses of sludge (6 g per 100 g of soil) and AMF, and the latter observed no effects.

Combined inoculation of NFB and AMF in substrates with sewage sludge for tree seedlings was assessed by [9]. Among several substrates tested, a sphagnum peat commercial formulation was considered favorable to form the symbiosis of *Samanea saman* with NFB, and the sewage sludge selected as favorable to form the symbiosis among *Samanea saman* and AMF. Seedlings produced in these substrates can potentialize the benefits of the symbioses, resulting in better tolerance and higher growth after planting in water-stress conditions because the AMF enhance the water absorption efficiency due to the small diameter of the hyphae [16,17], and the NFB enhance the plants' growth [18]. Due to the occurrence of short droughts, the seedlings can be subjected to water-stress conditions after planting, even during the rainy season [19]. In these situations, the planting takes place in moist soil, and seedlings must withstand a water-deficit period until precipitation or irrigation.

Commonly known as rain tree, *Samanea saman* (Jacq.) Merr. is a leguminous species with natural occurrence in tropical forests of Brazil, Peru, Paraguay, and Bolivia [20]. The species is extensively used in forest restoration [21,22], has medicinal properties [23], the fruits are suitable for cattle feeding [20], and it can associate with several nitrogen-fixing bacteria genera and families [24]. *Samanea saman* tolerates flooded environments, resists periods of drought and is flexible regarding soil fertility, showing profitable growth under low nutrient and pH levels [25].

The study's main objective was to evaluate the initial growth and tolerance to water stress after planting *Samanea saman* seedlings produced with different substrates and inoculation patterns. The specific objectives were (1) to verify the leaf water potential (Ψf) during the water stress period and at the beginning of rehydration; (2) to evaluate the dynamics of seedlings' height growth during the water stress period and after rehydration; and (3) to assess the biomass gain of plants at the end of the rehydration period.

The study's novelty is that it evaluated the combined effects of substrate and inoculation (with NFB and AMF) and how these nursery inputs can affect seedlings' performance under water-stress conditions after planting. Few studies were found assessing inoculation of NFB and AMF in substrates containing sewage sludge. Similar studies generally evaluate variable proportions of sludge as a substrate component when the present study evaluated a substrate composed of 100% sludge. Considering the importance of *Samanea saman* to restore degraded soils, it is crucial to generate more information about its seedling production, post-planting performance, and drought-stress tolerance.

It was observed that seedlings produced with the sludge substrate were bigger before planting and maintained their higher height and biomass under water-stress conditions. For planting in areas subjected to periodic droughts, it is recommended to use seedlings produced with sludge as substrate. Even though not considered the most favorable for symbiosis with NFB and AMF, the standard substrate showed the best response for the inoculation, maintaining seedlings' growth during and after the water-deficit period. The seedlings produced in the commercial substrate are not indicated for planting in areas subjected to periodic droughts. This manuscript can contribute to future studies assessing the NFB and AMF inoculation in tree seedlings, different substrates for seedlings production, drought tolerance of *Samanea saman*, and the interaction among these factors.

2. Materials and Methods

The experiment was conducted for 66 days in a greenhouse in Seropédica, Rio de Janeiro, Brazil (23 K, 635,524.60 mE, 7,482,447.04 mS). The region's climate is type Aw, according to Köppen. When the temperature inside the greenhouse exceeded 35 °C, the humidification system was automatically activated. The temperature and relative humidity inside the greenhouse were measured every 30 min during the experiment. The mean maximum and minimum temperatures and humidity were 34.7 and 20.6 °C and 25.9 and 21.7%. The general mean temperature was 25.9 °C, and the humidity 22.7%.

The experiment was assembled in randomized blocks with a factorial design (3 × 3) consisting of three substrates and three inoculation patterns, summing nine treatments with four replications (blocks).

The *Samanea saman* seedlings used for planting were produced during 113 days with 280 cm^3 plastic tubes as containers. The substrates were (a) standard: 67% of clay subsoil plus 33% of cattle manure; (b) commercial: composed mainly of sphagnum peat plus vermiculite and carbonized rice husk; and (c) sludge: 100% of sewage sludge from the Ilha do Governador Wastewater Treatment Plant (WWTP), provided by the Companhia Estadual de Águas e Esgoto do Rio de Janeiro (CEDAE). The Ilha WWTP receives only domestic wastewater and performs secondary treatment of sewage by activated sludge process. The sludge treatment consists of densification with centrifuges, anaerobic digestion for stabilization, and dewatering in open-air drying beds where the material stays for at least 90 days until it reaches a moisture content below 30% [12]. The sewage sludge heavy metal contents were evaluated before the seedling's production (Table 1).

Table 1. Heavy metal total contents of the sewage sludge used as a substrate to produce *Samanea saman* seedlings with different substrates and inoculation patterns.

