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Evaluation of biochar in the root nodulation of *Acacia mangium* Willd. in a nursery

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Abstract Organic matter stabilized in the form of biochar has benefits in a nursery for the physico-chemical and biological properties of substrates derived from forest propagation. With biochars from *Acacia mangium* Willd. (BAM) residue increases for agricultural production in nurseries have been reported. In this research, we evaluated the effect of different levels of this biochar and its mixture with a synthetic fertilizer (SF) on the root nodulation of *A. mangium* seedlings. Nine treatments were established to assess the effects on volume and nodule count. Two analyses were performed on a volume using the Kruskal-Wallis test and on counting using the Chi-square test of homogeneity distribution. The results of a subsequent comparison between treatments highlighted higher nodule volumes in treatments with BAM and a mixture with SF compared to those of only SF. The distribution of the nodule count was not homogeneous among treatments. The treatment 80 ton·ha^{-1} of BAM and 50% of SF produced a greater number of nodules compared to the control. All treatments with higher nodule volumes contained BAM to some degree. Biochar seems likely to contribute to increased volume and nodule count, which could suggest greater potential in the population of microorganisms associated with the development of nodules in seedlings.

Keywords Biochar · Pyrolysis · Organic matter · Substrate · Nodulation · *Acacia mangium*

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Introduction

The forest nursery is the area where forest seedlings are grown under special care until they reach an optimal size to be taken to the field, the objective being production and supply for forestry or timber production projects (CONIF 2002; CONPES 3680 2010). Depending on the type of management given, seedlings can develop greater growth, vigor, and health in the field, which translates into advantages in terms of reforestation and the lumber industry (Cobas 2001). In recent years in forest nurseries, the practice of replacing the sowing substrate with materials with similar characteristics to natural soil has increased, to provide optimal means of growth for seedlings (Santiago 2002; Roldán et al. 2005). Among these materials are vermiculite, sand, composting, sawdust, and charred rice husk, besides some other materials (Hartmann et al. 2002) that can provide the nutrition and moisture necessary for seedling roots (Kratz et al. 2012). Biochars are materials that improve the physico-chemical properties in the substrate also, known as pyrolyses or biocarbon. These are solid products of fine and porous grain obtained from the thermal conversion of biomass with a temperature about < 700 °C in the absence or low levels of oxygen. Incineration of these materials is through pyrolysis, which consists of the burning of organic materials in a limited oxygen environment (Sakhiya et al. 2020). Biochar acts as a matrix

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from which nutrients are slowly released (Altland and Krause 2012). By their low apparent density and high porosity, improve the relationships of the water that contributes to seedling growth in nurseries (Marimon-Junior et al. 2012). In this sense, biochar (for example from wood) could work as an alternative for materials like vermiculite in substrates, given its low apparent density, high porosity, and presence of important contents of nutritional elements such as potassium (K), phosphorus (P), and magnesium (Mg) (Angst et al. 2013; Gundale and DeLuca 2006). Although there is little research regarding the influence of biochar on substrates, research such as those of Santiago and Santiago (1989), Elad et al. (2010), Dumroese et al. (2011), and Tian et al. (2012) have analyzed factors such as nutrient leaching, seedling growth, and systemic resistance to diseases in which favorable responses have been obtained due to the effect of the addition of biochar.

Biochar influences the water dynamics of the substrate with the generation of micropores that measure smaller than 30 μm and macropores measuring greater than 75 μm (Brady and Weil 2002; Keech et al. 2005). Macropores are essential in gas exchange because they influence the mineralization of nutrients given the aerobic conditions that are generated by such exchanges. The varied pores of the biochar indirectly influence nutrient transformations, improving the chemical and biological properties of the soil through the habitat and substrate providing for different populations of microorganisms (Lehmann et al. 2011; Gao and DeLuca 2016; McCormack et al. 2013).

