

Interaction of Substrates and Inoculants for *Samanea Saman* (Jacq.) Merr Seedling Production

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Abstract

The objective of this study was to evaluate the effect of the inoculation with nitrogen-fixing bacteria (NFB) and arbuscular mycorrhizal fungi (AMF) and substrates in the production of *Samanea saman* (rain tree) seedlings. Three substrates and three inoculation treatments were combined in a randomized block design with six repetitions. The Sewage Sludge substrate favored symbiosis with AMF (promoting twelve times more sporulation), while the Commercial substrate favored symbiosis with NFB (promoting 70% more nodulation). The Sewage Sludge substrate provided the best development of *S. saman* seedlings, as it promoted higher biomass compared to the Commercial and Standard substrates (14% and 150% respectively). The most significant response to inoculation occurred in the seedlings produced in the Standard substrate, in which the inoculated seedlings had higher biomass than those that received fertilization and those that received neither inoculation nor fertilization (20% and 50% respectively).

Keywords: Arbuscular mycorrhizal fungi, Nitrogen-fixing bacteria, Biosolids, Root symbiosis, Rain tree.

1. INTRODUCTION AND OBJECTIVES

At the 11th United Nations Framework Convention on Climate Change held in Paris in 2015, Brazil committed to ingrestoring 12 million hectares of forest by 2030 (Scaramuzza et al., 2016). The cost to reach this goal is estimated to range between R\$ 31 and R\$ 52 billion depending on the projected scenario (Kishinami & Shigueo, 2016). Considering the sizeable amount of national investment planned for the next ten years, it is essential to research to develop techniques capable of reducing the forest restoration plantations costs. These include the inoculation of microorganisms that promote plant growth and nutrition, as nitrogen-fixing bacteria (NFB) and arbuscular mycorrhizal fungi (AMF).

NFB enable the use of atmospheric nitrogen, reducing the use of fertilizers and increasing the growth of forest legumes. Additionally, in mixed plantations, N fixing plants provide higher amounts of this nutrient and positively influence non-fixing plants (de Faria et al., 2020). On the other hand, AMF increase the absorption of water and nutrients of their host

plants as they function as an efficient extension of the root system, facilitating the establishment of seedlings in the field (Hoffman & Lucena, 2006). In forest plantations, the presence of AMF balances the growth of plant species, avoiding the dominance of the most adapted individuals (Santos, 2008).

Several authors have studied the effect of NFB and AMF inoculation on forest leguminous. Reports include increased height and diameter of *Mimosa caesalpiniaefolia* (Mendes et al., 2013), higher biomass production of *Mimosa scabrella* seedlings (Primieri et al., 2015) and improved development of *Acacia mangium* seedlings (Andraus et al., 2020).

Samanea saman (rain tree) is a leguminous species with natural occurrence in tropical forests of Brazil, Peru, Paraguay, and Bolivia (Lorenzi, 1998). It is widely used in forest restoration projects (dos Santos & Manesch, 2016; Góes & Quintela, 2015), It has medicinal properties (Prasad et al., 2008), and the fruits used for cattle feeding (Lorenzi, 1998). *S. saman* tolerates flooded environments and resists periods of drought. It is also flexible regarding soil fertility, showing profitable growth under low nutrient and pH levels

(Barwick et al., 2004). When it comes to its symbiosis with rhizobia, *S. saman* is considered a promiscuous species, able to associate with several bacterial genera and families capable of fixing atmospheric nitrogen (de Faria et al., 2020). Inoculation with *Bradyrhizobium elkanii* BR 6205 and BR 6212, is however indicated for seedling production (Brasil, 2011). Although seed germination rate is low, it exceeds 90% when seeds are subjected to chemical scarification with sulfuric acid (Nascimento & Oliveira, 1999). Seedlings can be produced in organic sandy soils (Lorenzi, 1998) and can reach 33cm in height, 5.2mm in the stem diameter and 4.6g of total dry mass at 195 days after sowing (Abaurre et al., 2020). Staples & Craig (2006) suggest that the seedlings are ready for planting between 90 and 150 days after sowing when they reach a height between 20-30cm and stem diameter greater than 4mm.

Abaurre et al. (2020) testing nine substrates identified that the symbiosis of *S. saman* with AMF and NFB is favoured when seedlings are produced in Carolina Soil Standard 0.7 and Sewage sludge substrate. Carolina Soil Standard 0.7 is a commercial substrate composed of sphagnum peat moss, vermiculite, and carbonized rice husk. The Sewage sludge substrate is supplied by the State Water and Sewage Company of Rio de Janeiro (CEDAE).

