Original Paper

Harnessing microbes: a new approach to carbon sequestration in cocoa agroforestry

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Abstract. *This study evaluated the carbon sequestration potential of fungal and bacterial strains in cocoa plantation soils in Ecuador's coastal region using a randomized complete block design. Four microbial treatments were tested:* Trichoderma longibrachiatum *(T1),* Trichoderma reesei *(T2),* Bacillus subtilis *(T3) and* Bacillus licheniformis *(T4). All microorganisms showed high viability, with bacterial and fungal colony-forming units exceeding* 10^8 *and* 10^6 *CFU/ml, respectively.* T. longibrachiatum *and* T.

reesei *significantly outperformed the bacterial treatments in carbon sequestration (p<0.0001).* T. reesei *achieved a 29% increase in carbon sequestration after the first application, while* B. subtilis *showed an 11.25% increase after four applications, though with decreasing efficacy.* B. licheniformis *maintained* NH_4^+ *at 19.00 ppm, Zn at 5.60 ppm, Mn at 5.20 ppm, and B at 0.61 ppm, while increasing P to 66.00 ppm, K to 1.89 Meq/100ml, Ca to 19.00 Meq/100ml, Mg to 5.10 Meq/100ml, S to 50.00 ppm, Cu to 5.20 ppm, and Fe to 54.00 ppm. Future research should focus on optimizing microbial dosages and application methods to enhance carbon capture and cocoa productivity.*

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1. Introduction

Greenhouse gas emissions from agriculture, primarily from livestock methane, are a significant contributor to climate change, accounting for approximately 18% of global emissions (Belezaca et al., 2022). Latin America, including Ecuador, is a major source of these emissions, with the agricultural sector being a key contributor (SELLCA, 2024; Paccha et al., 2023). Ecuador's agricultural practices, often characterized by deforestation and excessive chemical use, exacerbate this issue (Racines, 2018; FAO, 2022). To mitigate these emissions, carbon sequestration in soil and plant biomass offers a promising strategy (Ortiz & Batioja, 2023). Forests and agroforestry systems, including cocoa cultivation, have demonstrated significant carbon capture potential (Leiva & Ramírez, 2021; Buitrago et al., 2018; Andrade et al., 2020), with soil microorganisms playing a crucial role in this process (Ahmed et al., 2019).

This study introduces a novel approach by focusing on the carbon sequestration potential of specific fungal and bacterial strains within cocoa plantation soils in Ecuador's coastal regions. Unlike previous research that broadly examines microbial impacts, our study aims to provide a detailed assessment of how Trichoderma and Bacillus strains can enhance carbon capture in this context-specific setting. By evaluating the viability and efficacy of these microorganisms, our research seeks to contribute new insights into optimizing microbial applications for improved soil health and carbon storage. The findings are intended to bridge gaps in the existing literature by offering practical recommendations for integrating microbial inoculation into cocoa agroecosystems, with the potential to enhance sustainability and contribute to multiple Sustainable Development Goals (SDGs), including climate action, life on land, zero hunger, economic growth, and partnerships for the goals.

The potential applications and positive societal outcomes of this research lie in demonstrating how specific microbial strains can enhance carbon sequestration in cocoa plantations. By providing actionable strategies for farmers and policymakers, the study aims to support efforts to reduce agricultural emissions and promote sustainable practices. Although the results are context-specific to Ecuador's coastal cocoa plantations, they offer a valuable model for similar agroforestry systems, paving the way for broader implementation and environmental benefits. Ultimately, this study evaluates the carbon sequestration potential of selected fungal and bacterial strains, contributing to the development of targeted strategies for improving soil health and advancing sustainability in cocoa cultivation.

2. Methodology

2.1. Description of the study area

This research was conducted at the Escuela Superior Politécnica Agropecuaria de Manabí Manuel Félix López (ESPAM MFL), a higher education institution situated in Bolívar Canton, Manabí Province, Ecuador (0°49'8.87"S, 80°10'53.03"W, figure 1). Located at 15 meters above sea level, the region experiences a tropical climate with average temperatures ranging from 20.60°C to 31.11°C, annual rainfall of 624 mm, and relative humidity of 82.42%.

2.2. Microorganism activation

A completely randomized design (CRD) was employed for this study, with four treatments and four replications per treatment, totaling 16 experimental units. Each treatment consisted of the application of a specific fungal or bacterial strain (Table 1).