Within the porosity of the substrate that maintains a microbiota, micro-sites are created for their survival. Additionally, if there are constant inputs of organic matter (for example from waste), positive feedback cycles are generated by the addition of nutrients to the soil (Whitman et al. 2015). In this research, we proposed to evaluate the effects on nodulation in seedling roots of *A. mangium* in a nursery of the substrate after the addition of biochar residues. Likewise, we analyzed two variables: the volume of nodules and the root count of *A. mangium* employing a non-parametric test of ANOVA Kruskal-Wallis (volume) and homogeneity of a Chi-square distribution (nodule count). Though there were no significant differences between the control and the other treatments, a difference was found between treatments with only a synthetic fertilizer and biochar treatments where the biochar provided a greater number and volume of nodules.

Materials and methods

Study location

The study was carried out in the Green Cooperation company, in a nursery of an *A. mangium* plantation located in the town of Planadas, department of Meta (Colombia). The coordinates were between 3° 05′ and 4° 08′ North latitude, and between 71° 05′ and 72° 30′ West latitude; with an average annual temperature of about 30 °C.

Establishment of the experiment

The planting of *A. mangium* seedlings was established according to the methodology of International Seed Testing Association—ISTA (2013). The seeds came from a single tree provided by the commercial plantation of the forestry company where the study was carried out. They and the substrate were treated with water at 80 °C for the elimination of potential pathogens. The seeds were dried at room temperature for 24 h. Five seeds were sown in each polythene bag whose dimensions were 10-cm long × 5 cm in diameter. At 18 days, the germination percentage was calculated according to the methodology of ISTA (2013), where a single germinated seedling per bag was selected and followed up for analysis.

The biochar (BAM) came from waste from the gutter and pruning in the *A. mangium* nursery produced under slow pyrolysis with a processing time of 14 h and temperatures between 350 and 400 °C in a pyrolysis furnace located at the plantation. This biochar was prepared according to the methodology of Jeffery et al. (2011). Once the biochar was produced, it was screened with 4.75 and 1 mm sieves that produced a particle size of approximately 1–5 mm. The established levels of BAM were used according to Jeffery et al. (2011) that estimate on average the application of 50 ton·ha^{-1} of this material for an 18–28% increase in crop yields on a global scale. The synthetic fertilizer (SF) used was composed of 15% total nitrogen, 15% soluble phosphorus, neutral ammonium citrate, and 15% water-soluble potassium. Fertilization was part of the nutrition plan in the commercial plantation. The reference SF levels were applied according to the following plan: 100% equivalent to 100 g/seedling and half of this (50%) to evaluate possible synergies with the BAM. The pH of the substrate was measured to analyze its influence on nodulation.

Experimental design

A simple factorial design was established in a completely random arrangement with 9 treatments (materials) and 3 repetitions. These were an *A. mangium* biochar (BAM), synthetic fertilizer (SF), and BAM + SF mixture with 3 dose levels for the biochar (BAM) of 0, 40, and 80 ton·ha^{-1} and 3 levels for the SF application at 0% 50%, and 100%. The 9 materials were obtained from the mixtures of BAM and SF as the control treatment where neither of the two components BAM and/or SF was incorporated. The treatment ratios were as follows: (1) T0: 0% SF + 0 ton·ha^{-1} of BAM (soil only); (2) T1: 50% SF + 0 ton·ha^{-1} of BAM; (3) T2: 100% SF + 0 ton·ha^{-1} of BAM; (4) T3: 0% SF + 40 ton·ha^{-1} of BAM; (5) T4: 50% SF + 40 ton·ha^{-1} of BAM; (6) T5: 100% SF + 40 ton·ha^{-1} of BAM; (7) T6: 0% of SF + 80 ton·ha^{-1} of BAM; (8) T7: 50% of SF + 80 ton·ha^{-1} of BAM; and (9) T8: 100% SF + 80 ton·ha^{-1} of BAM.

Statistical analysis

The data were analyzed according to the non-parametric ANOVA Kruskal-Wallis for the volume of nodules and Chi-square distribution homogeneity for nodule counts. Because the nature of the response associated with the counts and for the fixed samples of each treatment was random, only the nodule count per treatment was according to size. Subsequently, a multiple comparison test of Nemenyi “a posteriori” was performed, once the non-parametric ANOVA was executed. This evaluated differences in the volume of nodules due to the effect of the treatments. All statistical analyses were assisted with free software R.

