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In Vitro Biodegradation of Tebuthiuron in Soil by Microbial Strains Obtained from an Agricultural Inoculant: Respiratory Responses and Comparative Efficiency

Victor Hugo Cruz[1]
https://orcid.org/0000-0003-3174-8890

Dhônata Marcos Perfeito[1]
https://orcid.org/0009-0007-2038-7002

Thais Lopes de Oliveira[1]
https://orcid.org/0009-0000-5377-4809

Yanca Araújo Frias[1]
https://orcid.org/0000-0003-1867-8533

João Pedro Maia[2]
https://orcid.org/0000-0001-5838-5757

Vitor Corrêa de Mattos Barretto[1]
https://orcid.org/0000-0002-9332-1094

Paulo Renato Matos Lopes[1]*
https://orcid.org/0000-0003-4886-5292

[1]Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Tecnológicas (FCAT), Dracena, São Paulo, Brasil; [2]AMTec® Bioagrícola Ltda., Uberlândia, Minas Gerais, Brasil.

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*Correspondence: prm.lopes@unesp.br; Tel.: +55-18-38217479 (P.R.M.L.).

HIGHLIGHTS

- Bioaugmentation enhances tebuthiuron degradation in sandy soil.
- *Pseudomonas* strain (M3) boosts CO_2 emissions by 42% over control.
- Fungal strain (M5) shows promise in herbicide bioremediation.
- Soil amendments and microbial inoculation synergize for remediation.

Abstract: Tebuthiuron, an herbicide widely used in crops such as sugarcane, can affect the sustainability of agroecosystems due to its high-water solubility and prolonged persistence in soil, thereby increasing environmental impact. Thus, bioremediation, a technique that utilizes microorganisms to degrade contaminants, employs bioaugmentation as one of its most effective strategies. Therefore, this study evaluated two main aspects: (1) the impact of tebuthiuron on soil microbial activity (through microbial respiration, measured by CO_2 emission) and (2) the efficiency of bioaugmentation (addition of commercial microorganisms) in mitigating these effects. The experiment was conducted for 60 days in the GAIA (Environmental Impact Action Group) laboratory at FCAT/Unesp-Dracena, using a dystrophic Red-Yellow Latosol. Tebuthiuron was applied according to the manufacturer's recommendations, at 2.0 L of commercial product ha^{-1} (1000 g of active ingredient ha^{-1}), which is the recommended dosage for sandy soils. Five microbial species, supplied by a commercial company, including bacteria and filamentous fungi (M1, M2, M3, M4, and M5), were used. Soil with native microbiota was also used as a control treatment (M0). The

methodology included the analysis of microbial respiration by quantifying CO_2 emission. The study results revealed that the application of tebuthiuron to the soil impacted the amount of CO_2 emitted by microbial respiration over time. Bioaugmented treatments showed a higher accumulated mean CO_2 compared to the control treatment. Treatment M3 obtained the highest accumulated mean CO_2 (1343 mg), representing a 42% increase relative to the control, followed by M5 (1256 mg, +33%) and M1 (1193 mg, +26%).

Keywords: bacteria; bioaugmentation; fungi; herbicide; CO_2 production.

INTRODUCTION

Tebuthiuron is a broad-spectrum systemic herbicide that is particularly notable for its ability to inhibit photosystem II, thereby preventing photosynthesis and ultimately leading to plant death [1]. According to [1], tebuthiuron effectively suppresses vegetation, including dicotyledonous species such as *Commelina benghalensis*, *Urochloa decumbens*, *Digitaria horizontalis*, and *Panicum maximum*.

In this context, the selection of tebuthiuron as the subject of study is justified by its extensive use in agriculture, particularly in sugarcane plantations, and its considerable environmental impact. This herbicide exhibits high water solubility, reaching 2.50 g L^{-1} at 25 ºC [2], a characteristic that facilitates the dispersion of its molecules into water bodies and their transport to different environmental areas, increasing the risk of contamination [2]. Additionally, tebuthiuron demonstrates soil persistence, remaining active for over 15 months, as documented by [3] and [4].

As a result, the prolonged persistence of this herbicide can impact soil and vegetation for extended periods, exacerbating environmental concerns and raising critical questions about its long-term effects on ecosystems [2]. Moreover, its application may also cause significant alterations in the microbiological conditions of the environment [5,6].

Furthermore, microorganisms play fundamentals roles in the health and functionality of terrestrial ecosystems [7]. These functions include organic matter storage, which provides essential nutrients for plants and enhances soil quality; nutrient cycling, which is vital for soil maintenance; and soil aggregation, which influences porosity and water retention [8]. Given the fundamental role of microorganisms in these processes, monitoring their activity is essential to understanding the environmental impacts of herbicide use, particularly tebuthiuron, on soil [9].

Thus, bioremediation, a technique that employs microorganisms to degrade contaminants, features bioaugmentation as one of its most effective strategies. This biotechnology involves the introduction of specialized microorganisms with specific metabolic pathways to transform herbicides and other pollutants into less toxic compounds [10]. According to these authors, when combined with the action of native microorganisms, bioaugmentation enhances decontamination, improves soil biochemical conditions, and accelerates pesticide degradation. However, maintaining microbial activity under adverse conditions, such as nutrient deficiency, inappropriate pH levels, or the presence of other contaminants, remains a significant challenge [11].

To address this issue, continuous monitoring of microbial respiration using the respirometry technique, developed by Bartha and Pramer [12], is essential. According to these authors, this technique measures the production of carbon dioxide (CO_2) by microorganisms, which serves as a direct indicator of respiratory activity and, consequently, the capacity to degrade compounds in the soil. The application of respirometry to monitor the biodegradation of the herbicide tebuthiuron has also been highlighted in several studies, underscoring the importance of this method for analyzing temporal changes in microbial activity and pesticide degradation [12,13,14,15].