Previous studies evaluated and recommended the most promising substrates to favor NFB and AMF inoculation in *S. saman* seedlings (Abaurre et al., 2020). It is necessary to advance the knowledge comparing the effects of inoculants and the traditional chemical fertilizers in seedlings' growth. For this reason, the present study evaluated the interaction of different substrates with or without the presence of NFB and AMF inoculation and fertilization to produce *S. saman* seedlings.

Besides evaluating the effects of plant growth-promoting microorganisms (PGPM), this study also evaluates the use of sewage sludge as a substrate for tree seedlings. Thus, the manuscript has the value of combining two trends (PGPM inoculation and waste reusing alternatives) in modern science (Fijalkowski & Kwarciak-Kzłowska, 2021, Freire et al., 2020).

2. MATERIALS AND METHODS

The experiment was conducted between December 2017 and April 2018, with a total duration of 130 days, in a

greenhouse located at the Federal Rural University of Rio de Janeiro, Seropédica, RJ, Brazil (22°45'33.29" S, 43°40'48.90" W). The greenhouse had the cooling system (air humidifying panel and exhaust fan) automatically turned on when the internal temperature reached 35°C and turned off when it reached 28°C.

The experiment was implemented in a randomized block design in a factorial scheme (3x3) corresponding to three substrates and three inoculation treatments with six replications, with each plot consisting of nine *S. saman* seedlings.

The inoculation treatments were: a) double inoculation with NFB and AMF; b) seedlings produced without inoculants and fertilized with 111 mg of N, 16 mg of P₂O₅ and 51 mg of K₂O per seedling, divided into ten weekly fertigrations between 31 and 93 days after sowing (Positive Control); and c) seedlings neither inoculated nor fertilized (Absolute Control). *Bradyrhizobium elkanii* BR 6205 and BR 6212 strains were used as the NFB, following the recommendation of the Ministry of Agriculture, Livestock, and Supply for the production of *Samanea saman* seedlings (Brazil, 2011). The peat inoculant containing the NFB strains (10⁷ CFU/g) was mixed with the seeds (1.0 g/kg) at the time of planting. Inoculation with AMF was done through application in each seedling of a mixed inoculant of nine species containing approximately five spores of each species of the following strains: A97 *Acaulospora colombiana*; A96 *Acaulospora morrowiae*; A38 *Acaulospora scrobiculata*; A2 *Dentiscutata heterogama*; A36 *Gigaspora candida*; A20 *Glomus formosanum*; A5 *Rhizophagus clarus*; A80 *Scutellospora calospora* and A75 *Scutellospora gilmorei*. The soil was used as a carrier for this inoculant and in addition to the spores counted, there were also fragments of colonized roots and hyphae (which were not counted). Both inoculants were provided by the Johanna Döbereiner Biological Resource Center (CRB-JD) of Embrapa Agrobiology.

The substrates used were: a) Standard: clay subsoil 66% and bovine manure 33% (V/V), which was used a control; b) Commercial: Carolina Soil Standard 0.7, composed of sphagnum peat moss, vermiculite, and carbonized rice husk; and c) Sewage sludge: sewage sludge supplied by the State Water and Sewage Company of Rio de Janeiro (CEDAE). The substrates used were autoclaved and chemically analyzed (Table 1).

Table 1. Chemical characteristics of the substrates analyzed through the soil fertility analysis methodology (Nogueira et al., 1998).

Substrate	pH (H ₂ O)	N _{pot}	P	K	Ca	Mg	H+Al	Al
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
Sewage sludge	5.1	409	388	189	3.6	1.3	10.2	0.1

Npot. = nitrogen potentially available to plants (nitrate + ammonium + nitrite + labile organic forms); Methodologies used in the analyses: Npot. (extraction in KCl, determination by acid digestion - Kjeldahl); Available P (extraction by Mehlich I, determination by colorimetry); Exchangeable K (Mehlich I extraction, flame photometry determination); Exchangeable Ca and Mg (extraction by KCl 1M and determination by atomic absorption spectrometry); Potential acidity (H+Al) and Al (extraction by KCl 1M and determination by titration).

The seedling containers used were 280 cm³ cone-tainers, superficially disinfected by immersion in a 3% sodium hypochlorite solution for 1 hour. The cone-tainers were supported on a 54-cell tray (6 rows of 9 cells). Each tray was only occupied by two experimental plots (two lines of 9 cells).