Variables analyzed

To determine the biological effect of BAM on the seedlings of *A. mangium*, the nodules generated at the roots of the seedlings were counted and measured; the volume of them was estimated, as an indirect effect of the BAM on this biological property. For the variable volume, the generated nodules were separated according to their volume or size into three respective groups: small nodules (SN), medium nodules (MN), and large nodules (LN). This grouping was obtained from the approximate calculated volume of ellipsoids or spheres, estimated according to the geometric shape of each nodule. The formula used to calculate these volumes was: $V = D\pi h$, where: V = volume; D = diameter; h = height. The analysis of the nodule volume data was based on the three groups described according to their reference size (Table 1), reported by Cruz and Myrna (2014) in *Phaseolus vulgaris* L. before inoculation with *Rhizobium* strains.

Regarding the second variable, the nodules were estimated quantitatively through counting evaluations in which the total number of nodules generated in the seedling roots was calculated as an average per treatment, according to the calculated size of these nodules (Table 1).

Results

Significant differences (p-value < 0.05) were found between treatments for the volume of nodules according to a Kruskal-Wallis ANOVA (Table 2), where the highest average nodule volume, according to the proposed groups, was found in the treatments with BAM + SF and only BAM according to the descriptive statistics (Table 5).

In general, the average volume of nodules obtained for the three established groups was found in the following decreasing ratio: LN group > MN group > SN group, where 77.2% of the LN group was larger in volume than the MN group and 96.9% more than the SN group (Fig. 1, Table 3).

From the Nemenyi multiple comparison test, T4 is significantly different from T2; T6 with respect to T1 and T2; T7 with respect to T1, T2, T4, T5, and T6; and T8 with respect to T4 and T5 for the volume of nodules. No significant differences were found between T0 and

Table 1 Average volume ranges for nodule groups

Group	Minimum	Maximum	Range
MN	21.6	44.7	23.1
LN	101.3	156.7	55.4
SN	2.3	6.0	3.7
Total	2.3	156.7	154.4

Table 2 Kruskal-Wallis test for the volume of nodules in *A. mangium* roots

Statistical test	GL	*p*-value
66,042	8	$3002\ e^{-11}$

the other treatments (Table 4). However, according to the descriptive statistics, the control treatment was found with a lower mean value for the average volume of nodules with respect to the estimated mean for this variable in T3, T4, T5, T6, and T7 (Table 5).

95.0% confidence intervals

Concerning the variable nodule counts, according to the Chi-square test, the null hypothesis of homogeneity of distribution of the counts for the treatments involved in the three nodule sizes was rejected, indicating a significant difference between treatments for this variable (Table 6).

According to counts and regardless of size, the largest number of nodules in general was shown in the treatment consisting of 80 ton·ha^{-1} of BAM + 50% of SF (T7), and the lowest number of nodules was found in the control treatment (T0). The counts with the highest numbers of LN were found in T4, T5, and T7, with 4.47%, 4.47%, and 13.40% of LN, respectively (Table 7). The T7 treatment had the highest proportion of LN (highest weighted average obtained), while T5 had the highest proportion of MN (Fig. 2, Table 7). The largest number of nodules by size was found within the MN group (Fig. 2, Table 7).

In general, the counts had a higher mean percentage of total nodulation in treatments with BAM alone or in combination with fertilizer (BAM + SF) (Table 7). In T7 treatment in comparison to T0 and T1 treatments, increased percentage differences of total nodulation were found with 17.18 and 8.24%, respectively. For treatments T7 and T8, made up of the mixture of SF and BAM in greater proportion, the T8 (a greater proportion in both SF and BAM) had a percentage decrease of 7.21% in the general nodule count with respect to T7 (Table 7). It is interesting to note that the measured pH values in the substrate of each treatment resulted in highly acidic pH for treatments T0, T1, and T2, medium acidic pH for T3, T4, T5, T7, and T8, and pH neutral for T6 (Table 8).

