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Leguminous Plants and Microbial Inoculation: An Approach for Biocatalytic Phytoremediation of Tebuthiuron in Agricultural Soil

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Abstract: Herbicides are important for weed control but can severely impact ecosystems, causing soil and water contamination, biodiversity loss, and harm to non-target organisms. Tebuthiuron, widely used in sugarcane cultivation, is highly soluble and persistent, posing significant environmental risks. Microbial inoculation has emerged as a sustainable strategy to mitigate such damage. This study investigated the phytoremediation potential of *Mucuna pruriens* and *Canavalia ensiformis* in tebuthiuron-contaminated soils, enhanced by fungal and bacterial inoculants. *Crotalaria juncea* served as a bioindicator plant, and *Lactuca sativa* was used in ecotoxicological bioassays. During a 140-day greenhouse experiment from September 2021 to March 2022, *M. pruriens* showed faster growth than *C. ensiformis* in uncontaminated soils but was more affected by tebuthiuron. Bacterial inoculants improved *M. pruriens* growth under stress, while fungal inoculants mitigated tebuthiuron's effects on *C. ensiformis*. *C. juncea* exhibited high sensitivity to tebuthiuron but grew beyond 100 cm with bacterial inoculants. Ecotoxicological assays showed that bacterial bioaugmentation significantly reduced soil toxicity. Natural attenuation further decreased tebuthiuron toxicity, and prior cultivation of *M. pruriens* enhanced soil detoxification. This integrated approach combining phytoremediation and bioaugmentation offers a sustainable method to degrade tebuthiuron, foster safer agriculture, and reduce environmental and health risks.

Keywords: bioaugmentation; bioremediation; *Canavalia ensiformis*; *Crotalaria juncea*; ecotoxicity; herbicide degradation; *Lactuca sativa*; *Mucuna pruriens*; sustainable agriculture

Citation: Cruz, V.H.; Moreira, B.R.d.A.; Valério, T.S.; Frias, Y.A.; Silva, V.L.d.; Morais, E.B.d.; Vasconcelos, L.G.d.; Tropaldi, L.; Prado, E.P.; Montagnolli, R.N.; et al. Leguminous Plants and Microbial Inoculation: An Approach for Biocatalytic Phytoremediation of Tebuthiuron in Agricultural Soil. *Agronomy* **2024**, *14*, 2805. https://doi.org/10.3390/agronomy14122805

Academic Editor: Dong Liu

Received: 28 October 2024
Revised: 20 November 2024
Accepted: 21 November 2024
Published: 26 November 2024

1. Introduction

The herbicide tebuthiuron raises significant environmental concerns due to its high water solubility (2.5 g L^{-1} at 25 °C) [1], prolonged soil persistence (log K_ow = 1.8) [2], and potential to contaminate terrestrial and aquatic ecosystems [3]. Given the increasing evidence of tebuthiuron's detrimental environmental impacts, there is an urgent need for innovative and sustainable remediation strategies. Phytoremediation has emerged as a promising approach, utilizing plants to detoxify environments contaminated by various pollutants, including heavy metals, hydrocarbons, dyes, and pesticides [4].

Our study investigates the tolerance and phytoremediation potential of two leguminous species, *Mucuna pruriens* (L.) DC. var. *pruriens* and *Canavalia ensiformis* L., in combination with microbial inoculants. These annual plants not only have the potential to degrade tebuthiuron but also fix atmospheric nitrogen, enriching the soil and improving its physical, chemical, and biological properties [5]. Previous studies have indicated that these species exhibit resilience to tebuthiuron, making them promising candidates for remediating contaminated environments.

Several studies have evaluated the potential of leguminous species for phytoremediation of tebuthiuron-contaminated soils. For instance, Mendes et al. [6] assessed the use of *Crotalaria spectabilis*, *C. ensiformis*, *Stizolobium aterrimum*, and *Lupinus albus* in soils treated with quinclorac and tebuthiuron. They found that all four species absorbed more tebuthiuron than quinclorac, with *C. ensiformis* identified as the most efficient species for remediating tebuthiuron-contaminated soils. Ferreira et al. [7] further advanced the field by identifying suitable phytoremediator organisms for tebuthiuron in agricultural soils. Their experiments involved *Cajanus cajan*, *C. ensiformis*, *M. pruriens*, and *Pennisetum glaucum*, which successfully removed tebuthiuron applied at 2 $L \cdot ha^{-1}$, enabling subsequent growth of *Crotalaria juncea* and *Lactuca sativa* in the presence of residual herbicide. They also highlighted the potential of repurposing vinasse as a source of organic carbon to enhance plant development and improve the ecological viability of phytoremediation.