Despite advances in understanding the effects of tebuthiuron, there is a lack of studies evaluating the efficacy of commercial inoculant microorganisms in their degradation, particularly when monitored by sensitive techniques such as respirometry. Previous studies have focused on native microbial communities [15] or field biodegradation [13], but they have not compared bacterial and fungal strains from inoculants under controlled conditions, mainly to the tebuthiuron-herbicide. This gap limits the development of optimized protocols for bioremediation. Based on the hypothesis that bioaugmentation accelerates tebuthiuron degradation, this study investigated: (1) the respiratory response of five commercial strains in contaminated soil, and (2) the relationship between CO_2 emissions and biodegradation efficiency, using respirometry as a metabolic indicator. Therefore, the primary objective of this study was to evaluate the capacity of agricultural inoculant microorganisms to degrade tebuthiuron in sandy soil, using cumulative CO_2 production as a parameter of microbial activity.

MATERIAL AND METHODS

The experiment was performed at the GAIA (Environmental Impact Action Group) laboratory, in the College of Agricultural and Technology Sciences of São Paulo State University "Júlio de Mesquita Filho" – FCAT/Unesp, Dracena Campus. The soil used was a dystrophic Red-Yellow Latosol, with no history of pesticide application, collected from the Experimental Farm at a depth of up to 20 cm, and subsequently sieved and stored. It is noteworthy that the soil was collected from an adjacent experiment and had not been previously cultivated. A granulometric analysis described the soil as containing 79.40% sand, 14.10% clay, and 6.50% silt, characterizing it as having a sandy-loam texture. Soil correction (Final) was performed based on the chemical composition prior to the experiment setup (Initial), as shown in Table 1.

Table 1. Chemical analysis of soil before (initial) and after (final) cultivation.

Parameters	Initial	Final	Unit
pH	4.3	7.6	
Organic Matter	7.0	7.0	g dm^{-3}
Potassium	1.1	1.6	g dm^{-3}
Calcium	2.0	28.0	g dm^{-3}
Magnesium	9.0	19.0	mmolc dm^{-3}
Hydrogen + Aluminum	22.0	6.0	mmolc dm^{-3}
Aluminum	9.0	0	mmolc dm^{-3}
Copper	0.1	0.1	mmolc dm^{-3}
Iron	1.0	1.0	mmolc dm^{-3}
Manganese	0.5	1.0	mmolc dm^{-3}
Zinc	0.1	0.30	mmolc dm^{-3}
Phosphorus	2.0	16.0	mg dm^{-3}
Boron	0.08	0.05	mg dm^{-3}
Sulfur	5.0	13.0	mg dm^{-3}
Sum of Bases	12.1	48.6	mg dm^{-3}
Cation Exchange Capacity	34.1	54.6	mg dm^{-3}
Base Saturation	35.0	89.0	%
Aluminum Saturation	43.0	0	%

The herbicide analyzed was tebuthiuron, acquired from a commercial establishment in the form of the product Combine 500SC (500 g a.i. L^{-1}, Dow AgroSciences Industrial Ltda.). Five microbial strains, components of a commercial agricultural inoculant, were supplied by AMTec® Bioagricola Ltda (Uberlândia, Brazil). These strains were previously isolated and characterized by the company during product development; phylogenetic identification remains its proprietary and is not disclosed here. For the experiments, they were coded as CMA 468, CCT 8332, CCT 8330, CCT 8331 (bacteria) and CCT 8328 (filamentous fungus). The phylogenetic identification is the private domain of the company (AMTec Bioagricola) and therefore cannot be disclosed.

Experimental planning

The experiment followed a completely randomized design with 6 treatments and 4 replicates, comprising a total of 24 experimental units. The treatments consisted of a native microorganism present in the soil at the collection site and five microbial inoculate, aiming to assess bioaugmentation, as outlined in Table 2. It is noteworthy that tebuthiuron was applied to all treatments.

Table 2. Treatments used for respirometric monitoring

Treatment	Code	Microbial group
M0	Control	Native
M1	CMA 468	*Bacterium*
M2	CCT 8332	*Bacillus mycoides*
M3	CCT 8330	*Pseudomonas nicosulfuronedens*
M4	CCT 8331	*Pseudomonas canadensis*
M5	CCT 8328	*Trichoderma yunnanemse*

Application of fertilizers and herbicides to the soil

Based on the analyses conducted, as shown in Table 1, soil fertility and acidity correction strategies were implemented to optimize subsequent processes. For 144 kg of soil, 52.50 g (947.92 kg·ha^{-1}) of limestone was applied to adjust the pH. To correct soil fertility, synthetic fertilizers were used: for 144 kg of soil, 25 g (451.4 kg·ha^{-1}) of single superphosphate was applied to supplement phosphorus, and 5.53 g of potassium chloride (99.85 kg·ha^{-1}) was applied to ensure adequate levels of phosphorus and potassium, respectively.

After soil correction, 4.0 L plastic pots were filled with soil to receive the application of tebuthiuron at the dose recommended by the product label. The herbicide was sprayed at a rate of 2.0 L of commercial product per hectare (1000 g of active ingredient ha^{-1}), which is the dosage recommended for sandy soils. Spraying was performed using a laboratory-scale automated sprayer, at a speed of 5 km h^{-1}, from a height of 0.75 m above the soil surface, with XR 8002 nozzles operating at a pressure of 2.0 bar and a flow rate of 0.65 L min^{-1}, spaced 0.5 m apart, and pressurized with CO_2. This setup resulted in a spray volume of 156 L ha^{-1}.