The seedlings were irrigated daily with autoclaved distilled water by applying 7.5 ml/cone-tainer. The height and stem diameter of the seedlings were measured 113 days after sowing. The plants were harvested 130 days after sowing, dried in a forced circulation oven (65-70°C) and weighed. For the root system, a sample of approximately 0.5 g of fresh thin roots was separated previously to drying to assess the root mycorrhizal colonization rate. These samples were clarified and stained according to Koske & Gemma (1989) and Grace & Stribley (1991), respectively. The colonization rate was estimated, according to McGonigle et al. (1990). The mass of the fresh roots sampled to evaluate colonization was used to correct the total root dry mass. For each substrate, the inoculation response was calculated by dividing the seedling total dry mass of the inoculated treatment by seedling total dry mass of the Absolute Control treatment.

AMF spores were extracted from 50 ml substrate samples through wet sieving (Gerdemann & Nicolson, 1967) and consecutive centrifugations in water and sucrose solution (Jenkins, 1964), after which the spores were counted under a stereomicroscope.

The data were subjected to normality (Shapiro-Wilk), and homogeneity of variance (Bartlett) tests using the R

software program. These were followed by the analysis of variance (two-way ANOVA), and Scott-Knott means comparison test at 5% probability of error, both conducted using SISVAR 5.6.

3. RESULTS AND DISCUSSION

The analysis of variance indicated a significant interaction between inoculation and substrates factors for most of the variables. Root colonization was the exception, as it was only significantly influenced by the inoculation treatments (Table 2).

Although the interaction substrate x inoculation was significant for shoot, root and seedling total dry mass, these variables showed the same response to the substrates regardless of the inoculation treatment, suggesting a strong and consistent difference between the substrates (Table 3). The Sewage sludge substrate increased biomass production the most, followed by the Commercial substrate. The Standard substrate promoted the lowest growth of the seedlings in relation with other two substrates.

Contrary to seedling growth, the greatest response of the inoculation on the biomass was verified for the Standard substrate of lower fertility, because in this substrate, seedling had a higher shoot and total dry mass when inoculated with NFB and AMF, followed by seedlings that received fertilization. The lowest growth for this substrate was verified in the seedlings from the Absolute Control.

Table 2. Summary of the two-way analysis of variance (p-value) for variables measured in *S. saman* seedlings produced in three substrates with three inoculation treatments.

Variables	Substrates	Inoculation	Substrates * Inoculation
Root dry mass	0.0000	0.0000	0.0037
Shoot dry mass	0.0000	0.0000	0.0192
Seedling total dry mass	0.0000	0.0003	0.0137
Height	0.0000	0.0757	0.0000
Stem diameter	0.0000	0.4333	0.0000
Nodule dry mass	0.0000	0.0000	0.0004
Root colonization	0.8530	0.0000	0.9575
Spore density	0.0000	0.0000	0.0000

For p-value ≤ 0.05, the effect was considered statistically significant.

Table 3. Shoot, root, and total dry mass of *Samanea saman* seedlings produced in three substrates with three inoculation treatments 130 days after sowing.

Substrates	Inoculation								
	NFB and AMF			Positive Control			Absolute Control		
Shoot dry mass (g) CV(%) = 7.2									
Sewage sludge	5.73	A	a	5.31	A	b	5.26	A	b
Commercial	5.05	B	a	4.84	B	a	4.70	B	a
Standard	2.83	C	a	2.04	C	b	1.68	C	C
Root dry mass (g) CV(%) = 10.2									
Sewage sludge	1.65	A	b	1.95	A	a	1.98	A	A
Commercial	1.41	B	b	1.73	B	a	1.40	B	B
Standard	0.70	C	b	0.89	C	a	0.63	C	B
Seedling total dry mass (g) CV(%) = 7.1									
Sewage sludge	7.38	A	a	7.25	A	a	7.24	A	A
Commercial	6.47	B	a	6.57	B	a	6.10	B	A
Standard	3.53	C	a	2.93	C	b	2.30	C	C

Means with equal letter, upper case comparing substrates (column) and lower case comparing inoculation treatments (row), do not differ statistically by Scott-Knott test (P≤0.05). CV (%) = coefficient of variation of the analysis of variance; NFB = nitrogen-fixing bacteria; AMF = arbuscular mycorrhizal fungi.