Fungal activation

Fungal strains were cultured on Potato Dextrose Agar (PDA) in Petri dishes. A sterile inoculation loop was used to transfer fungal suspensions to the plates, which were subsequently incubated in the dark at room temperature for 24 hours. Following incubation, fungal colony growth and development were assessed.

Bacterial activation

Bacteria were initially cultured in a liquid peptone water medium before being transferred to nutrient agar plates. These plates were incubated at 37°C for 24 hours under aerobic conditions. Bacterial colony growth and development were monitored.

Microorganism reproduction

To increase microorganism populations, serial dilutions were prepared according to the protocol outlined by the Ministry of Agriculture and Livestock of Ecuador (2014) and Mero (2019). Briefly, activated microorganisms were suspended in sterile water, centrifuged, and resuspended in peptone water. Serial dilutions ranging from 10^6 to 10^8 CFU/mL were prepared.

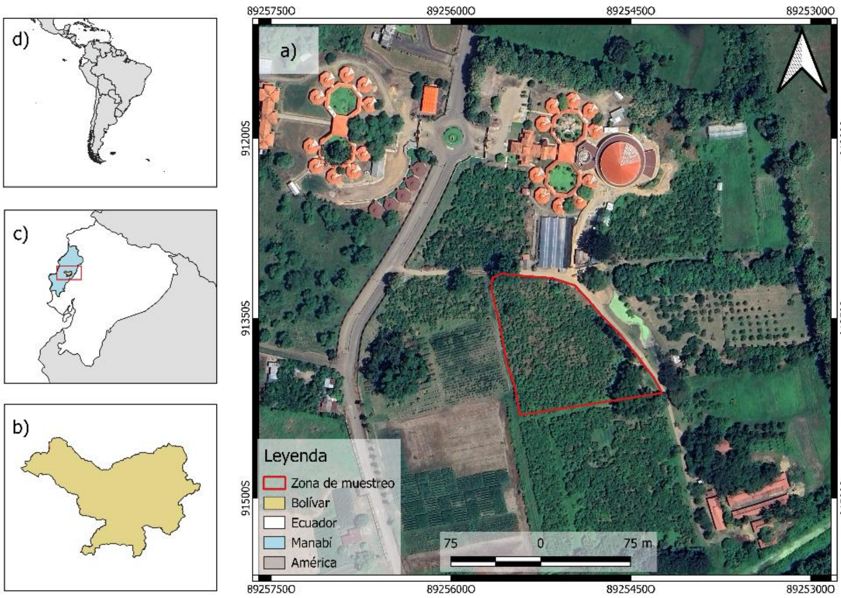

Figure 1. a) Geographic location of the sampling area, b) Bolívar, c) Ecuador, d) South America.

2.3. Soil analysis

To assess soil quality, samples were collected from ESPAM MFL cocoa plantations before and seven days after experimental treatments. These samples were submitted to the Instituto Nacional de Investigaciones Agropecuarias of

Ecuador (INIAP) for analysis of pH, organic matter, NH4, P, K, Ca, Mg, S, Zn, Cu, Fe, Mn, and B. Standard laboratory methods were employed, including potentiometry, Walkley-Black, atomic absorption, modified Olsen, micro Kjeldahl, and Yuan procedures (Table 2).

Table 1. Applied treatments.

Treatment	Code	Concentration
Trichoderma longibrachiatum	T1	1 x 10^8 ufc/ml
Trichoderma reesei	T2	1 x 10^6 ufc/ml
Bacillus subtilis	T3	1 x 10^8 ufc/ml
Bacillus licheniformis	T4	1 x 10^7 ufc/ml

Dilution concentration : 1 x 108 ufc/ml

Table 2. Parameters analyzed in the study and their classification criteria.