Discussion

According to complementary descriptive statistics to the inferential test there were no significant difference between the control and the other treatments for the volume of nodules (Table 4), there was a greater number of nodules in plants under the influence of treatments with SF and treatments with BAM in the substrate. Thus, it can be inferred that treatments that included BAM to some degree had an additional effect on the formation of nodules. Besides, it is important to compare the greatest nodule gain with the use of 50% less SF between treatments T7 and T8 (with the same amount of BAM), which could result in a lower economic and environmental cost related to the use of synthetic fertilizers that are widely accepted in nurseries (Ramírez y Rosales, 2009).

This relationship between the addition of biochar and increased nodule formation in plant roots is reported by several authors (Xiang et al. 2017; Yusif et al. 2016). Roldan et al. (2005) find a significant increase in the biological fixation of nitrogen by rhizobia (which translates into an increase in nodules) with the addition of biochar in the substrate at levels of 30, 60, and 90 g/kg,

Fig. 1 Nodule formation in seedling roots of *A. mangium*

Table 3 Descriptive statistics for the volume of nodules found

Group	*N*	Mean	Median	Variance	Standard deviation	Coefficient of variance
MN	118	30,396	30.4	17.80	4.22018	13.8837%
LN	93	129,626	128.7	137.35	11.7197	9.0412%
SN	80	4145	4.0	0.588	0.767026	18.5049%

compared to treatments with only conventional fertilization based on the addition and/or increase of chemical synthetic fertilizers, as shown in this study. However, studies of nodulation in clover plants with the application of biochar materials reveal a reduction in nodules with increases in nitrogenase activity (Quilliam et al. 2013).

Our results showed that the treatment T7 (80 ton·ha^{-1} of BAM and 50% of SF) was found with the highest weighted mean for the formation of the amount and volume of nodules compared to the other experimental treatments. This could be explained by the activity of the microorganisms present in the substrate with BAM in synergy with the synthetic chemical fertilization that determined the presence and magnitude of the nodules formed (Anderson et al. 2011; Fernández-Pascual et al. 2002). Authors such as Beedy et al. (2010) and Pezzolla et al. (2013) report that with the application of organic materials such as biochars, nutrient retention (and most likely assimilation) contributed by synthetic fertilizers are chemically improved when applied in both tropical and temperate regions.

However, O'Neill et al. (2009) and Liang et al. (2010) find twice as many populations of microorganisms in Amazonian soils with a high content of pyrogenic carbon than in adjacent Amazon soils with a low content of this element. Likewise, Anderson et al. (2011) show increases of up to 15% in some taxonomic groups of microorganisms in response to biochar additions to pasture soils. The effect of biochar on populations of soil or substrate microorganisms is evident, as may have happened in this particular study with *A. mangium*, where an increase in nodules (in number and size) in its roots confirmed the influence of BAM.

Studies by Steinbeiss et al. (2009), Lehmann et al. (2011), and Ameloot et al. (2013) indicate that one of the factors that could increase the community of soil microorganisms in biochar treatments is the increase of habitat generating conditions for the microorganisms. DeLuca et al. (2009) and Gundale and Deluca (2007) link the increase in nitrification processes to the high porosity of the biochar.

It could be considered that this positive change in the biological and/or physico-chemical properties of the substrate occurred notably with the application of BAM in the T7 treatment. The synergy of this material with the SF applied in a certain proportion obtained the greatest number of nodules (Lehmann et al. 2011). The treatments that resulted with the highest average nodule volume (T7, 80 ton·ha^{-1} of BAM and 50% of SF; T6, 80 ton·ha^{-1} of BAM without SF; T3, 40 ton·ha^{-1} of BAM without SF and T4, 40 ton·ha^{-1} of BAM and 50% of SF)

Table 4 Statistical differences between treatments for nodule volume by pairs of treatments (Nemenyi test)

	T0	T1	T2	T3	T4	T5	T6	T7	T8
T0	–								
T1		–							
T2			–						
T3				–					
T4			*		–				
T5						–			
T6		*	*				–		
T7		*	*		*	*	*	–	
T8					*	*			–

[*] Significant difference p-value < 0.05 make it meaningful

Table 5 Average nodule volume per treatment arrange table and its title properly

Treatment	*N*	Median	Standard error (*s* grouped)
T0	9	43.4778	15.7926
T1	35	29.9257	8.00829
T2	16	13.7875	11.8444
T3	18	61.1611	11.167
T4	44	59.4432	7.14246
T5	49	57.8449	6.76824
T6	23	72.7913	9.87893
T7	59	90.1136	6.16805
T8	38	20.3316	7.68568

Table 6 Chi-square test of homogeneity of distribution for the count of nodules in *A. mangium* roots

Test	Statistic	GL	*p* value
Chi-square	201,676	16	0.0000

contain biochar to some degree, and this determined the material as the fundamental factor for an increase or improvement of populations of microorganisms and therefore of expressed nodules.