Fertilization favoured rooting in the Standard and Commercial substrate, whereas in the Sewage sludge substrate (with greater fertility) this effect was not verified. The highest growth of the seedling produced in the Sewage sludge substrate was probably due to higher N and P availability (Table 1). Similar results have been documented for by Delarmelina et al. (2014), Gonçalves et al. (2014), Cabreira et al. (2019), Silva et al. (2019) and Lima Filho et al. (2019) for *Ateleia glazioviana*, *Sesbania virgata*, *Schizolobium parahyba*, *Luehea divaricata*, and *Ceiba speciosa*, respectively.

Fertilization of the Positive Control treatment increased rooting of the seedlings grown in the Commercial and Standard substrates, but not of those produced in the Sewage sludge, probably due to the high fertility of the latter. In the

Standard substrate which had the lowest fertility, the effect of inoculation with NFB and AMF was evident as it increased the shoot and seedling total biomass.

The results of the seedling height and stem diameter are shown in Table 4 and reflect the initial fertility of the substrates (Table 1). The highest values of height and stem diameter corresponded to the seedlings grown in the Sewage sludge, the substrate with the highest N and P levels. Seedling height represents one of the leading indicators of initial growth in the field (Caldeira et al., 2014) while stem diameter is one of the leading indicators of root growth and survival after field planting (Santos et al., 2000). Therefore, these two non-destructive variables are the most used to assess the seedling quality of forest species (Ferreira et al., 2019).

Table 4. Height and stem diameter in *Samanea saman* seedlings produced with three substrate and inoculation treatments at 113 days after sowing.

Substrates	Inoculation								
	NFB and AMF			Positive Control			Absolute Control		
Height (cm) CV (%) = 4.0									
Sewage sludge	35.2	A	c	39.1	A	a	37.4	A	b
Commercial	34.2	A	a	32.3	B	b	33.8	B	a
Standard	28.2	B	a	24.8	C	b	23.4	C	b
Stem diameter (mm) CV (%) = 3.8									
Sewage sludge	5.1	A	b	5.5	A	a	5.7	A	a
Commercial	5.0	A	a	5.1	B	a	4.9	B	a
Standard	4.1	B	a	3.7	C	b	3.7	C	b

Means with the equal letter, upper case comparing substrates (column) and lower case comparing inoculation treatments (row), do not differ statistically by Scott-Knott test (P≤0.05). CV (%) = coefficient of variation of the analysis of variance; NFB = nitrogen-fixing bacteria; AMF = arbuscular mycorrhizal fungi.

The most significant height was verified for *S. saman* seedlings grown in the Sewage sludge and the Commercial substrate with inoculation (Table 4). In the Sewage sludge, the height exceeded 35 cm for all the inoculation treatments. It

corresponds with results obtained by Gonçalves et al (2014), who suggested plant height as an excellent parameter to indicate seedling quality. This result indicates that in this substrate, the seedlings need less time to reach the proper

height to be transferred to the field, thereby reducing the cost of production (Simões et al., 2012).

The height in plants grown in the Standard substrate was lower than in the other substrates, even when mineral fertilizer was supplied (Table 4). The main component of the Standard substrate is clay subsoil (66%). Compaction of this substrate and consequently reduced gas exchange, associated to low nutrient availability might explain these results (Table 1), as they are essential factors for seedlings development in cone-tainers (Kämpf, 2005, de Oliveira et al., 2018).

Considering that *S. saman* seedlings are ready to be planted in the field when they reach a height between 20 and 30cm and Stem diameter greater than 4 mm (Staples & Craig, 2006) it is possible to observe in Table 4 that all seedlings produced on the substrates Commercial and Sewage sludge, had the

indicated parameters, however, for the Standard substrate, only the seedlings that received inoculation managed to reach the ideal stem diameter. Therefore, the use of inoculants is necessary for the production of *S. saman* seedlings in the Standard substrate.

Inoculation with NFB and AMF promoted greater nodulation in the seedlings in the Sewage sludge and Standard substrates (Table 5). Mycorrhizal colonization percentages varied from 10% to 13% in seedlings that received inoculation, which is low (less than 20%) according to the classification proposed by Carneiro et al. (1998). On the other hand, spore production was favored in the Sewage sludge, while nodulation was favoured in the Commercial substrate (Table 5). There was no AMF sporulation or colonization in seedlings that did not receive inoculation.