The dosage used in this study was also adopted in respirometry experiments to assess herbicide biodegradation under controlled conditions [13,15].

Respirometric bioassays

Based on the commercial microbial inoculant's package insert, application should occur in moist soil. Therefore, the soil's liquid saturation point was determined prior to adding the recommended dose of each microbial isolate, incorporated at 20 mL kg^{-1} of soil. This measurement was used to calculate the volume of inoculant and deionized water to be added to each respirometer.

Subsequently, respirometric flasks were prepared, each containing a chamber with 50 grams of tebuthiuron-amended soil, along with 1 mL of the standardized microbial strain and 7.5 mL of deionized water to maintain appropriate soil moisture. The device's side chamber was reserved for 10 mL of 0.2 mol potassium hydroxide (KOH) solution. This compound plays a crucial role in capturing CO_2 released during the degradation process. It's important to note that the KOH solution was periodically replaced throughout the experiment to prevent saturation of the medium, as indicated by the reading points on the x-axis of Figures 1 and 2. The bioassays monitored biodegradation for 60 days with incubation under controlled Biochemical Oxygen Demand (BOD) conditions at a constant temperature of 28 ± 2°C, in the absence of light.

The procedure for measuring CO_2 release involved reading the electrical conductivity (EC) using an OneSense Cond 2500 conductimeter. This method includes four steps: (1) First, a syringe was inserted into the potassium hydroxide (KOH) solution and withdrawn from the respirometer's side arm; (2) Next, the sensor was washed twice with 10 mL of boiled, CO_2-free deionized water; (3) After washing, 1 mL of 0.25 mol barium chloride ($BaCl_2$) solution was added to the withdrawn samples, and then the reading was taken. To ensure the internal environment was refreshed, air was injected into the respirometer; (4) Finally, a new portion of the KOH solution was added to the flask to continue the monitoring process.

Microbial CO_2 production was indirectly quantified (Equation 1; R^2 ~ 0.9999) by converting the electrical conductivity values from mS to mg of CO_2 [16]. The equation used was validated as described by [16], ensuring its applicability to the experimental system. All reagents used in the experiment, including potassium hydroxide (KOH) and barium chloride ($BaCl_2$), adhered to established analytical standards to guarantee the quality and reproducibility of the results.

$$GC\mathrm{D}_2 = 1554.8 - 95.726 * EC \quad \text{(Equation 1)}$$

Where: GCD_2 = generation of carbon dioxide in mg; and EC = electrical conductivity in mS.

Standardization of the Inoculum

The Shapiro-Wilk and Bartlett tests were applied to assess data normality and homoscedasticity, respectively, assuming a 5% significance level. Subsequently, the experimental data were subjected to analysis of variance (ANOVA, $p < 0.05$) and Tukey's multiple comparison test ($p < 0.05$) to evaluate differences in CO_2 production among the different microbial strains. Additionally, a logarithmic model was fitted to the respirometry data to describe the kinematic behavior of CO_2 production (Equation 2). All statistical analyses were performed using R software.

$$y = a * \ln(x) + b \quad \text{(Equation 2)}$$

Where: GCD_2 = y: cumulative CO_2 (mg) (dependent variable); x: time (days) (independent variable); a: slope coefficient of the logarithmic curve; and b: intercept coefficient of the logarithmic curve.

RESULTS AND DISCUSSION

Figure 1 illustrates the weekly CO_2 production over a 60-day period. Overall, soil samples treated with tebuthiuron exhibited higher microbial respiration rates in the initial weeks, including the non-inoculated control (M0). This early response may be linked to prior soil conditioning (Table 1), which raised the pH to 7.6, and increased the availability of nutrients, such as calcium and magnesium, creating a less stressful environment for microbiota. Over time, CO_2 release gradually declined, reflecting reduced substrate availability for microbial metabolism. Among the tested isolates, the bacterial strain CCT 8330 (M3) stood out, demonstrating significantly higher respiration rates, particularly between days 30 and 40.

These findings align with [13], who reported that a synergistic bacterial consortium from sugarcane soils mineralized up to 2141 mg of CO_2 over 90 days, reaching a maximum specific rate of 89.6 mg CO_2 day^{-1}. These values are comparable to the peak rate of 45–60 mg CO_2 day^{-1} observed for CCT 8330 in this study, suggesting that this individual strain's respiratory efficiency rivals that of complex microbial consortia. Furthermore, [13] fitted their data to the Gompertz model, supporting the applicability of sigmoid functions to describe tebuthiuron mineralization in tropical soils.