Another important factor that could influence the increase or improvement of the populations of beneficial microorganisms in this study is the pH of the substrate with the addition of BAM in treatments T3, T4, T5, T6, T7, and T8, where pH shows near neutral and neutral values compared to the strongly acid values of T0, T1, and T2 (Table 8) that could have increased the viability of microorganisms and nodule formation (Graber et al. 2011; McCormack et al.2013). In general, it is known that many beneficial bacteria increase their populations with neutral pH (Padan et al. 2005). Taking into account the latter and the pH values more favorable to the growth of bacteria in treatments with BAM and SF in this study, from the productive point of view the use of these components or inputs in productive systems of *A. mangium* is justified. This is especially true in the defined proportion of 50% of SF + 80 ton·ha^{-1} of BAM where greater quantity and volume of nodules were obtained, compared with the absence of its application where strongly acidic pH and lower nodulation were obtained (T0, T1, and T2). The contribution of nutrients with the biochar could be another of the factors to consider that could have contributed to an increase in the biological properties of the substrate for treatments where there was a greater development of nodules. This is because the communities of microorganisms can use the nutrients of these materials as an energy source for survival (Warnock et al. 2007; Lehmann et al. 2011).

Comparing the control treatment (T0) with the T8 treatment (with a greater amount of BAM and SF—80 ton·ha^{-1} of BAM and 100% of SF), where a higher production of medium nodules was obtained in the T0 can be explained due to the inhibition of nodulation in T8 because of a high level of nitrates provided by the applied SF. This is because of the union of nitrate with specific receptors of microorganisms such as *Rhizobium* that prevents nodulation (Cheng et al. 2011). This can also explain the results of greater nodulation in the T7 treatment using 50% less of the SF (80 ton·ha^{-1} of BAM and 50% of SF). Concerning the significant formation of nodules in the control (T0), it is known that *A. mangium* has a high capacity for establishing symbiotic relationships, particularly with the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, and *Mesorhizobium* (Thies et al. 2015).

Some research has also proven that high levels of nitrates cause a decrease in nitrogenase (a fundamental enzyme in the conversion of natural nitrogen to nitrogen available to plants) due to an increase in resistance to oxygen diffusion, a key element either by absence or excess. A high amount of oxygen can inhibit component II of nitrogenase (Fernandez-Pascual et al. 2002). Another cause of reduced nodule formation in some experimental treatments could be due to the high applications of nitrates that cause alterations in the morphology of the nodular cortex, causing obstructions in the middle cortex due to glycosidic substances (De Lorenzo et al. 1994).

Taking into account that the highest volumes and nodule counts were found in the T7 treatment (80 ton·ha^{-1} of BAM and 50% of SF). These application levels of BAM and SF could be proposed as an alternative to the current application of synthetic fertilizers in *A. mangium* production systems in the tropics to increase the nodulation levels in the seedlings. With the development and application of BAM, the amount of organic waste produced in this agroecosystem could be reduced; and, also, through the contribution of nutrients from this material, a reduction of the amount of application of soluble fertilizers (nitrates) is possible (Ramírez and Rosales 2009). Where negative environmental externalities to other ecosystems are reduced, a more closed system where nutrients from the entire agroecosystem are used is possible.

Particularly, *A. mangium*, a legume, could interact and enhance soil biota, generating changes in the feedback cycles between the inclusion of waste (biochar), the plant, and the soil. However, it is important to keep in mind that this association depends on factors such as the type of biochar, its proximity to the root, the characteristics of the plant, and the physico-chemical properties of the soil (Altieri 1999).