	As	**Ba**	**Cd**	**Pb**	**Cu**	**Cr**	**Ni**	**Zn**
Reference	mg kg^{-1}							
Sludge	0.12	217.12	1.35	119.21	202.45	55.40	79.17	920.05
Conama *	41.00	1300.00	39.00	300.00	1500.00	1000.00	420.00	2800.00

* Maximum heavy metal contents allowed by the Conama resolution nº 498/20 [26] for class A sewage sludges. The analysis used the acid digestion method, and the chemical elements were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

The inoculation treatments were (a) inoculated: double inoculation with nitrogen-fixing bacteria (NFB) and arbuscular mycorrhizal fungi (AMF); (b) positive control: no inoculation and chemical fertilization with 111 mg of N, 16 mg of P_2O_5, and 51 mg of K_2O per seedling, divided in ten weekly fertigations between the 31st and 93rd day after sowing; and (c) control: without inoculation or fertilization. The NFB were *Bradyrhyzobium elkanii* from the strains BR 6205 and BR 6212 recommended for *Samanea saman* by [27]. The NFB inoculant was mixed with the seeds of the inoculated treatment just before sowing. The AMF inoculation was performed with a mixed inoculant containing approximately five spores of nine different species from the following lineages: A97 *Acaulospora colombiana*; A96 *Acaulospora morrowiae*; A38 *Acaulospora scrobiculata*; A2 *Dentiscutata heterogama*; A36 *Gigaspora cândida*; A20 *Glomus formosanum*; A5 *Rhizophagus clarus*; A80 *Scutellospora calospora*; and A75 *Scutellospora gilmorei*. Both inoculants were provided by the Centro de Recursos Biológicos Johanna Döbereiner from Embrapa Agrobiologia.

The morphological characteristics of the seedlings produced in each treatment are presented in Table 2.

Table 2. Morphological parameters before planting of *Samanea saman* seedlings produced with different treatments of substrate and inoculation.

Substrate Treatment	Inoculation Treatment								
	Control	**Positive Control**	**NFB + AMF**	**Control**	**Positive Control**	**NFB + AMF**	**Control**	**Positive Control**	**NFB + AMF**
	Nodule dry mass (mg) CV = 21.9%			Height (cm) CV = 4.0%			Roots dry mass (g) CV = 10.2%		
Standard	170 Bb	64 Bc	380 Ba	23.4 Cb	24.8 Cb	28.2 Ba	0.63 Cb	0.89 Ca	0.70 Cb
Commercial	584 Aa	243 Ab	656 Aa	33.8 Ba	32.3 Ba	34.2 Aa	1.40 Bb	1.73 Ba	1.41 Bb
Sludge	150 Bb	25 Bc	372 Ba	37.4 Ab	39.1 Aa	35.2 Ac	1.98 Aa	1.95 Aa	1.65 Ab
	Root colonization (%) CV = 130.0%			Collar diameter (mm) CV = 3.8%			Shoot dry mass (g) CV = 7.2%		
Standard	0 Ab	0 Ab	13 Aa	3.7 Cb	3.7 Cb	4.1 Ba	1.67 Cc	2.04 Cb	2.83 Ca
Commercial	0 Ab	0 Ab	13 Aa	4.9 Ba	5.1 Ba	5.0 Aa	4.70 Ba	4.84 Ba	5.05 Ba
Sludge	0 Ab	0 Ab	10 Aa	5.7 Aa	5.5 Aa	5.1 Ab	5.26 Ab	5.31 Ab	5.73 Aa
	Spore density CV = 142.0%			Leaf area (cm^2) CV = 14.2%			Total dry mass (g) CV = 7.1%		
Standard	0 Ab	0 Ab	33 Ba	129 Bb	192 Ba	252 Ba	2.30 Cc	2.93 Cb	3.53 Ca
Commercial	0 Ab	0 Ab	21 Ba	417 Aa	438 Aa	444 Aa	6.09 Ba	6.57 Ba	6.47 Ba
Sludge	0 Ab	0 Ab	411 Aa	375 Ab	452 Aa	503 Aa	7.24 Aa	7.25 Aa	7.38 Aa

Inoculation treatments: NFB + AMF (seedlings inoculated with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi); positive control (non-inoculated seedlings fertilized with 111 mg of N, 16 mg of P_2O_5 and, 51 mg of K_2O); control (no inoculation and no fertilization. Substrate treatments: sludge (sewage sludge from Ilha do Governador Wastewater Treatment Plant provided by Companhia Estadual de Águas e Esgoto do Rio de Janeiro—CEDAE); commercial (commercial substrate composed mainly of sphagnum peat plus vermiculite and carbonized rice husk); standard (67% clay subsoil plus 33% of cattle manure). CV: coefficient of variation. Equal letters, upper-case comparing substrates (columns), and lower-case comparing inoculation patterns (rows) do not differ from each other by the Scott-Knott test at 5% of probability.

The seedlings were planted in 12 liters plastic pots (with 25 cm of diameter and height) containing soil from degraded pastureland to simulate outplanting conditions. The soil used to fill the pots was extracted from the "Terraço" experimental area located in Embrapa Agrobiologia, Seropédica, Rio de Janeiro, Brazil (23 K, 636,877.09 mE, 7,483,438.23 mS). It is classified as Argissolo Vermelho Amarelo distrófico [28] (Ultisol, [29]). The granulometric composition of the soil was 49.2% of sand (2.0–0.05 mm), 16.4% of silt (0.05–0.002 mm), and 34.4% of clay (<0.002 mm). Before being placed in the pots, the soil was sieved on a 1 cm mesh diameter. There was no supplementary fertilization. Samples of the soil and the substrates were collected to evaluate their chemical fertility (Table 3). The physical properties of the substrates were also evaluated (Table 4).

After planting the seedlings, irrigation was performed until the soil reached the field capacity, simulating the ideal planting conditions. Then the seedlings went through 35 days without receiving water, simulating a condition of post-planting drought. On the 36th day, the rehydration began with weekly irrigations to maintain the soil at field capacity, simulating precipitation return.