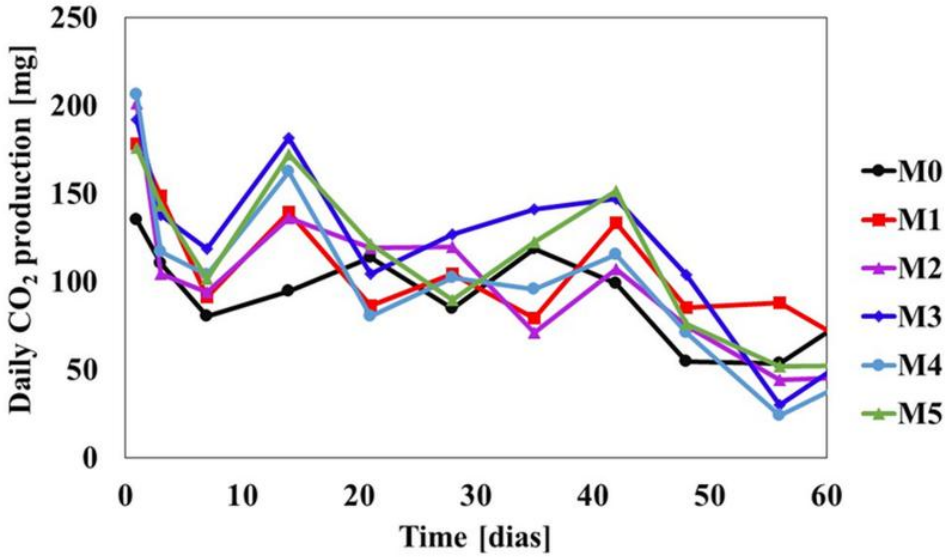

Figure 1. Weekly CO_2 production over 60 days of microbial respiration in tebuthiuron-contaminated soil under controlled conditions. Caption: M0 – native microbiota; M1 – bacterial strain; M2 – *Bacillus mycoides*; M3 – *Pseudomonas nicosulfuronedens*; M4 – *Pseudomonas canadensis*; M5 – *Trichoderma yunnanemse*.

The analysis of cumulative CO_2 production over time (Figure 2), conducted through mathematical modeling, revealed the classic phases of microbial growth: (a) Lag Phase: The initial adaptation period of microorganisms to the environment, with no significant increase in respiratory activity. In this phase, soil acidity correction (reduction of Al^{3+} to 0 mmolc dm^{-3}) likely minimized herbicide toxicity, allowing for more efficient adaptation of the introduced strains (M3 and M5); (b) Log (Exponential) Phase: Characterized by accelerated microbial growth and a rapid increase in CO_2 production. The peak activity in this phase coincided with the high availability of phosphorus (16.0 mg dm^{-3}) and sulfur (13.0 mg dm^{-3}) in the amended soil (Table 1), critical nutrients for the synthesis of degrading enzymes; (c) Stationary Phase: Where growth rate stabilizes due to nutrient depletion or accumulation of toxic byproducts; and (d) Decline Phase: Marked by reduced microbial activity due to resource exhaustion or buildup of inhibitory substances. The logarithmic modeling fit the data well ($R^2 > 0.88$), confirming its utility in describing respiratory dynamics and biodegradation in tebuthiuron-treated soils.

Recent literature highlights *Pseudomonas aeruginosa* and *P. glycinae* as the most tebuthiuron-tolerant bacterial species, with minimum inhibitory concentrations of up to 185 µL mL^{-1} in selective medium [17]. The robustness of these strains was attributed to the presence of monooxygenases and amidases that catalyze the N-demethylation of the phenylurea ring, an initial step also described for the degradation of the molecule by strains employed in this study. These findings support the hypothesis that CCT 8330 may possess physiological pathways like those of *Pseudomonas*, justifying its superior performance during the Log phase.

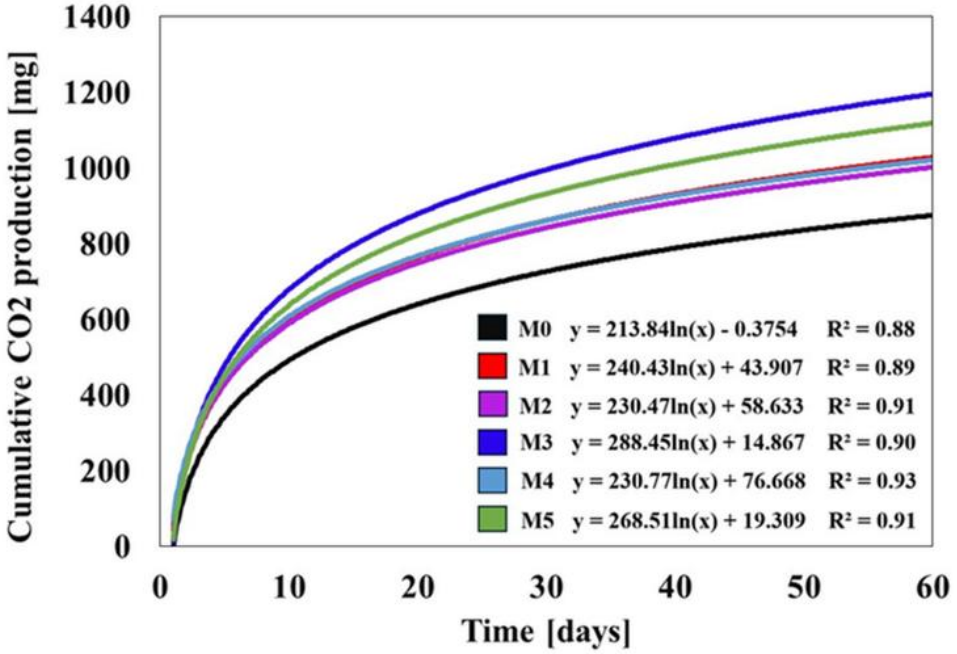

Figure 2. Cumulative CO_2 production over 60 days of microbial respiration in tebuthiuron-contaminated soil under controlled conditions. Caption: M0 – native microbiota; M1 – bacterial strain; M2 – *Bacillus mycoides*; M3 – *Pseudomonas nicosulfuronedens*; M4 – *Pseudomonas canadensis*; M5 – *Trichoderma yunnanemse*.

