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Original article

Effect of Bacillus megaterium and Trichoderma harzianum on the growth of Eucalyptus globulus seedlings in a nursery

Efecto de Bacillus megaterium y Trichoderma harzianum en el crecimiento de plántulas de Eucalyptus globulus en vivero

Efeito de Bacillus megaterium e Trichoderma harzianum no crescimento de mudas de Eucalyptus globulus em viveiro

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ABSTRACT

Sustainable forest management requires inputs that enhance seedling quality during the nursery phase. This study evaluated the effect of *Bacillus megaterium* and *Trichoderma harzianum* as plant growth promoters in *Eucalyptus globulus* seedlings. The experiment was conducted at the NOVOPAN company nursery (Imbabura, Ecuador) using a completely randomized design with four treatments: T1 (*B. megaterium*), T2 (*T. harzianum*), T3 (combination of both), and T4 (control with foliar fertilizer). The variables evaluated included plant height, root length, leaf dry weight, and root dry weight. Data were analyzed using ANOVA and Duncan's multiple range test ($p < 0.05$). Treatment T1 showed the best results, with increases of 43.41% in plant height and 16.56% in root length compared to the control. It also showed significantly higher accumulation of aerial and root biomass. The combination of both microorganisms (T3) did not demonstrate a synergistic effect. The results indicate that B. megaterium is an effective biofertilizer in the nursery stage of E. globulus, promoting vigorous early growth and reducing dependence on chemical fertilizers. This contributes to the adoption of sustainable forest management practices.

Keywords: *Eucalyptus globulus, Bacillus megaterium, Trichoderma harzianum*, biofertilizers, plant growth, forest nurseries.

RESUMEN

El manejo sostenible de plantaciones forestales requiere insumos que mejoren la calidad de las plántulas durante su fase de vivero. Este estudio evaluó el efecto de la aplicación de *Bacillus megaterium* y *Trichoderma harzianum* como promotores del crecimiento en plántulas de *Eucalyptus globulus*. El experimento se llevó a cabo en el vivero de la empresa NOVOPAN (Imbabura, Ecuador), bajo un diseño completamente aleatorizado con cuatro tratamientos: T1 (B. megaterium), T2 (*T. harzianum*), T3 (combinación de ambos) y T4 (control con fertilizante foliar). Se evaluaron altura de planta, longitud de raíz, peso seco foliar y peso seco radicular. Los datos fueron analizados mediante ANOVA y prueba de Duncan ($p < 0{,}05$). El tratamiento con *B. megaterium* (T1) mostró los mejores resultados, con aumentos del 43,41 % en altura y 16,56 % en longitud radicular respecto al control. Además, presentó mayor acumulación de biomasa aérea y subterránea. La combinación de microorganismos (T3) no evidenció efecto sinérgico. Se concluye que *B. megaterium* puede ser considerado un biofertilizante eficaz en viveros de *E. globulus*, promoviendo un crecimiento vigoroso en la etapa inicial y reduciendo la necesidad de fertilizantes químicos, lo que contribuye al manejo forestal sostenible.

Palabras clave: *Eucalyptus globulus, Bacillus megaterium, Trichoderma harzianum,* biofertilizantes, crecimiento vegetal, viveros forestales.

RESUMO

O manejo florestal sustentável requer insumos que melhorem a qualidade das mudas durante a fase de viveiro. Este estudo avaliou o efeito da aplicação de *Bacillus megaterium* e *Trichoderma harzianum* como promotores de crescimento em mudas de *Eucalyptus globulus*. O experimento foi realizado no viveiro da empresa NOVOPAN (Imbabura, Equador), utilizando um delineamento inteiramente casualizado com quatro tratamentos: T1 (*B. megaterium*), T2 (*T. harzianum*), T3 (combinação de ambos) e T4 (controle com fertilizante foliar). As variáveis avaliadas foram: altura da planta, comprimento da raiz, massa seca foliar e massa seca radicular. Os dados foram analisados por meio de ANOVA e do teste de Duncan ($p < 0,05$). O tratamento T1 apresentou os melhores resultados, com aumentos de 43,41% na altura da planta e 16,56% no comprimento da raiz em comparação ao controle. Também apresentou maior acúmulo de biomassa aérea e radicular. A combinação dos microrganismos (T3) não evidenciou efeito sinérgico. Os resultados indicam que *B. megaterium* é um biofertilizante eficaz na fase de viveiro de *E. globulus*, promovendo crescimento vigoroso na fase inicial e reduzindo a necessidade de fertilizantes químicos. Isso contribui para a adoção de práticas sustentáveis no manejo florestal.