The water volume to be provided by the irrigations was estimated with four replications of a water retention test, which was conducted before the experiment implementation. The test's first part consisted of weighing 1 kg of the soil, putting it in a pot with drainage holes, and slowly adding 1 L of water. In the second part, the sample was weighed after 3 h, so the water that was not retained by the soil could drain. The retained water volume was calculated by subtracting the first weight from the second. Thus, the soil could retain a mean value of 190 mL kg^{-1} of water. This value was multiplied by 12 (considering that the pots contained 12 kg of soil), resulting in 2.28 L of water per pot, which was the volume of water supplied in each irrigation.

Table 3. Chemical fertility (methodology in [30]) and pH of the substrates and components used to produce *Samanea saman* seedlings with different substrates and inoculation patterns.

Substrate	pH (H_2O)	N_{pot}	P	K	Ca	Mg	H + Al	Al
		mg L^{-1}				cmol$_c$ dm^{-3}		
Standard	7.4	98	81	729	3.2	1.8	1.3	0.0
Commercial	5.4	141	193	313	3.5	15.0	5.5	0.3
Sludge	5.1	409	388	189	3.6	1.3	10.2	0.1
Soil	5.2	69	13	64	1.6	0.8	3.8	0.04

Npot = potentially available nitrogen (nitrate + ammonium + nitrite + organic labile forms). Methodology: N_{pot} (potassium chloride—KCl) extraction, acid digestion determination—Kjeldahl); available P (Mehlich I extraction, colorimetric determination); exchangeable K Mehlich I extraction, determination by flame photometry); exchangeable Ca and Mg (KCl 1M extraction, determination by atomic absorption spectroscopy); H + Al and Al (KCl 1M extraction and titration determination).

Table 4. Physical properties (methodology in [31]), organic carbon, and organic matter of substrates used to produce *Samanea saman* seedlings with different substrates and inoculation patterns.

Substrate	EC	BD	TP	AP	EAW	WBC	AW	UW	C_{org}	OM
	(mS/cm)	>(kg/m^3)	%							
Standard	0.85	932.49	60.84	12.43	18.43	3.76	22.19	26.22	5.70	9.82
Commercial	1.03 *	183.28 *	83.84 *	23.66 *	21.79 *	3.93 *	25.72 *	34.46 *	21.58	37.21
Sludge	2.97 *	453.48 *	83.21 *	15.25 *	28.32 *	7.76 *	36.08 *	31.88 *	12.61	21.74

EC: electrical conductivity obtained in 1:5 solution (V:V); BD: bulk density; TP: total porosity; AP: air-filled porosity; EAW: easily-available water; WBC: water buffering capacity; AW: available water; UW: unavailable water; C_{org}: organic carbon (determined by dry combustion method); OM: organic matter (calculated with C_{org} using the "van Bemmelen" factor). * Data presented in [9] that evaluated the same batch of these substrates.

The plants' height was measured at 3, 10, 20, 24, 31, 43, 52, 59, and 66 days after planting. The daily height increment (DHI) was calculated for every measurement interval. At 66 days after planting, we took the seedlings to the laboratory, where we separated the shoot from the roots, dried them in a forced-air oven (65–70 °C), and weighed them on a precision scale. Total dry mass was obtained by summing the roots' and shoots' mass. To obtain the increment in root dry mass, we subtracted the value measured in the seedlings before planting by the one obtained from the plants at 66 days after planting.

The leaf water potential (Ψf) was measured at 0, 7, 16, 22, 28, 37, and 38 days after planting. The first five measurements were during the water stress period, and the last two were at the beginning of the rehydration. The Ψf was assessed before dawn between 04:30 a.m. and 05:30 a.m. using a Scholander pressure chamber.

The water stress was maintained until 35 days after planting, when the Ψf measures reached values under −40 bar (minimum pressure measured by the Scholander chamber). At this moment, the rehydration period started, and the experiment was kept until 66 days after planting when the height's daily mean increment of some treatments indicated the return of normal plant growth.

The data were submitted to a two-way analysis of variance (ANOVA). The experimental unit consisted of five replications per block for all the variables, except for Ψf, which had only one replication per block. The residues' normality (Shapiro–Wilk) and the homogeneity of variances (Bartlett) were tested to check the ANOVA assumptions. When the F test showed differences between treatments, their means were compared using the Scott-Knott test with a 5% error probability. The software SISVAR 5.6 (Daniel Furtado Ferreira, Departamento de Estatística, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil) was used for all the analyses.

3. Results

The interaction between the factors (substrates and inoculation patterns) was significant for height's growth and increment at all measurement periods. Immediately after planting (0–3 days), the soil was very moist, allowing plants to grow in height (Table 5). The seedlings from sludge and commercial treatments showed higher leaf area before planting (Table 1), were in a substrate with higher nutrients contents (Table 2), and consequently presented more significant growth them seedlings from the standard substrate (Table 5).

Table 5. Height total values and increment at 3, 20, 31, 52, and 66 days after planting *Samanea saman* seedlings produced with different inoculation patterns and substrates under water restriction (until the 36th day) and rehydration (after the 36th day).