During the Lag Phase (days 0–10), a short and rapid period of adaptation and transition was observed, where cells increased in size and prepared for cellular division under herbicide exposure. The effect was less pronounced in treatment M0 due to the absence of specialized microbiota. The lower activity in M0 can be attributed to the lack of adapted inoculum, combined with the soil's original low fertility (initial pH of 4.3 and reduced calcium and magnesium levels). In contrast, treatments M3 and M5 exhibited a more prolonged Lag phase with high metabolic activity, synthesizing new enzymes to adapt to the environment, indicating degradation efficiency. This reflects the microorganisms' adaptation time required to establish themselves in the soil.

The Log Phase (days 10–30) was marked by an exponential increase in CO_2 production, indicating intense microbial metabolic activity. Strain M3 (CCT 8330) stood out during this stage, demonstrating higher efficiency in utilizing tebuthiuron as a substrate. This performance was enhanced by the high in base saturation (89%), which improved cation exchange capacity (54.6 mmolc dm^{-3}), favoring the retention of nutrients essential for microbial metabolism. This efficiency may be linked to the presence of specific enzymes that facilitate the breakdown of the herbicide's chemical bonds, as well as the strain's ability to utilize intermediate metabolites generated during tebuthiuron degradation. Furthermore, the availability of essential nutrients (Table 1) likely supported the biochemistry of degradation and active microbial respiration. The high CO_2 release during this period directly reflects the degradation and biotransformation of tebuthiuron into less toxic byproducts.

Therefore, theses the results validated the initial hypothesis: the bacterial strain (M3) exhibited the highest CO_2 production (1343 mg), indicating a high degradation capacity. This performance even surpassed that of the fungus (M5), suggesting that specific bacterial enzymes may be more effective in breaking down the respective herbicide [18]. Furthermore, the Log phase coincided with the peak CO_2 emission, corroborating studies that associate respiratory activity with biodegradation [13].

Although bacteria stood out, filamentous fungi also showed relevant potential. Classic studies conducted by [19] demonstrate that *Trichoderma* spp. strains are capable of metabolizing phenylurea herbicides through hydroxylation followed by N-demethylation, albeit with lower CO_2 mineralization rates than those observed for *Pseudomonas*. Additionally, more recent investigations with commercial inoculants indicate that microbial inoculation can reduce the residual phytotoxicity of tebuthiuron in lettuce bioassays, simultaneously acting in the partial degradation of the compound and the mitigation of oxidative stress in seedlings [20]. These results support the good performance of treatment M5 in the present study, even in low organic matter soil.

During the Stationary Phase (days 30–50), native microorganisms (M0) showed an early stabilization of respiratory activity. On the other hand, treatments M3 and M5 sustained CO_2 production for a longer period, demonstrating greater resilience and capacity to continue the mineralization of tebuthiuron byproducts. The

presence of micronutrients, such as zinc and manganese (Table 1) may have acted as enzymatic cofactors, prolonging the metabolic activity in these strains. This could be related to the nutritional support offered by the corrected soil, with high levels of calcium (28.0 mmolc.dm^{-3}) and magnesium (19.0 mmolc.dm^{-3}), both essential for maintaining cellular integrity and the functioning of oxidative enzymes involved in herbicide degradation [21].

Although the study demonstrates the effectiveness of strains M3 and M5 under controlled conditions (28 ± 2°C and constant humidity), it is important to highlight that tropical soils in natural environments are subject to significant thermal and hydric fluctuations. In the field, the sandy loam texture of the soil (79.4% sand) could increase the leaching of tebuthiuron, reducing its availability for microbial degradation. These variations can alter the dynamics of microbial activity and, consequently, the rate of tebuthiuron biodegradation. Studies such as those by [22] and [23] reinforce that humidity and temperature are critical determinants for the stability of degrading enzymes and the survival of introduced microorganisms. Therefore, the results obtained here represent an idealized scenario, and future field validations are essential to evaluate the resilience of these strains under dynamic environmental conditions.

The Decline Phase (partially observed on day 60), treatment M0 exhibited a sharp decrease in respiratory activity, whereas M3 and M5 still maintained residual levels of CO_2 release, indicating potential degradation of intermediate metabolites of tebuthiuron. The sustained activity in M3 and M5 can be attributed to a combination of factors: (i) high base saturation (89%) maintained pH stability, preventing additional stress; (ii) increased micronutrient content (such as Zn and Mn) supported residual enzymatic activity. This continuous degradation in amended environments may have been stimulated by the presence of micronutrients such as zinc (from 0.1 to 0.3 mg.dm^{-3}) and manganese (from 0.5 to 1.0 mg.dm^{-3}), both of which are cofactors of enzymes involved in the breakdown of persistent aromatic compounds [18,24,25].

These results highlight a synergistic relationship between soil chemical amendments, the introduction of microorganisms with degradation potential, and increased respiratory activity as an indicator of tebuthiuron biodegradation. The previously acidic and nutrient-poor soil (pH 4.3; 2.0 mmolc dm^{-3} of Ca) was transformed into an environment favorable to active microbial respiration after amendments (pH 7.6; 28.0 mmolc dm^{-3} of Ca), resulting in the conversion of the herbicide into less harmful byproducts for the agroecosystem. The improvement in soil conditions, evidenced by the increase in cation exchange capacity (from 34.1 to 54.6 mmolc dm^{-3}), created a chemically stable environment for microbial activity. The accumulated CO_2 production, particularly in M3 and M5 treatments, represents not only intensified microbial activity but also the effective transformation of tebuthiuron, reinforcing the potential of bioaugmentation as a tool for the remediation of contaminated areas.

The comprehensive review by [26] highlights that *Pseudomonas*, *Arthrobacter*, *Flavobacterium*, and *Bacillus* are the most frequently associated with pesticide bioremediation, corroborating the findings of this study for CCT 8330 and the consortium of [14]. This perspective reinforces the importance of selecting strains belonging to these taxonomic groups for the development of commercially applicable bioinoculants.