Table 5. Dry nodule mass, root mycorrhizal colonization rate, and AMF spore density of *Samanea saman* seedlings grown in three substrates with three inoculation treatments 130 days after sowing.

Substrates	Inoculation								
	NFB and AMF			Positive Control			Absolute Control		
Nodule dry mass (mg) CV (%) = 21.9									
Sewage sludge	372	B	a	25	B	c	150	B	b
Commercial	656	A	a	243	A	b	584	A	a
Standard	380	B	a	64	B	c	170	B	b
Root colonization (%) CV (%) = 130.0									
Sewage sludge	10	A	a	0	A	b	0	A	b
Commercial	13	A	a	0	A	b	0	A	b
Standard	13	A	a	0	A	b	0	A	b
Spore density CV (%) = 142.0									
Sewage sludge	411	A	a	0	A	b	0	A	b
Commercial	22	B	a	0	A	b	0	A	b
Standard	33	B	a	0	A	b	0	A	b

Means with equal letter, upper case comparing substrates (column) and lower case comparing inoculation treatments (row), do not differ statistically by Scott-Knott test ($P \leq 0.05$). CV (%) = coefficient of variation of the analysis of variance; NFB = nitrogen-fixing bacteria; AMF = arbuscular mycorrhizal fungi.

Fertilization of plants within the Positive Control reduced nodulation in the seedlings grown in all substrates. It was because the NFB and AMF activities are more evident in the absence of nutrients N and P, respectively. The presence of these nutrients inhibits the formation of the respective symbioses since the energy cost to perform these symbioses is very high compared to the high availability of these nutrients in the environment (de Faria et al., 2010, de Faria et al., 2020, Smith & Smith, 1997, Dobereiner, 1984).

Nodules were verified in plants within the Positive and Absolute Controls, which did not receive inoculation (Table 5). However, the dry nodule mass in plants of these treatments was lower than in those of the inoculated treatment. Inoculation with NFB and AMF promoted greater nodulation of the seedlings in plants grown in the Sewage sludge and Standard substrate, but not in those produced in

the Commercial substrate, where the nodulation was equal to that of the Absolute Control.

There was no contamination by mycorrhizal fungi, the presence of which was only verified in the treatment with NFB and AMF (Table 5). AMF spores are large and attached to the mycelium, which makes it challenging to move through air and water. The spores of the inoculated species in the present study vary between 60 μm (*Acaulospora morrowiae*) and 280 μm (*Gigaspora candida*) in diameter (INVAM, 2020). Also, several AMF species produces form sporocarps containing many spores, which are much larger than individual spores, and some species preferentially sporulate within roots (Smith & Smith, 1997). Thus, the contamination by AMF is more complicated than by NFB.

The results obtained herein are supported by the cited literature and indicate the Sewage sludge as a useful and low-

cost substrate. Sewage Treatment Stations have large quantities of this waste, and the reuse of it in the production of seedlings contributes to sustainability by reusing a material that would be destined for dumping ground. However, an analysis of the fertility of different Sewage sludges must be conducted to avoid nutritional excess or scarcity for the seedlings.

Even though the Standard substrate favoured plant growth in response to inoculation (Table 3 and 4), results regarding NFB nodulation and AMF sporulation were inferior compared to those of the other two substrates. Symbionts bring greater benefits to the host plant in low fertility substrates (Nogueira et al., 2010), the Standard substrate proves to be ideal for the functioning of these symbioses, presenting a more significant response to inoculation.

The substrates of greater fertility (Sewage sludge and Commercial), enabled the formation of larger seedlings and restricted the response to inoculation. In these two substrates, seedling within the inoculated treatment did not exceed the results obtained for these variables within the Positive and Absolute Controls. The Sewage sludge and the Commercial substrate promoted AMF sporulation and NFB nodulation, respectively, which can be useful when transplanting the seedlings to the field.

4. CONCLUSIONS

The Sewage sludge substrate provides more significant seedling development and is conducive to symbiosis with AMF allowing high sporulation;

The Commercial substrate favors symbiosis with NFB, increasing nodulation of *S. saman* seedlings and showing little benefit from the application of fertigation;

The Standard substrate promotes the smallest growth of the seedlings, proving to be an unsuitable substrate for seedling production in cone-tainers. Despite producing the smallest *S. saman* seedlings, this substrate evidences the effect of inoculation with NFB and AMF on plant growth. However, it does not prove to be the best for the mutual benefit of the symbionts.

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