Substrate Treatments	Inoculation Treatments					
	Height (cm)			Daily Mean Height Increment (mm)		
	Control	Positive Control	NFB + AMF	Control	Positive Control	NFB + AMF
	3 days after planting CV = 4.84%			between 0 and 3 days after planting CV= 36.88%		
Standard	26.6 Bb	28.0 Cb	31.6 Ba	10.7 Ba	10.6 Ba	11.4 Ba
Commercial	39.5 Aa	37.4 Ba	40.0 Aa	18.9 Aa	16.9 Aa	19.2 Aa
Sludge	41.9 Aa	44.7 Aa	42.5 Aa	15.1 Aa	18.5 Aa	24.2 Aa
	20 days after planting CV = 4.06%			between 3 and 20 days after planting CV= 17.53%		
Standard	30.6 Bc	34.5 Cb	42.4 Ba	2.3 Bc	3.8 Bb	6.3 Aa
Commercial	48.9 Aa	48.6 Ba	51.1 Aa	5.6 Aa	6.6 Aa	6.6 Aa
Sludge	47.6 Ab	51.6 Aa	50.3 Aa	3.3 Ba	4.1 Ba	4.6 Ba
	31 days after planting CV = 4.93%			between 20 and 31 days after planting CV= 52.19%		
Standard	31.9 Bc	35.1 Cb	41.1 Ba	1.2 Aa	0.6 Aa	−1.1 Ab
Commercial	45.0 Aa	43.6 Ba	46.0 Aa	−3.6 Ca	−4.5 Ca	−4.7 Ba
Sludge	47.1 Ab	50.1 Aa	45.7 Ab	−0.4 Ba	−1.3 Ba	−4.2 Bb
	52 days after planting CV = 5.44%			between 31 and 52 days after planting CV= 51.85%		
Standard	35.4 Cb	37.5 Cb	46.2 Ba	1.7 Aa	1.1 Aa	2.4 Aa
Commercial	46.8 Ba	46.8 Ba	47.5 Ba	0.8 Aa	1.5 Aa	0.7 Aa
Sludge	51.4 Aa	54.5 Aa	52.2 Aa	2.1 Aa	2.1 Aa	3.1 Aa
	66 days after planting CV = 6.62%			between 52 and 66 days after planting CV= 21.27%		
Standard	44.8 Cb	45.9 Bb	56.4 Ba	6.7 Aa	6.1 Aa	7.3 Aa
Commercial	50.4 Ba	50.7 Ba	52.3 Ba	2.6 Ba	2.8 Ba	3.4 Ba
Sludge	64.6 Aa	64.7 Aa	61.4 Aa	9.4 Aa	7.3 Aa	6.6 Aa

Inoculation treatments: NFB + AMF (seedlings inoculated with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi); positive control (non-inoculated seedlings fertilized with 111 mg of N, 16 mg of P_2O_5 and, 51 mg of K_2O); control (no inoculation and no fertilization. Substrate treatments: sludge (sewage sludge from the Ilha do Governador WWTP, provided by CEDAE); commercial (commercial substrate composed mainly of sphagnum peat plus vermiculite and carbonized rice husk); standard (67% clay subsoil plus 33% of cattle manure). CV: coefficient of variation. Equal letters, upper-case comparing substrates (columns), and lower-case comparing inoculation patterns (rows) do not differ from each other by the Scott-Knott test at 5% of probability.

At the beginning of the water stress period (3–20 days), the height increment was reduced in all treatments to less than 1/3 of the growth measured in the first three days (Table 5), demonstrating the water deficit's influence in reducing the plants' growth. The plants from the commercial substrate (favorable to nodulation according to [9]) had a higher increment in height than those of other treatments.

The plants whose seedlings were produced in the sludge substrate showed an increment in height similar to those grown in the standard substrate (Table 5). However, with inoculation, plants from the standard substrate showed a higher increment than those from the sludge. The plants from the sludge and commercial substrates did not show the effects of fertilization nor inoculation in the height increment between 3 and 20 days after planting. For plants from the standard substrate, the inoculation increased the height increment, surpassing plants whose seedlings were fertilized in the nursery.

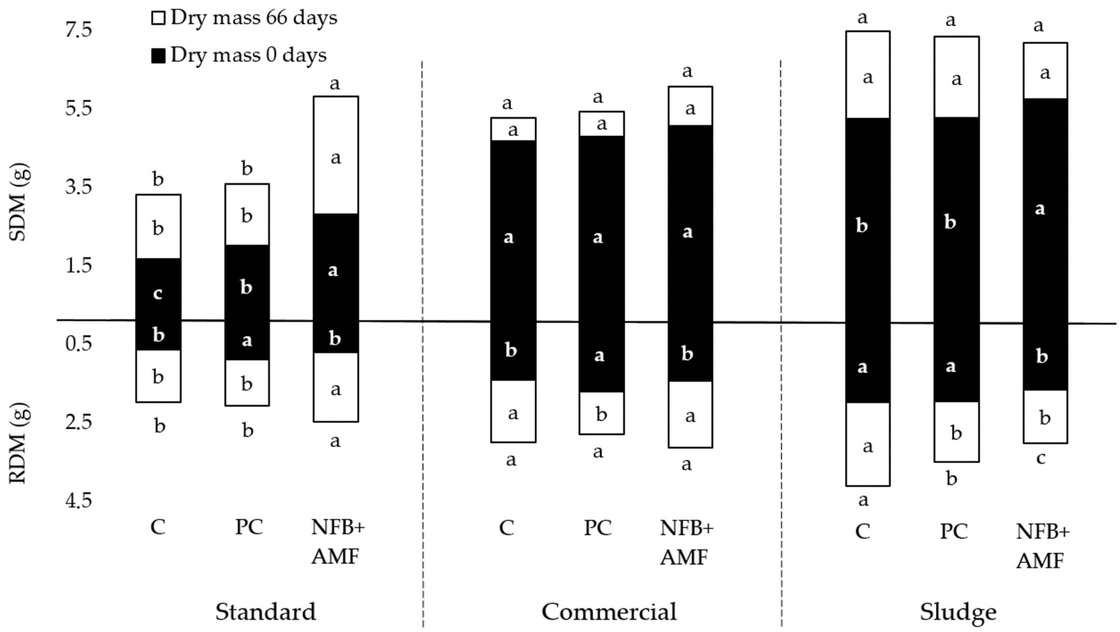
At the end of the water-deficit period (20–31 days), the water restriction effects were drastic on the height increment (Table 3). The plants of most treatments presented negative values due to apical wilting. The non-inoculated plants from the standard substrate were the only ones to present a positive height increment during this period since, before planting, these seedlings had the lowest values of biomass, height, and diameter (Table 1). The inoculation that favored seedlings' growth in the sludge and standard substrates