Parameter	Class criteria			Unit
	High	Medium	Low	
NH_4	> 30,1	12,1 - 30,0	< 12,0	ppm
P	> 15,0	8,0 - 14,0	<1,0 - 7,0	
K	> 0,4	0,2 - 0,4	< 0,2	Meq/100ml
Ca	> 4,1	2,0 - 4,0	< 2,0	
Mg	> 2,1	0,8 - 2,0	< 0,8	
S	> 16,0	5,0 - 15,0	< 5,0	ppm
Zn	> 7,1	3,1 - 7,0	3,0	
Cu	> 4,1	1,1 - 4,0	1,0	
Fe	> 41,0	21,0 - 40,0	20,0	
Mn	> 15,1	5,1 - 15,0	5,0	
B	> 0,6	0,2 - 0,6	< 0,2	
Organic matter	> 6,1	6,0 - 3,1	< 3,0	%

2.4. Carbon uptake assessment

Carbon uptake was determined by measuring soil respiration rates (CO_2 release) before and after treatment application. Laboratory analyses, including titration and CO_2 estimation, were conducted to assess soil biological activity, carbon storage, and the impact of treatments on carbon sequestration.

3. Results and Discussion

Microorganism viability

Both fungal (*Trichoderma longibrachiatum* and *Trichoderma reesei*) and bacterial (*Bacillus subtilis* and *Bacillus licheniformis*) strains exhibited high viability, consistently exceeding 10^6 and 10^8 CFU/mL, respectively. These findings align with previous research by Bolaños et al. (2014); Bampidis et al. (2023); Hernández et al. (2019); González et al. (2023).

Nutrient availability

Trichoderma and *Bacillus* application significantly enhanced soil nutrient availability. Initially low levels of NH_4, Zn, Fe, and Mn increased following treatments, with NH_4 and Zn reaching medium levels and Fe attaining high levels. These results corroborate findings by Abdelmoaty et al. (2022); Andrade et al. (2023) regarding Trichoderma's role in organic matter decomposition and nutrient release. Bacillus demonstrated a more limited effect on Mn, increasing it only to medium levels. Mg, S, and Cu levels increased to high levels with both treatments, while B exhibited variable responses. P, K, Ca, and organic matter remained consistently high. Araújo et al. (2022) support the hypothesis of increased nutrient use efficiency following microbial inoculation. Soil pH was neutralized from 6.7 to 7.3 in all treatments (table 3).

Table 3. Results of soil parameter analysis before and after each treatment.

Parameter	Pre application	T1 (*T. longibrachiatum*)	T2 (*T. reesei*)	T3 (*B. subtilis*)	T4 (*B. licheniformis*)
NH_4	8,00 **L**	29,00 **M**	22,00 **M**	24,00 **M**	19,00 **M**
P	66,00 **H**	57,00 **H**	57,00 **H**	56,00 **H**	66,00 **H**
K	0,98 **H**	1,79 **H**	1,87 **H**	2,00 **H**	1,89 **H**
Ca	11,00 **H**	18,00 **H**	18,00 **H**	19,00 **H**	19,00 **H**
Mg	1,00 **M**	4,10 **H**	5,40 **H**	4,70 **H**	5,10 **H**
S	15,00 **M**	55,00 **H**	60,00 **H**	55,00 **H**	50,00 **H**
Zn	1,00 **L**	5,80 **M**	6,10 **M**	6,00 **M**	5,60 **M**
Cu	2,60 **M**	4,80 **H**	4,70 **H**	4,40 **H**	5,20 **H**
Fe	13,00 **L**	54,00 **H**	56,00 **H**	46,00 **H**	54,00 **H**
Mn	3,20 **L**	4,80 **L**	4,90 **L**	5,80 **M**	5,20 **M**
B	0,55 **M**	0,98 **H**	0,57 **M**	0,30 **L**	0,61 **M**
Organic matter	7,00 **H**	8,00 **H**	7,90 **H**	8,00 **H**	7,80 **H**
pH	6,7 **H**	7,3 **N**	7,3 **N**	7,3 **N**	7,3 **N**

*__H=High; M=Medium; L=Low__ (criteria in Table 2).

All microbial treatments significantly enhanced soil carbon sequestration ($p<0.05$) compared to the control. *Trichoderma reesei* initially exhibited the highest carbon capture rate (29.00 mg*kg-s), followed closely by *Trichoderma* *longibrachiatum*. *Bacillus subtilis* and *Bacillus licheniformis* showed lower carbon sequestration rates. These findings align with previous research highlighting the potential of *Trichoderma* species for carbon sequestration (Obando & Vélez, 2023; Conrado et al., 2019; González et al., 2023).