Palavras-chave: *Eucalyptus globulus, Bacillus megaterium, Trichoderma harzianum*, biofertilizantes, crescimento vegetal, viveiros florestais.

INTRODUCTION

Sustainable forest management has evolved toward intensive systems that maximize timber production per unit area, incorporating agricultural practices into plantation management. This approach requires highly specialized forest nurseries that produce seedlings with optimized root systems using techniques such as chemical pruning with copper salts and the use of containers designed to induce aerial root pruning. These systems can improve new root generation by up to 27%. Within this framework, forest nurseries represent an essential component of the production chain, as they are responsible for supplying seedlings with optimal morphological and physiological characteristics that ensure successful establishment in the field. Seedling quality, especially regarding root and foliar development, is a determining factor in the probability of survival and initial growth within permanent plantations (Aldrete *et al.*, 2024).

In Ecuador, forest plantations cover 258,658 hectares, equivalent to 2% of the country's total forested area. These are located primarily in the Sierra region (30%), where the *Eucalyptus species predominates. globulus,* used in the timber industry and charcoal production (Giles Álvarez and Gutiérrez Juárez, 2023).

Among the cultivated species, *Eucalyptus *Pseudomonas globulus** stands out as a highly productive forest species, capable of establishing itself in areas with degraded soils and under a wide range of edaphoclimatic conditions. This versatility consolidates it as a key alternative for commercial afforestation programs in the Ecuadorian highlands, standing out for its accelerated growth rate and economic appeal (Guallpa-Calva *et al.*, 2019, 2016). The productive efficiency of forest plantations depends fundamentally on three critical factors: the appropriate site selection, the genetic quality of the plant material, and the application of suitable silvicultural techniques (*Rojo,* 2021). In Ecuador, forest plantations are concentrated mainly in the provinces of Pichincha, Imbabura, and Cotopaxi, constituting the main source of timber at the national level, accounting for approximately 79% of production. These areas lead in forested area, with exotic species predominating, especially eucalyptus, which represents about 70 % of total planted (Giles Álvarez and Gutiérrez Juárez, 2023). However, various factors, such as altitude and limited root system development, negatively affect the growth and productivity of this species, highlighting the need to implement practices that promote optimal development from the initial stages of cultivation, addressing the three critical factors mentioned above (Guallpa-Calva *et al.,* 2019).

The use of chemical fertilizers in forest plantations generates significant environmental impacts, such as the consumption of non-renewable energy resources and the contamination of soil and water bodies. In response to this problem, sustainable alternatives are being promoted, such as the use of plant growth-promoting microorganisms (PGPMs), which improve nutrient absorption and reduce dependence on chemical inputs without compromising productivity. (Chávez-Díaz *et al.,* 2020; González Ulibarry, 2019).