Conclusions

BAM is a material that, when incorporated into substrates, provides characteristics that enhance the

Table 7 Summation of means and counting percentages in each nodule treatment per group arrange data of table properly

Treatments		MN	LN	SN	Total number of nodules and % by treatments
T0	Number of nodes	8	1	0	9
	% of presence	2.75%	0.34%	0.00%	3.09%
T1	Number of nodes	24	2	9	35
	% of presence	8.25%	0.69%	3.09%	12.03%
T2	Number of nodes	6	0	10	16
	% of presence	2.06%	0.00%	3.44%	5.50%
T3	Number of nodes	2	8	8	18
	% of presence	0.69%	2.75%	2.75%	6.19%
T4	Number of nodes	31	13	0	44
	% of presence	10.65%	4.47%	0.00%	15.12%
T5	Number of nodes	36	13	0	49
	% of presence	12.37%	4.47%	0.00%	16.84%
T6	Number of nodes	2	12	9	23
	% of presence	0.69%	4.12%	3.09%	7.90%
T7	Number of nodes	9	39	11	59
	% of presence	3.09%	13.40%	3.78%	20.27%
T8	Number of nodes	0	5	33	38
	% of presence	0.00%	1.72%	11.34%	13.06%
Total number of nodules and % by size		118	93	80	291
		40.55%	31.96%	27.49%	100.00%

formation of nodules in *A. mangium* seedlings and can generate similar effects on other leguminous plants or forest species in commercial nurseries. All experimental treatments with higher counts and volumes of nodules obtained in this study with *A. mangium* contained BAM to some degree. This type of biochar was a conducive factor for increasing the formation of nodules in the roots of the seedlings

Although the mechanisms by which biochar acts for the benefit of soil microorganisms are not entirely clear, this research suggested an increase in the population of microorganisms, translated into a greater number and size of nodules in *A. mangium*, because the BAM provided favorable conditions for the habitat and development of these populations of beneficial microorganisms. However, it is necessary to carry out specific studies of

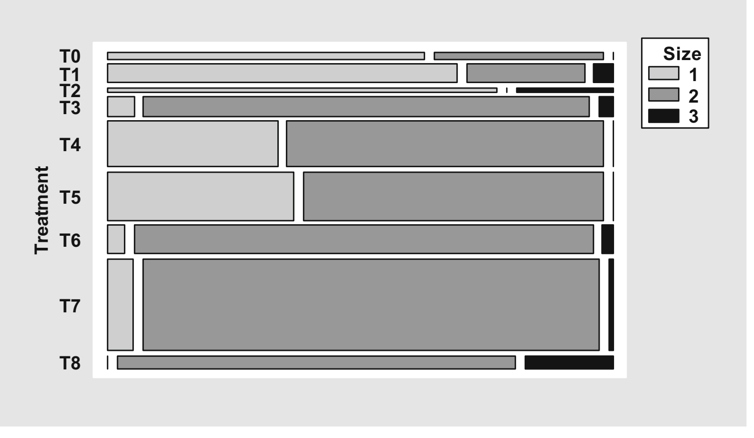

Fig. 2 Mosaic for treatments according to nodule volume group (group 1 = medium nodules (MN), group 2 = large nodules (LN), and group 3 = small nodules (SN))

Table 8 Mean pH values in substrates of the experimental treatments

Analyses	0	1	2	3	4	5	6	7	8
*pH	3.4	3.5	3.2	5.7	5.5	5.9	7.0	5.7	5.6

*logH+

qualitative and quantitative characterization of microorganisms associated with *A. mangium* under the effect of BAM, both in semi-controlled nursery conditions (substrates) and in natural conditions (cultivation soils).

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Code availability Not applicable.

Authors' contributions Conceptualization, G.R.M.; methodology, G.R.M.; software, E.D.C.; validation, E.D.C. and N.J.C.P.; formal analysis, E.D.C.; writing, G.R.M. and N.J.C.P.; writing—review and editing, E.D.C. and N.J.C.P. All authors have read and agreed to the published version of the manuscript.

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Declarations

Ethics approval We declare ethical approval in the performance of this work.

Consent to participate We consent to participate in the submission of this document.

Consent for publication We consent for publication of this document.

Conflict of interest The authors declare no competing interests.

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