While *Trichoderma longibrachiatum* maintained a consistent carbon capture throughout the experimental period, *Trichoderma reesei* exhibited a slight decline after the initial peak. Both *Bacillus* species showed limited carbon sequestration capacity (figure 2). Although both *Trichoderma* and *Bacillus* species are beneficial soil microorganisms, our results indicate that Trichoderma*,* particularly *Trichoderma longibrachiatum*, is more effective in promoting carbon sequestration under the studied conditions. While Mohammed et al. (2017) reported the positive impact of these microorganisms on plant growth and disease control, our study focused specifically on their carbon sequestration potential.

The results obtained suggest that the use of *Trichoderma longibrachiatum* as a biofertilizer could be a promising strategy to increase carbon storage in agricultural soils, thus contributing to mitigating the effects of climate change. Further studies are recommended to evaluate the long-term impact of this microorganism on soil carbon dynamics and its interaction with other soil factors.

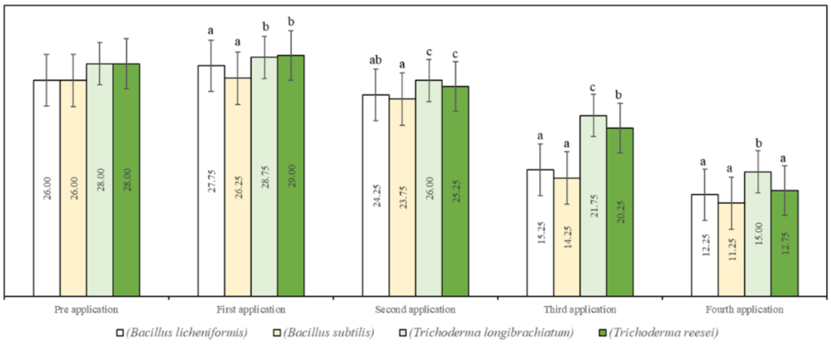

Figure 2. Carbon sequestration rates (mg CO_2-C kg^{-1} soil) among treatments over four weeks. Error bars represent standard error. Means with the same letter are not significantly different ($p > 0.05$) according to Tukey's HSD test.

Trichoderma longibrachiatum and *Trichoderma reesei* demonstrated superior carbon sequestration capabilities compared to *Bacillus subtilis* and *Bacillus licheniformis* throughout the four-week study. However, a general decline in carbon capture was observed across all treatments after the initial week. This reduction is likely attributed to factors such as plant and microbial carbon uptake, as well as ongoing soil organic matter decomposition (Pérez et al., 2021; Zamora et al., 2020).

While Trichoderma treatments showed promise in enhancing carbon sequestration, the results indicate the need for further research to optimize application rates and assess long-term effects. Additionally, exploring alternative strategies to sustain carbon capture in cocoa plantation soils is warranted. The observed decrease in carbon sequestration compared to initial levels and findings from previous studies (Ortiz, 2019; Barrezueta, 2019) suggests that cocoa agroecosystems may require specific carbon management practices to maintain soil fertility and productivity.

4. Conclusions

A comprehensive analysis of the research data indicates that Trichoderma and Bacillus strains exhibited high viability, with bacterial and fungal colony-forming units exceeding 10^8 and 10^6 CFU/ml, respectively, suggesting their potential for successful establishment in field conditions. Although microbial inoculation led to initial improvements in soil properties. Such as *Bacillus licheniformis* maintaining NH_4^+ at 19.00 ppm, Zn at 5.60 ppm, Mn at 5.20 ppm and B at 0.61 ppm, while increasing P to 66.00 ppm, K to 1.89 Meq/100ml, Ca to 19.00 Meq/100ml, Mg to 5.10 Meq/100ml, S to 50.00 ppm, Cu to 5.20 ppm and Fe to 54.00 pp, the overall impact on carbon sequestration was limited. Specifically, *Trichoderma reesei* achieved a 29% increase in carbon sequestration after the first application, while Bacillus subtilis showed an 11.25% increase after four applications, though with diminishing returns. These findings suggest that while microbial inoculation can positively influence soil health, a more holistic approach is necessary to achieve sustained carbon storage within complex cocoa agroecosystems. To optimize microbial applications and maximize their benefits, future research should focus on integrated management strategies that consider the specific conditions of cocoa plantations and the intricate interactions within the soil ecosystem.

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