These microorganisms, which naturally inhabit soils, establish beneficial interactions with plant roots, promoting not only plant growth but also ecosystem stability. Their networked functioning improves soil fertility, and, in the context of climate change, their application as natural inoculants increases crop resistance, optimizes the absorption of essential nutrients such as phosphorus, and significantly contributes to mitigating negative environmental impacts (Rosabal Ayan). *et al.,* 2021). Among these beneficial microorganisms, plant growth-promoting rhizobacteria (PGPR) stand out for establishing mutualistic relationships with plants that significantly enhance their development. These bacteria improve nutrient uptake through various biological mechanisms such as nitrogen fixation, phosphate solubilization, and phytohormone production (Posada Castaño *et al.,* 2021). Additionally, they activate defense responses in plants, reducing the need for synthetic fungicides and insecticides. The efficacy of plant growth-promoting microorganisms depends on the compatibility between the strain, the crop, and the environmental conditions (Tripathi *et al.,* 2024). This efficacy is expressed through various physiological and biochemical mechanisms, including the modulation of ion transporters in the roots and the secretion of organic acids, processes that favor the solubilization and availability of essential soil nutrients, particularly phosphorus and iron (Khoso *et al.,* 2024). In particular, *Trichoderma Harzianum* is a saprophytic species with recognized biocontrol activity, widely used in agriculture. Its mechanism of action includes mycoparasitism, competition for nutrients and space, and the production of lytic enzymes such as chitinases and glucanases, which degrade the cell walls of phytopathogens (Acurio-Vásconez and García-Bolívar, 2024).

In addition to bacteria, some fungi play a key role in promoting plant growth. The genus *Trichoderma,* widely present in agricultural soils, stands out for its antagonistic action against phytopathogens through the production of hydrolytic enzymes, antifungal metabolites, and the induction of systemic resistance in plants (Ramírez-Valdespino *et al.*, 2025). Within this group, *Trichoderma harzianum* is a saprophytic species widely used in biocontrol, whose mechanism of action includes mycoparasitism, competition for nutrients and the synthesis of lytic enzymes such as chitinases and glucanases, capable of degrading the cell walls of pathogens (Ramírez-Valdespino *et al.*, 2025).

Despite advances in sustainable forest management, challenges persist in the nursery phase, where seedling quality is a determining factor for successful field establishment. Traditionally, chemical fertilizers have been used to enhance growth, but their application has significant environmental impacts, such as soil and water pollution. In this context, plant growth-promoting microorganisms, such as *Bacillus, offer valuable insights. Megatherium* and *Trichoderma Harzianum,* are emerging as sustainable alternatives. However, studies on their application in forest species, particulary *Eucalyptus, are lacking. Studies on the use of *C. globulus** are limited and do not comprehensively address their individual and synergistic effects. This study contributes to the development of scientific knowledge on the use of these microorganisms, evaluating their potential to improve seedling quality in nurseries, reducing dependence on chemical inputs, and promote sustainable forest management practices.

The objective of this study was to determine the effect of *Bacillus Megatherium* and *Trichoderma harzianum,* applied individually and in combination, as growth promoters in *Eucalyptus seedlings globulus* during the nursery phase. For this purpose, morphological parameters indicative of quality were analyzed, such as height, root development and foliar biomass

MATERIALS AND METHODS

Molecular identification of microorganisms

The strains evaluated were obtained from the strain collection of the Life Sciences Laboratory at the Salesian Polytechnic University, previously identified to the genus level by microscopic visualization. The bacterial microorganism was cultured on TSB (Trypticase Soy Agar) medium, incubated at 30 °C and 100 rpm for 24 hours. The fungus was cultured on PDA (Potato Dextrose Agar) medium and incubated at 26 °C for seven days. DNA extraction from both microorganisms was performed following the methodology described by Páramo Aguilera (2021) and Vásconez *et al.* (2020).

Polymerase chain reaction (PCR)

For molecular identification of the bacterial microorganism, the 16S region was amplified using the 27F (5'-AGAGTTTGATCCTGGCTCAG-3') primers (Dos *Santos et al.*, 2019). In the case of the fungus, the ITS1 (5' TCGGTAGGTGAACCTGCGG-3') primers were used. (Hammad *et al.*, 2021). PCR for the bacteria was performed following the protocol described by Acurio *et al.* (2020), which included an initial denaturation at 95 °C for two minutes, followed by 24 cycles of denaturation at 95 °C for 30 seconds, annealing at 58 °C for one minute, an initial extension at 72 °C for two minutes, and a final extension of 10 minutes at 72 °C, with subsequent maintenance at 4 °C. For the amplification of fungal DNA, the methodology described by [reference missing] was used. *Rigobelo et al.* (2024)By making modifications to the thermocycler conditions, an initial denaturation is performed at 94 °C for five minutes, 30 cycles of denaturation at 94 °C for one minute, hybridization at 53 °C for one minute, an initial extension at 72 °C for one minute and a final extension of ten minutes at 72 °C, followed by maintenance at 4 °C.