However, despite the strong performance of the logarithmic model in initial data, it is important to note that it assumes continuous exponential microbial growth, which does not accurately represent real environments with resource limitations or metabolite inhibition [27,28]. In field conditions, soil heterogeneity (14.1% clay and 6.5% silt) and variations in nutrient availability could limit the direct applicability of the model. Therefore, for more realistic predictions, models such as Monod, Haldane, or Michaelis-Menten are recommended, as they consider the influence of substrate (herbicide) concentration on microbial growth. [29] also emphasizes the importance of using statistical models and machine learning algorithms to monitor the interaction between pesticides and soil microbiota. Nonetheless, despite these limitations, the mathematical model employed in this study provided satisfactory preliminary results, supported by statistical analyses that demonstrated the impact of tebuthiuron on native and bioaugmented microbial communities.

Additionally, Figure 3 shows that, at day 60, the highest respiration rate was recorded in the treatment with the bacterial strain CCT 8330 (M3), reaching 1343 mg of CO_2, followed by the fungal strain (M5), with 1256 mg of CO_2. This difference may be related to the greater efficiency of bacteria in utilizing available nutrients in the amended soil (such as phosphorus and sulfur, which increased by 8- and 2.6-fold, respectively, as shown in Table 1) for the degradation of recalcitrant compounds. The other three bacterial species exhibited similar behavior, with lower metabolic activity. The control soil without inoculation (M0) showed the lowest CO_2 production (943 mg), highlighting the positive effect of microbial inoculation on tebuthiuron biodegradation.

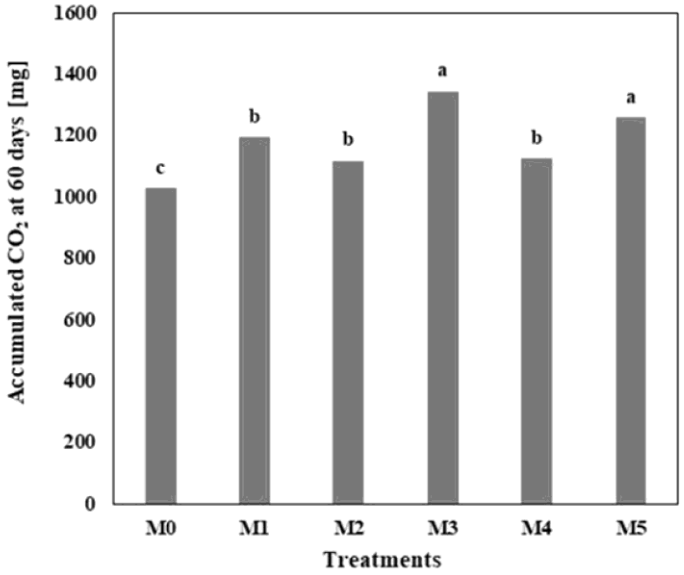

Figure 3. Mean comparison of cumulative CO_2 production after 60 days of microbial respiration under controlled conditions. Caption: M0 – native microbiota; M1 – bacterial strain; M2 – *Bacillus mycoides*; M3 – *Pseudomonas nicosulfuronedens*; M4 – *Pseudomonas canadensis*; M5 – *Trichoderma yunnanemse*.

These results make significant contributions to future perspectives in the field, as highlighted by [10] in a recent review. The authors noted that the fungal group remains underexplored as a biodegradative agent for pesticides in soil. It is noteworthy that the performance of the CCT 8328 (M5) fungus was remarkable, considering that the amended soil had a low organic matter content (7.0 g dm^{-3}), a factor that typically limits fungal growth. In this context, the second-highest respiration rate observed in the M5 treatment underscores the potential of these microorganisms, which can be harnessed in the development of biotechnological tools applied to agricultural soils.

Furthermore, abiotic factors such as pH, moisture, temperature, and organic matter content can influence microbial activity and, consequently, the success of bioremediation [22,23]. In this study, adjusting the pH to 7.6 (Table 1) likely optimized the activity of microbial enzymes, such as dehydrogenases and oxidases, involved in tebuthiuron degradation. For instance, pH and carbon content directly affect the activity of degradative enzymes and the persistence of herbicides like linuron, an effect potentially applicable to tebuthiuron [21,30,31]. Although the organic matter content remained constant (7.0 g dm^{-3}), it provided basic support for the microbiota without excessively affecting tebuthiuron bioavailability, which could be reduced at higher organic matter levels due to its affinity for organic compounds [32].

Although native microorganisms are adapted to adverse soil conditions, the presence of tebuthiuron compromises their metabolic activity, particularly in M0, where no bioaugmentation was applied. Initial chemical analysis of the soil revealed high acidity (H+Al = 22 mmolc dm^{-3}) and low base saturation (35%), conditions that, even after amendment, may have limited the resilience of the native microbiota to the stress caused by the herbicide. The reduction in microbial respiration and mineralization may indicate that the herbicide's toxicity inhibits a significant portion of the native microbiota. This response is exacerbated by the chemical complexity of tebuthiuron, which can form toxic byproducts during degradation, negatively impacting microbial metabolism [32].

In practical terms, biostimulation through the addition of organic matter and nutrients can enhance microbiological activity. However, tebuthiuron exhibits a high affinity for organic matter, which reduces its bioavailability and hinders degradation [32]. Balancing the addition of organic matter to stimulate the microbiota with the potential sorption of the herbicide is particularly critical in sandy soils, such as those in this study (79.4% sand), which inherently have a low contaminant retention capacity. This behavior may contribute to the herbicide's persistence in soil and increase the risk of environmental contamination, particularly in aquatic ecosystems [5,6]. Previous studies have shown that, even with biostimulants like vinasse, tebuthiuron mineralization is a slow, time-dependent process [13,14,15]. Moreover, high herbicide doses are toxic to microorganisms, compromising enzymatic activity and soil respiration [33].