(Table 2) resulted in plants that showed growth reduction when the water stress reached extreme values near the permanent wilting point (20–31 days).

The plants returned to grow in height at the rehydration period (31–52 days), showing the recovery after irrigation (Table 5). However, they presented low increments and no evidence of the treatments' effects for substrates, fertilization, or inoculation. At the end of the rehydration period (52–66 days), the height increment reached values close to the ones observed at the beginning of water stress (3–20 days). In this final period, the plants from sludge and standard substrates showed a higher height increment. The seedlings produced with the commercial substrate were less tolerant to the drought simulation and did not recover their total growth after irrigation reestablishment. During this last phase, the inoculation treatments did not influence the plants' height increment.

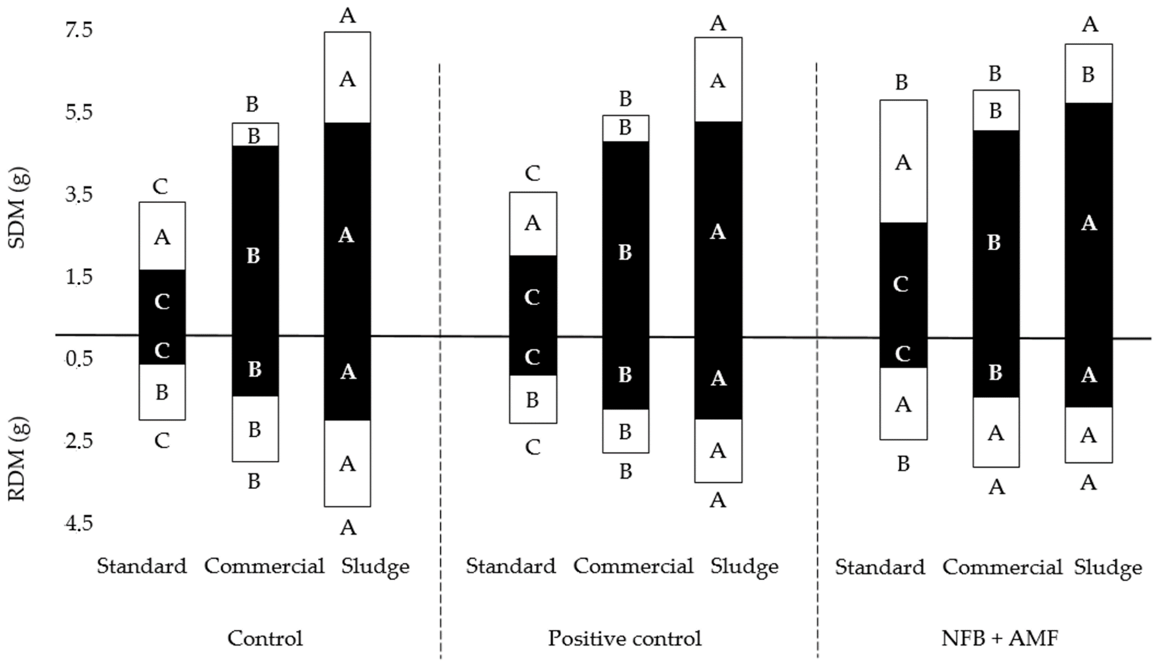
The interaction between the factors (substrates and inoculation patterns) was significant for the plants' shoot, root, and total dry mass. The standard substrate promoted the more significant inoculation benefit effect on the plants' biomass growth (Figure 1). The plants produced with the commercial substrate presented lower biomass growth during the experiment. However, when inoculated, the seedlings from this substrate showed higher root growth. The plants' rooting increased with the inoculation in the standard and commercial substrates and decreased for the sludge.

Regarding the leaf water potential (Ψf) after planting *Samanea saman* under water-stress conditions, for 0, 7, and 22 days after planting, there was no interaction between the substrate and inoculation patterns (Table 6). While at 16, 28, 37, and 38 days after planting, there was an interaction between the factors (Table 7). The results show that the longer the plants remain in a water-deficit condition and the bigger the seedlings before planting, the lower Ψf will be. The plants produced with the standard substrate were the ones that suffered less with the water stress. On the other hand, the seedlings produced in the commercial substrate suffered more water stress. The sludge presented intermediate results, with a significant increase of Ψf after rehydration at 37 and 38 days after planting (Table 7).