The PCR products were visualized on agarose gels to verify their quantity and apparent quality and were subsequently sent for sequencing to the company MacroGen-Korea (Kocić) *et al.*, 2019). The similarity of the sequences was compared using the NCBI's (National *Center for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) program. Biotechnology Information)*. Multiple sequence alignment was performed using UGENE v.1.30.0 software, employing the MUSCLE algorithm, and the phylogenetic tree was constructed using the Neighbor Joining method (Herrera *et al.*, 2024)

Auxin production

To evaluate the indoleacetic acid (IAA) synthesis capacity of the fungal microorganism, a potato dextrose broth (PDB) culture medium supplemented with L-tryptophan (1 mg ml $^{-1)}$ was prepared. The fungus was inoculated into a flask containing 50 mL of the medium at a concentration of 10^7 conidia/L, incubated at 20 ° C and 100 rpm for seven days. For the bacteria, tryptone soy broth supplemented with L- tryptophan (1 mg ml $^{-1}$) was used, inoculating test tubes with 6 ml of the medium and incubating at 30°C and 100 rpm for two days. The modified methodology of (Acurio Vásconez *et al. 2020)* was used.

For the quantification of IAA, the study by Mestre *et al.* (2025) was used as a reference. Salkowski reagent was prepared with 15 mL of $FeCl_3 \cdot 6H_2O$ (0.5 M), 300 mL of H_2SO_4 (98%), and 500 mL of distilled water. After the incubation period, the samples were centrifuged at 3,000 rpm for 15 minutes. Then, 1 mL of the supernatant was taken and mixed with 4 mL of Salkowski reagent and allowed to stand in the dark at room temperature for 30 minutes. Quantification was performed using a calibration curve with IAA (*Bacillus* and *Trichoderma*) concentrations between 10 and 50 (mg ml-1), measuring the absorbance at 530 nm in a JASCO V-730 spectrophotometer. Ten samples per microorganism were analyzed and an independent t-test with 95% confidence was applied to determine significant differences.

The trial was conducted at the NOVOPAN company nursery, located in the province of Imbabura, Cotacachi Canton, Imantag Parish (0°25'33'' N, 78°12'16'' W), at an altitude of 2,295 m above sea level. The area has temperatures between 4 °C and 16 °C, with an average annual rainfall of 582.27 mm. (Decentralized Autonomous Government of the Imantag Parish, 2023) A substrate composed of ground and decomposed pine bark was used, mixed with rice hulls in a 90:10 ratio, arranged in plastic trays with 96 holes each.

The experimental design included three treatments and a control group (control), which consisted of the application of Metalosate foliar fertilizer multimineral (0.5 ml L^{-1}) (Table 1). Treatments were applied weekly for four months, using a concentration of 1×10^{8} CFU ml^{-1} for both *Bacillus megaterium* as for *Trichoderma harzianum.* Each tray of seedlings was considered an experimental unit, and each treatment was replicated three times.

***Table 1. -** Treatments used*

Treatments	*Description*
T1	*Bacillus megaterium*
T2	*Trichoderma harzianum*
T3	*B. megaterium + T. harzianum*
T4	*Control*

The variables were evaluated for each replicate. Forty seedlings were randomly selected, avoiding plants at the edges of the trays. Seedling height, root length, leaf dry weight, and root dry weight were assessed. The collected data were subjected to a one-way analysis of variance with Duncan's multiple range test, with a 95% confidence level. To

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determine dry matter, the aerial parts of the plants were separated and dried in paper bags in an oven at 72 °C for 48 hours. The weight was then recorded using a GRAM SPX-1000D electronic balance. This procedure was repeated for root dry matter. The measurement was performed once at the end of the four-month experimental period.