The environmental persistence of tebuthiuron also poses a risk to groundwater quality. Studies have detected its presence in aquifers, such as the Guarani Aquifer, at depths exceeding 50 meters [34,35]. In this

study, despite the sandy soil texture, amendment with limestone (947.92 kg ha^{-1}) and nutrient addition may have partially reduced the risk of leaching, as the elevated pH favored the formation of stable complexes between the herbicide and residual Fe and Al oxides. In sandy soils, the low contaminant retention capacity increases leaching risk. Although higher clay and organic matter contents reduce this mobility, tebuthiuron sorption is strongly influenced by the decomposition state of organic matter [36,37,38].

Moreover, metabolites formed during tebuthiuron degradation may be more toxic than the original molecule [39], reinforcing the need for effective remediation strategies. The presence of micronutrients such as Cu and Fe (1.0 mg dm^{-3} each, Table 1) may have influenced the degradation pathway, potentially determining the toxicity of the metabolites formed. Sorption reduces the herbicide's availability for microbial degradation, prolonging its half-life (DT50) and contributing to its classification as a high environmental risk substance [1].

Additionally, to reduce the environmental persistence of tebuthiuron, bioaugmentation can be employed, a technique proposed in this study, as a bioremediation strategy for soils contaminated with recalcitrant pesticides like tebuthiuron. The selection of strains M3 and M5 proved strategic, as both tolerated the initial soil conditions (low pH and high Al saturation) and responded positively to the corrections made. The inoculation of specific microbial strains, whether fungal or bacterial, can promote the degradation of persistent toxic compounds, as evidenced by [40]. When these strains are previously isolated with proven potential for biodegrading the target pollutant, as demonstrated by [10] and [13], the efficiency of treating tebuthiuron-contaminated soil is significantly maximized. The results obtained in these studies confirm this premise, showing that the inoculants used were effective both in tolerance and removal of tebuthiuron residues under controlled conditions. This highlights the potential for applying the technique in contaminated agricultural fields, to validate, on a real scale, the efficiency and effectiveness of the introduced microorganisms.

In this context, it is pertinent to cite the study by [41], in which the degradation rate of the insecticide chlorpyrifos was higher in open fields compared to a greenhouse environment. In the present study, the prior correction of the soil with limestone and fertilizers (Table 1) may have created a chemical "memory effect" that, even in the field, could sustain the activity of strains M3 and M5 for a longer period. This difference was attributed to variations in environmental conditions, such as temperature, humidity, and exposure to solar radiation, which can favor the performance of microorganisms in natural conditions. This reinforces the importance of testing inoculants in different agroecosystem scenarios to confirm their robustness and adaptability.

Therefore, the microorganisms selected in this work showed promise as bioremediation agents for tebuthiuron. The combination of bioaugmentation with chemical correction of the soil (especially the increase in base saturation to 89%) created synergies that would hardly be achieved with only one of the strategies alone. Their introduction into the soil elevated bioaugmentation to a new level, expanding the prospects for using commercial inoculants in managing areas contaminated with pesticides. However, to optimize their efficiency, it is essential to deeply understand the ecology, mechanisms of action, and interactions of these strains with the native microbiota and the environment [42]. Such knowledge is fundamental to guiding future research and practical applications of bioaugmentation in agriculture.

Nonetheless, the implementation of this technique must be carried out with careful planning and systematic monitoring, considering its potential ecological impacts. Continuous monitoring of parameters such as pH, organic matter content, and availability of micronutrients (such as Zn and Mn) will be crucial in field applications, as these variables directly influence the stability of introduced microbial strains. The introduced strains compete with native microorganisms for nutrients and space, which may, in some cases, lead to the suppression of already established beneficial populations in the soil. This interference can compromise essential ecological functions and soil health in agroecosystems, as warned by [43].

Thus, bioaugmentation enhanced microbial respiration and tebuthiuron degradation compared to the control soil, confirming its potential as a biotechnological tool for restoring contaminated soils. This study suggests that bioremediation protocols for tebuthiuron in sandy soils should include: (i) prior correction of soil acidity and fertility (as performed in Table 1); (ii) selection of strains adapted to low organic matter content; and (iii) monitoring of Ca, Mg, and micronutrient levels throughout the process. To maximize efficiency and mitigate risks, it is recommended to combine bioaugmentation with complementary techniques—such as biostimulation, phytoremediation, or biochar application—which have proven effective in remediating persistent organic pollutants [44,45]. However, field validation must consider thermal variations, soil heterogeneity, competition with native microbiota, and the reduced bioavailability of herbicide in the presence of organic matter or clay [22,23,36]. Field-scale trials must simultaneously monitor degradation rates and ecotoxicological indicators in non-target organisms, ensuring robust and environmentally safe bioremediation protocols.

The results highlight a synergistic relationship between soil chemical amendments, as microbial strains M3 and M5 increased respiratory activity as an indicator of tebuthiuron biodegradation. Initially, the acidic and nutrient-poor soil (pH 4.3; 2.0 mmolc dm^{-3} of Ca) was transformed through amendments into a more favorable environment for active microbial respiration (pH 7.6; 28.0 mmolc dm^{-3} of Ca), which facilitated the conversion of tebuthiuron into less harmful byproducts for the agroecosystem.