(**a**)

Figure 1. *Cont.*



(**b**)

Figure 1. Root dry mass (RDM) and shoot dry mass (SDM) of *Samanea saman* plants, in which seedlings were produced with different inoculation patterns and substrates. Equal lower-case letters for inoculation patterns (**a**) and equal upper-case letters for substrate (**b**) do not differ from each other by Scott-Knott test at 5% of probability. Letters inside the black bars refer to seedlings' dry mass before planting (CV = 7.2 and 10.2% respectively for SDM and RDM), inside white bars to their increment during the 66 days of the experiment (CV = 23.2 and 17.2% respectively for SDM and RDM), and out of the bars for the total dry mass at 66 after planting (CV = 12.1 and 9.1% respectively for SDM and RDM). Inoculation treatments: NFB + AMF (seedlings inoculated with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi); PC = positive control (non-inoculated seedlings fertilized with 111 mg of N, 16 mg of P_2O_5, and 51 mg of K_2O); C = control (no inoculation and no fertilization). Substrate treatments: sludge (sewage sludge from the Ilha do Governador WWTP, provided by CEDAE); commercial (commercial substrate composed mainly of sphagnum peat plus vermiculite and carbonized rice husk); standard (67% clay subsoil plus 33% of cattle manure).

Table 6. Leaf water potential (Ψf in bar) at 0, 7, and 22 days after planting *Samanea saman* when there was no interaction between the factors: substrates and inoculation patterns.

	Inoculation Treatments			Substrates		
Ψf (bar)	**Control**	**Positive Control**	**NFB + AMF**	**Standard**	**Commercial**	**Sludge**
0 days (CV = 17.4%)	−2.9 a	−2.7 a	−3.1 a	−2.8 a	−3.1 a	−2.7 a
7 days (CV = 16.6%)	−3.1 a	−3.6 a	−3.5 a	−3.7 b	−3.5 b	−3.0 a
22 days (CV = 16.1%)	−12.7 a	−12.9 a	−17.2 b	−7.5 a	−20.2 c	−15.2 b

Inoculation treatments: NFB + AMF (seedlings inoculated with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi); positive control (non-inoculated seedlings fertilized with 111 mg of N, 16 mg of P_2O_5, and 51 mg of K_2O); control (no inoculation and no fertilization. Substrate treatments: sludge (sewage sludge from the Ilha do Governador WWTP, provided by CEDAE); commercial (commercial substrate composed mainly of sphagnum peat plus vermiculite and carbonized rice husk); standard (67% clay subsoil plus 33% of cattle manure). CV: coefficient of variation. Equal letters in the same row do not differ by the Scott-Knott test at 5% probability.

Table 7. Leaf water potential (Ψf in bar) at 16, 28, 37, and 38 days after planting *Samanea saman* when there was an interaction between the factors: substrates and inoculation patterns.

Substrates	**Inoculation Treatments**		
	Control	**Positive Control**	**NFB + AMF**
	Ψf at 16 days after planting (CV = 12.3%)		
Standard	−5.3 Aa	−5.2 Aa	−5.9 Aa

Table 7. *Cont.*

Substrates	Inoculation Treatments		
	Control	Positive Control	NFB + AMF
Commercial	−7.7 Ba	−6.9 Ba	−6.4 Aa
Sludge	−7.1 Ba	−6.6 Ba	−8.2 Bb
	Ψf at 28 days after planting (CV = 5.2%)		
Standard	−26.5 Aa	−31.2 Ab	−38.5 Cc
Commercial	−38.5 Ca	−37.8 Ba	−37.2 Ba
Sludge	−31.5 Ba	−38.5 Bb	−33.6 Aa
	* Ψf at 37 days after planting (CV = 10.8%)		
Standard	−10.2 Aa	−28.2 Ab	−37.5 Cc
Commercial	−36.0 Bb	−37.4 Bb	−20.0 Aa
Sludge	−33.4 Bb	−33.2 Bb	−26.0 Ba
	* Ψf at 38 days after planting (CV = 10.7%)		
Standard	−8.1 Aa	−16.8 Bb	−9.5 Aa
Commercial	−18.0 Ca	−30.5 Cc	−24.0 Cb
Sludge	−15.0 Bb	−10.2 Aa	−16.2 Bb

* After the irrigation return. Inoculation treatments: NFB + AMF (seedlings inoculated with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi); positive control (non-inoculated seedlings fertilized with 111 mg of N, 16 mg of P_2O_5, and 51 mg of K_2O); control (no inoculation and no fertilization. Substrate treatments: sludge (sewage sludge from the Ilha do Governador WWTP, provided by CEDAE); commercial (commercial substrate composed mainly of sphagnum peat plus vermiculite and carbonized rice husk); standard (67% clay subsoil plus 33% of cattle manure). CV: coefficient of variation. Equal letters, upper-case comparing substrates (columns), and lower-case comparing inoculation patterns (rows) do not differ from each other by the Scott-Knott test at 5% of probability.

4. Discussion

The initial growth in height after planting is essential, allowing the plants to compete better for space and sunlight with spontaneous invasive fast-growing species [32]. For this reason, the average height of tree species is one of the success indicators of forest restoration projects [33]. For seedlings' nursery evaluation, height is a non-destructive morphological parameter that is easy to measure and can indicate water stress. In [34], the authors mentioned that, for tree species, water stress influences more the growth in height than in diameter, since, under water deficit, the plant reduces the cellular turgidity and inhibits the cellular elongation and expansion reducing the height's growth [35].

The plants from sludge and commercial substrates presented more significant growth in the experiment's early stages, responding to the higher availability of nutrients in the substrates (Table 3) and the seedlings' higher leaf area (Table 2). These characteristics promoted an increase in CO_2 assimilation [36], more efficient utilization of solar energy to photosynthesis [37], higher production of photoassimilates, and higher transpirational flux, permitting better assimilation of the water with the nutrients [38].