RESULTS AND DISCUSSION

Molecular identification of microorganisms

In the phylogenetic tree generated from the 16S rRNA region sequence (Figure 1), the bacterial isolate was placed within a monophyletic clade composed exclusively of strains identified as *Priestia megaterium,* formerly known as *Bacillus megaterium* (Biedendieck) *et al.*, 2021). This grouping included the type strain IAM 13418, used as a taxonomic reference, and showed virtually no phylogenetic distances (0.0000–0.0014), reflecting high genetic similarity among the analyzed sequences. The isolate's position within this group confirms its identity as *P. megaterium,* with no evidence of phylogenetic ambiguity. Additionally, a strain classified as *Bacillus sp.* clustered closely within the same clade, suggesting a possible classification pending update in the database. These results are consistent with those reported by Liu *et al.* (2023) and Gupta *et al.* (2020), who highlight the usefulness of molecular tools such as phylogenetic analysis in the accurate identification of microorganisms with agricultural potential.

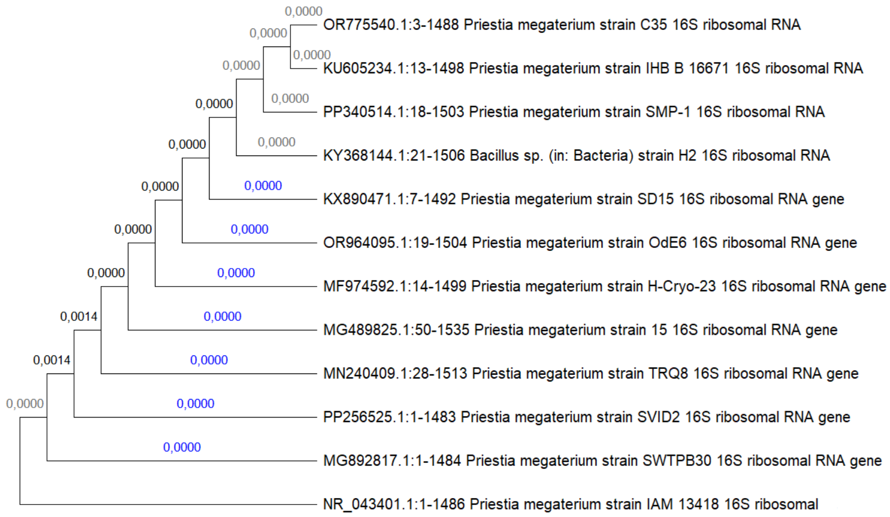

Figure 1. *- Phylogenetic relationship based on the sequence of the 16S rRNA region*

The phylogenetic tree constructed from the ITS1 sequence of the fungal isolate showed a clear and consistent grouping with reference strains of *Trichoderma harzianum,* confirming its taxonomic identity (Figure 2). The obtained sequence was positioned within a clade closely related to well-characterized accessions such as HG904098.1, KP340273.1, and KP340282.1, with minimal evolutionary distances (≤ 0.006), demonstrating high genetic similarity and low divergence with respect to other strains of the same species. The robustness of the clustering, along with the results obtained in the BLAST analysis, validates that the isolate unequivocally corresponds to *T. harzianum* . This finding is consistent with that reported by Cai and Druzhinina (2021), who indicate that the use of molecular tools, such as multigene sequencing and phylogenetic analysis, allows for a more precise and reliable delimitation of species within the genus *Trichoderma,* especially in cases of cryptic species or previously ambiguous taxonomies.