Although this study demonstrates the efficacy of M3 and M5 strains under controlled laboratory conditions, these factors represent an idealized environment that does not fully replicate the complexity of natural environment. Their real-world performance may hinge on environmental variables, such as the sandy soil texture (79.4% sand) could exacerbate tebuthiuron leaching in field. Moreover, some parameters variation common in tropical soils (pH, moisture, and temperature) might disrupt microbial activity and enzyme stability [22,23]. Notably, soil composition (e.g., clay or organic matter content) can modulate herbicide sorption and persistence, potentially altering degradation rates [36–38].

The wide range of ambient temperatures found in tropical regions can affect the metabolic rates and enzymatic efficiency of inoculated strains. Hence, changes in soil moisture can alter oxygen availability and herbicide diffusion, especially in sandy soils with low water retention. Variations in soil pH may impact microbial survival and modify the ionization state of the herbicide, potentially reducing its accessibility for degradation. Furthermore, the sandy-loam texture increases the risk of herbicide leaching under rainfall or irrigation, which reduces the residence time of pesticide in the root zone and limits microbial exposure. Conversely, soils richer in clay or organic matter may enhance herbicide sorption, further restricting bioavailability.

Moreover, competition from native microbiota and potential nutrient limitations in non-amended crop soils can modulate the performance of inoculated strains. This underscores the importance of integrating biostimulation strategies, such as nutrient amendments, to enhance microbial resilience in situ.

These factors collectively underscore the need for future validation under non-idealized conditions, such as greenhouse and field trials, to assess the robustness, resilience, and biodegradation efficacy of these strains under more variable and realistic environmental conditions. These evaluations are fundamental for implying laboratory findings into effective, scalable, and environmentally safe bioremediation strategies suitable for dynamic tropical agroecosystems.

It is important to emphasize that this study was conceived as an exploratory and hypothesis-driven investigation aimed at evaluating the biodegradation potential of commercial microbial inoculants under controlled conditions, using microbial respiration as a sensitive metabolic indicator. Although chromatographic techniques are traditionally employed to quantify tebuthiuron residues and metabolites in soil, the choice not to apply such analyses in the present study was deliberate and methodologically justified. Respirometry provides an integrative and highly responsive measure of microbial activity, reflecting the mineralization and transformation processes occurring during herbicide biodegradation, as widely recognized in pesticide–soil interaction studies. In this context, cumulative CO_2 production was used as a functional proxy to compare the relative efficiency of different microbial strains in stimulating tebuthiuron degradation. The results obtained here establish a robust physiological and kinetic basis for subsequent, more targeted studies, in which chromatographic quantification of tebuthiuron and its metabolites will be incorporated to elucidate degradation pathways and mass balance under laboratory and field conditions.

CONCLUSION

The study demonstrated that bioaugmentation with selected microorganisms, particularly the bacterial strain CCT 8330 (M3), enhanced the biodegradation of tebuthiuron, as reflected by the high CO_2 production during phases of peak respiratory activity. Mathematical modeling confirmed the degradation efficiency, with logarithmic adjustments accurately describing the microbial growth phases. The Log phase (days 10–30) stood out as a period of intense metabolic activity. Additionally, the fungal strain (M5) showed significant performance, reinforcing the potential of microbial groups that remain underexplored in the bioremediation of herbicides.

Therefore, it can be concluded that bioaugmentation may serve as an effective strategy to reduce the persistence of tebuthiuron in the soil. The combination of adapted microorganisms, nutritional support, and suitable physicochemical conditions proved promising for the remediation of contaminated areas. However, field application requires further assessments, considering ecological interactions and potential effects on non-target organisms. The integration of techniques, such as biostimulation and phytoremediation may optimize the process, contributing to sustainable solutions for the recovery of agricultural soils impacted by pesticides.

FUTURE PERSPECTIVES

The inoculation of microorganisms for bioremediation of soils contaminated with tebuthiuron involve multiple and complementary research, development, and application pathways. A priority lies in validating the performance of strains M3 (*Pseudomonas nicosulfuronedens*, CCT 8330) and M5 (*Trichoderma yunnanense*, CCT 8328) under natural conditions, including greenhouse and field trials across diverse soil types, climatic regimes, and environmental contexts. Thus, it should explicitly account for environmental variability, which can modulate tebuthiuron sorption, leaching potential, and microbial accessibility.

Hence, future studies should explore integrative strategies such as biostimulation through targeted nutrient amendments, bioaugmentation with microbial consortia to harness synergistic degradation effects, and phytoremediation using plants that enhance tebuthiuron breakdown or uptake. The application of biochar can further improve water retention and contaminant bioavailability, while nanocatalysts and immobilized enzymes represent promising technologies for enhancing degradation in challenging soils.

Predictive modeling will also play a critical role. The integration of mathematical models with artificial intelligence and machine learning tools could improve the prediction of degradation kinetics under dynamic and complex environmental conditions. These approaches would facilitate the design of adaptive remediation protocols that anticipate seasonal and site-specific variability.

Advanced metagenomics, transcriptomics, and proteomics applied to strains M3 and M5 will also allow the identification of key genes, enzymes, and regulatory mechanisms involved in tebuthiuron degradation, supporting targeted genetic engineering to enhance biocatalytic efficiency.

Finally, any large-scale application must be accompanied by ecotoxicological monitoring to ensure environmental safety. Bioassays with different test-organisms are essential to evaluate the ecotoxicity of tebuthiuron metabolites and to verify the absence of secondary environmental risks. Extending these validated strategies to other persistent herbicides, and adapting microbial strains to extreme conditions, will substantially broaden the applicability of microbial bioremediation in sustainable agroecosystem management.

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