Between the 3rd and 20th day after planting, the commercial substrate plants (that showed higher nodulation before planting; Table 2) had higher height growth than those produced with sludge. Higher growth of nodulated plants facing initial water-stress conditions was found by [18], in which a higher number of nodules in peanut plants favored their growth in a water-deficit situation. However, [39] discussed the influence of water restriction on different bean genotypes and found that the nodules' dry mass did not reduce the plants' water stress.

The plants produced with the standard substrate inoculated with nitrogen-fixing bacteria (NFB) and arbuscular mycorrhizal fungi (AMF) showed a higher height increment in the initial period of water restriction (3–20 days). Among the benefits of inoculation, the nodulation with NFB can increase plants' growth, and the association with AMF can reduce the effect of water stress due to the efficiency of hyphae in absorbing water from soil micropores [17,40,41]. This result shows that even in a substrate that is not considered favorable to symbiosis [9], there may be an after-planting beneficial effect of seedling inoculation.

When the soil water deficit increased to the extreme (20–31 days), there was an intense restriction in the plants' growth. According to [36], cupuaçu plants (*Theobroma grandiflorum*) submitted to water stress after planting presented an 86% height increment reduction than plants from an irrigated treatment. The authors also observed that the height growth had negative values in some progenies due to the shoot apical wilting. Studying Indian neem (*Azadirachta indica*) after planting, [42] also found that intense water stress reduced the plant height increment. In the present experiment conditions, when the water restriction was intense (20–31 days), the seedlings with a larger leaf area before planting (Table 2) were less prepared for water stress. The leaf area reduction is a morphological response of the plants to water-deficit conditions [43], together with the shoot dry mass reduction [44].

The commercial substrate has a hydrophobic behavior (difficult rehydration) when it reaches low humidity [45]. This physical property is common in substrates composed mainly of sphagnum peat. This substrate rehydration problem harmed the roots' water absorption and intensified water stress damages in plants.

Tree species are fast to recover their growth when submitted to rehydration after a water-deficit period. In [46], the authors mention that after a water restriction period, African mahogany (*Khaya ivorensis*) seedlings returned to grow three days after the irrigation was restarted. The present study verified similar results with *Samanea saman* and [47] for the cupuaçu tree (*Theobroma grandiflorum*).

Despite having a larger leaf area before planting (Table 2), the seedlings produced with sludge resisted more than 20 days of water restriction without reducing their height increment (Table 5). Higher available water (retained at tensions between 1 to 10 KPa) was observed in the sludge than in the commercial and standard substrates (Table 4). The authors of [11,12], among other studies, also verified the higher water retention capacity of substrates composed mainly of sludge. The information cited above justifies the water-deficit resistance and the height and biomass growth results observed in the present study for plants produced with the sludge substrate.

The microporosity increase is another effect of using sewage sludge as a substrate component to produce tree seedlings [2,11,12]. This parameter is related to the water retention capacity and can justify the growth regain after water restriction that we observed for plants produced with sludge. According to [48], the higher microporosity results in substrate rehydration improvements, considering both its effects in increasing the water retention capacity and decreasing the drainage of irrigation water. Such results indicate the use of substrates composed mainly of sewage sludge to produced seedlings for planting in areas subjected to short droughts or other temporary water restriction situations.

The sludge substrate also presented higher N and P contents than other substrates (Table 3), which could have nurtured plants along with the experiment, increasing their growth in biomass (Figure 1). Other studies show that the application of sewage sludge in the soil can reduce or even replace chemical fertilization in agricultural production systems due to its high nutrient content [49,50]. The higher rooting of plants produced with sludge (Figure 1) may have also enhanced water absorption from the soil and reduced the damages caused by water restriction [51]. In the sludge substrate, the high contents of N and P could have restricted AMF and NFB symbiosis benefits during the experiment [52] because when nutritional conditions are optimal, the microorganisms behave like a drain of photoassimilates of the plants [53].

The seedlings in the standard substrate had a lower leaf area before planting (Table 2), which resulted in lower water loss by transpiration [54], maintaining the water from the after-planting irrigation in the soil for more time, reducing the effects of the water restriction [55], and enabling higher growth of shoot biomass during the water restriction period.

The higher roots biomass in plants from the standard substrate combined with inoculation contributed to their recovery in shoot growth after rehydration. The presence of AMF hyphae could have enhanced the water absorption efficiency [17] and potentialized the effects of NFB in nitrogen biological fixation [56]. In the study of [57], the biomass of sabiá (*Mimosa caesalpiniaefolia*) after outplanting was superior when the seedlings were inoculated with NFB and AMF together.

In substrates with lower P contents, [58] mention more significant AMF inoculation effects in the seedlings' survival and growth after outplating. We observed similar results in the present study for seedlings produced in the standard substrate combined with AMF and NFB inoculation. The seedlings produced with this substrate did not present the better morphological characteristics before planting (Table 2) but showed higher increments after outplanting (Table 5), even in adverse water-deficit conditions. For this reason, seedlings produced in this treatment should be considered of good quality [59].

The plants from commercial substrate invested more in roots biomass than in shoot biomass. As observed by [44,60], in water-stress conditions, the plants allocate photoassimilates to roots instead of the shoot to enhance their capacity of absorbing water from the soil. The commercial substrate's hydrophobic behavior in low humidity conditions [45] harmed the roots' water absorption and intensified the water-stress damage, reducing the biomass growth [61]. When combined with inoculation, the commercial substrate plants presented higher root biomass (Figure 1). Like the standard substrate, for the commercial, the presence of symbiotic microorganisms enhanced the plants rooting in water-stress conditions.