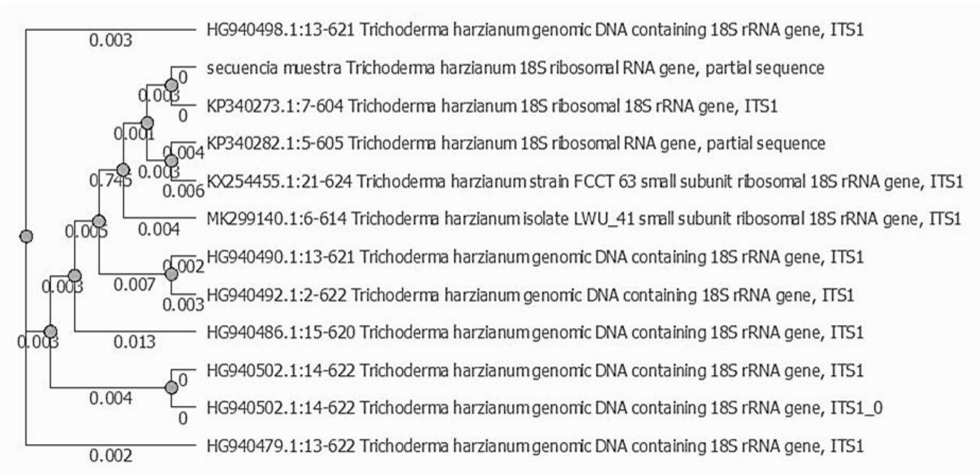

Figure 2. *- Phylogenetic tree of the species Trichoderma harzianum from the sequence of the 16S rRNA region and ITS1*

Auxin production

The results obtained demonstrate that *Bacillus Megaterium* showed a significantly higher production of IAA (11.32 ± 0.51 µg/ mL) compared to *Trichoderma harzianum* (9.64 ± 0.23 µg/ mL) ($p < 0.0001$; Table 2). This finding is consistent with previous studies that highlight *Bacillus* spp. as efficient producers of auxins, with a proven ability to stimulate plant development through the synthesis of phytohormones such as IAA Bader *et al.*

(2020). Similarly, these results support the key role of auxins in promoting plant growth, especially in early developmental stages (Acurio-Vásconez and García-Bolívar, 2024). However, it is particularly noteworthy that *T. harzianum,* despite producing lower concentrations of IAA, also induced plant growth-promoting effects. This observation suggests the activation of physiological mechanisms in addition to auxin synthesis, such as the production of other phytohormones, improved nutrient uptake, or the modulation of defense responses in the host plant. In this regard, Urías-García *et al.* (2022) demonstrated that *T. harzianum* can significantly promote the growth of tomato plants, even under field conditions, through multifactorial mechanisms not necessarily dependent on high concentrations of IAA, thus reinforcing its potential as a comprehensive biostimulant (Figure 3).

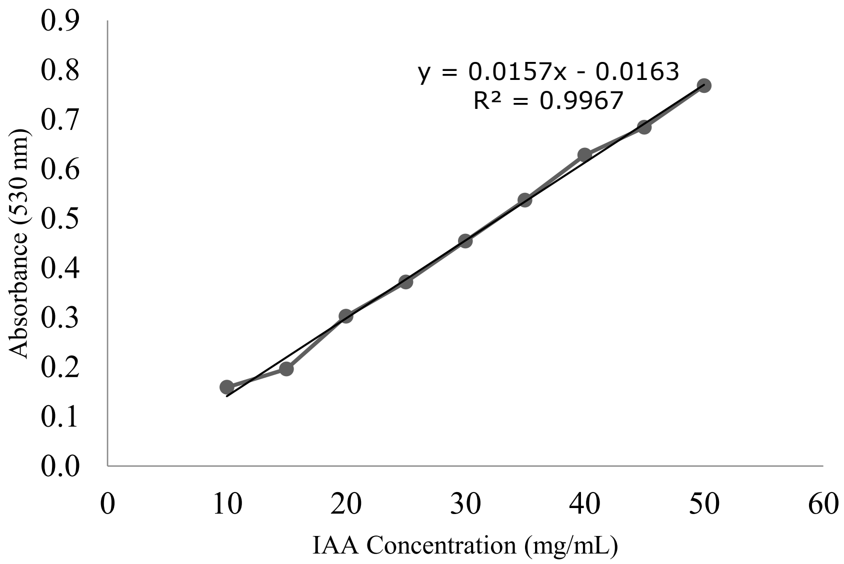

Figure 3. - *Calibration curve of indoleacetic acid (IAA)*

Table 2. - *Indoleacetic acid (IAA) production capacity*

Microorganism	(µg ml^ -1)
Trichoderma harzianum	9.64 ± 0.23 B
Bacillus megaterium	11.32 ± 0.51 A

Different letters indicate significant differences (P < 0.05), n=10.

Nursery evaluation

Bacillus treatment *Megaterium* demonstrated a significantly greater growth-promoting effect compared to the other treatments and the control (T4). In plant height, T1 recorded 207.72 mm, exceeding the control by 43.4%. This finding is consistent with previous studies that attribute to *Bacillus* spp. the ability to stimulate plant growth through multiple mechanisms, such as the production of phytohormones (auxins, gibberellins), phosphate solubilization and synthesis of bioactive metabolites that would modulate root development (Laishram *et al*., 2025).

In terms of root length, T1 reached 159.33 mm, 16.6% greater than the control, reinforcing the role of *B. megaterium* in root development, which is crucial for water and nutrient absorption. This finding is consistent with previous studies suggesting efficiency in activating key physiological pathways for cell expansion and nutrient uptake. (Zhang *et al*., 2023).

Regarding leaf dry weight (2.21 g), T1 showed an increase of 169 % and 83.33 % in root dry weight (0.33 g) Compared to the control, this shows a greater accumulation of biomass and physiological efficiency in the seedlings. These results coincide with those reported by Joseph *et al.* (2025), who demonstrated that *Trichoderma Harzianum* significantly promotes growth in *Eucalyptus,* improving both shoot and root dry mass. The observed effect can be attributed to increased nutrient availability, hormonal stimulation, and photosynthetic activity, reinforcing the potential of this fungus as a biostimulant in nurseries.

Trichoderma treatment *Harzianum* showed intermediate performance, with significantly lower values than T1, but higher than the control in leaf dry weight (2.04 g), indicating some promoting capacity, although less effective than *B. megaterium.* According to Pršić and Ongena (2020), this could be because its benefits are more evident in later growth stages or under biotic stress, where its role as a biocontrol agent is critical.

The combination of *B. megaterium* + *T. harzianum* (T3) did not show synergy, with results similar to or lower than the control in root dry weight (0.16 g), supporting the hypothesis of a possible mutual inhibition in the expression of growth-promoting genes, as proposed Karuppiah *et al.* 2019). On the other hand, its potential as a bioinoculant for nurseries is highlighted, especially in systems where rapid biomass accumulation is a priority. However, the lack of synergy in T3 underscores the need to optimize microbial consortia, considering metabolic compatibility and ecological niches (Santoyo *et al.*, 2021) (Table 3).

Table 3. - *Variables evaluated in Eucalyptus seedlings globulus*

Treatments	*Floor height (mm) ± DE*	*Root length (mm) ± DE*	*Leaf dry weight (g) ± SD*	*Root dry weight (g) ± SD*
T1 (B. megaterium)	*207.72 ± 36.6 A*	*159.33 ± 11.48 A*	*2.21 ± 0.47 A*	*0.33 ± 0.06 A*
T2 (T. harzianum)	*157.29 ± 11.7 B*	*131.94 ± 0.74 B*	*2.04 ± 0.72 B*	*0.214 ± 0.10 AB*
T3 (B. megaterium + T. harzianum)	*141.62 ± 20.0 B*	*143.61 ± 3.97 B*	*1.85 ± 0.30 BC*	*0.16 ± 0.03 B*
T4 (Control)	*144.84 ± 4.56 B*	*136.69 ± 4.89 B*	*0.82 ± 0.05 C*	*0.18 ± 0.03 B*

Different letters indicate significant differences ($P < 0.05$), one-way ANOVA, n=3.

CONCLUSIONS

The application of *Bacillus megaterium* It proved to be a biostimulant for *Eucalyptus globulus in the* nursery, outperforming *Trichoderma harzianum* and the control in all variables evaluated. Its individual application is recommended, while the combination with *Trichoderma harzianum* showed no synergistic effects, suggesting the need for further studies to elucidate microbial interactions.

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The authors declare no conflicts of interest.

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The authors have participated in the writing of the work and analysis of the documents.