The planting soil texture also influenced the response of treatments to water stress and rehydration. In general, soils with a clay loam texture have a decent water retention capacity, which provides a gradual reduction in water availability after the irrigation interruption [62]. This characteristic was beneficial in the present study, considering that it permitted the water stress evaluation for a longer time and in more detail. The soil could also maintain its high moisture after irrigation, allowing the rehydration of the plants.

The plants' responses to water-stress conditions vary according to the species, cultivar, exposure time, edaphic factors, among others [63]. The leaf water potential (Ψf) is an essential parameter for studying water stress due to its relation with stomatal conductance, gas exchanges, transpiration, and soil water availability [64]. When the soil reaches a water potential inferior to -15 bar, the plants cannot absorb water from the soil, reaching the permanent wilting point [61]. However, this same author mentions that some species can survive in soils with -60 bar of Ψf. In our study, *Samanea saman* absorbed water from the soil without reaching the permanent wilting point in pressures lower than -15 bar in all treatments (Tables 6 and 7), showing that this is species is tolerant to water stress.

The fast recovery of the Ψf after irrigation restart corroborates with the study of [65]: analyzing plants of pajeú (*Triplaris gardneriana*), seven days after the irrigation's return, the plants presented the same Ψf than plants from the treatment without water stress. The recovery of Ψf after irrigation confirms the tolerance of *Samanea saman* to water stress since the damages caused during the 35 days that plants stayed without irrigation did not restrict their physiologic functions of absorbing and transporting water from the roots to shoot [46]. Considering that until the 20th day, the plants have not stopped growing in height (Table 5), it is possible to state that the Ψf reached before this period (-7.8 bar) did not affect *Samanea saman* growth.

The seedlings produced with the standard substrate were the ones that suffered less from the water stress. The lower leaf area and height of seedlings before planting (Table 2) possibly justify this result since these morphological characteristics positively correlate with transpiration and soil water consumption after planting [66].

The commercial substrate produced the seedlings that most suffered from water stress. The sphagnum peat's hydrophobic behavior can explain this result since after reaching low humidity, the substrates based on this material have difficulties rehydrating [45]. In the commercial substrate, the inoculation favored the plants' resistance to water stress, corroborating the studies of [40,67,68].

Studying the water stress in corn plants, [69] concluded that using humic acids from sewage sludge improved the Ψf, softened the physiological effects, and enhanced plants' morphological growth. However, the present study's results show that the sludge substrate had favorable effects on Ψf only at the beginning of water stress (at seven days after planting), at 28 days when combined with inoculation, and after rehydration (at 37 and 38 days). Before planting, the higher leaf area and height of the seedlings may have caused higher transpiration and, consequently, lower Ψf during the water-stress period.

It is also essential to state the limitations of using inoculants in tree seedlings' production. For NFB inoculants, it is worth mentioning the specificity of the relation between leguminous species and the different species and strains of NFB [27], affecting the competition for symbiotic zone colonization between the inoculated bacteria and the ones that are naturally present in the planting soil [70]. Besides, there is a limited number of institutions producing and commercializing NFB inoculants for tree species. Regarding the AMF, the scale production is hampered by the obligation of a host for multiplication since it is impossible to produce inoculants in a culture medium without functional roots [71]. In addition, the high standards required by specific laws that regulate the licensing of production and commercialization of biological inoculants in Brazil [27,72–74] reduce the feasibility of using the AMF inoculation for tree species.

It is crucial to evaluate the sludge properties before using it as a nursery substrate since their chemical and physical characteristics vary among different batches from the same or different WWTPs [4,12]. The limitations can occur due to high salinity, toxic elements, high density, poor stability, among other factors [4,6,48]. Regarding physical properties, for [11], the high density and microporosity of substrates with 60–80% sludge hindered the aeration and drainage, harming the growth of the roots. According to [75], higher proportions of sludge in the substrate harm root aggregation and complicate removing seedlings from the container.

Although the high salinity of sludges can be a significant issue to produce seedlings of ornamental plants [4], forest species can present better tolerance [2,48], which shows that it is essential to consider the requirements and characteristics of each cultivated species [6]. The authors of [75] observed for *Mimosa scabrella* seedlings that even small proportions (10%) of sludge had adverse effects on the growth compared with a commercial substrate composed of pine bark and vermiculite. On the other hand, for *Eucalyptus benthamii*, the authors observed similar growth for the commercial and substrates containing up to 50% sludge. Considering the growth in the nursery and after planting, the sludge used in the present study can be considered adequate to produce *Samanea saman* seedlings.

From a circular economy perspective, the transformation of a residue in a resource and the nutrient recycling makes it interesting to use the sewage sludge as a substrate [3].The other substrates evaluated in the present study have components that generate environmental impacts during their extraction, namely, the sphagnum peat and clay subsoil. Meanwhile, using sewage sludge as a substrate can be considered a sustainable alternative compared to the landfilling of this material [76]. From a nursery perspective, using substrates composed mainly of sewage sludge can reduce production costs, especially with commercial substrates and chemical fertilizers [2,10]. In the present study, similar results for fertilized (positive control) and control (no inoculation and no fertilization) seedlings before and after planting suggests that chemical fertilizers may be avoided to produce *Samanea saman* seedlings with the sludge substrate.

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