Contrastingly, Frias et al. [8] investigated the efficacy of *M. pruriens* as a phytoremediator in soil supplemented with vinasse and found it ineffective at removing tebuthiuron. *M. pruriens* was exposed to tebuthiuron at 0.5, 1, 1.5, and 2 $L \cdot ha^{-1}$ and vinasse at 75, 150, and 300 $m^3 \cdot ha^{-1}$. The herbicide caused phytotoxicity, severely inhibiting germination and growth. The addition of vinasse exacerbated damage to both photosynthetic and non-photosynthetic structures, reducing biomass production. Consequently, neither *C. juncea* nor *L. sativa* could grow in the presence of residual pesticide.

Despite its promise, phytoremediation faces challenges, as its success depends on factors such as soil characteristics, climate, and co-contaminants [9]. To optimize phytoremediation effectiveness in addressing tebuthiuron contamination, a comprehensive understanding of the interactions between selected plant species and the herbicide is essential [7,8]. To enhance remediation efficiency, we incorporate bioaugmentation as a pivotal strategy. Bioaugmentation involves introducing microorganisms into a contaminated environment to accelerate pollutant degradation [10]. By introducing selected microbial strains with high pesticide degradation capabilities, resilience, and adaptability [11], we establish a symbiotic alliance that expedites pesticide degradation in soil. The integration of phytoremediation and bioaugmentation offers a powerful synergy [9], significantly improving tebuthiuron degradation efficiency and overall remediation outcomes.

Our approach emphasizes safety and efficacy by evaluating the environmental toxicity levels of tebuthiuron during the remediation process. We designed tests to realistically predict the behavior of substances in the environment. Specifically, we employed *C. juncea* as a bioindicator species sensitive to tebuthiuron after prior cultivation of *M. pruriens* and *C. ensiformis*. Additionally, *L. sativa* was used in ecotoxicological assays to assess residual toxicity. These complementary experiments aimed to verify the presence of tebuthiuron in the soil [7–16].

Therefore, this research aims to investigate the tolerance and phytoremediation potential of *M. pruriens* and *C. ensiformis*, in conjunction with microbial inoculants, for the remediation of tebuthiuron-contaminated agricultural soil. Our findings will advance our understanding of the viability and effectiveness of these techniques in addressing tebuthiuron contamination and the associated ecological concerns. Innovative and sustainable approaches are crucial for the successful remediation of tebuthiuron-contaminated soils.

2. Materials and Methods

2.1. Soil, Tebuthiuron, and Microbial Inoculant

The soil used in this study was classified as a Dystrophic Red-Yellow Oxisol. This soil was sourced from an agricultural facility in the Dracena region with no recent history of phytosanitary treatments, ensuring minimal prior contamination. Upon acquisition, the soil was transported to a greenhouse, air-dried, sieved through a 2.0 mm mesh, and stored in hermetically sealed plastic containers for chemical characterization. Soil samples were collected both before and after the experiment to determine chemical composition (Table 1). For each analysis period, data from all treatments were averaged to present the overall soil properties. Additional soil samples were collected at the end of the experimental period for further chemical analysis.

Table 1. Soil chemical analysis at 0 and 70 DAS of *C. ensiformis* and *M. pruriens*.

Attributes	Unit	0 DAS	70 DAS	Indication
pH (H_2O)	-	4.0	7.5	Increased
Organic matter	g dm^{-3}	4.0	10	Increased
Potassium	mmol dm^{-3}	0.3	1.6	Increased
Calcium	mmol dm^{-3}	6	51	Increased
Magnesium	mmol dm^{-3}	2	23	Increased
Hydrogen + Aluminum	mmol dm^{-3}	33	8	Decreased
$Aluminum^{3+}$	mmol dm^{-3}	13	0	Decreased
Phosphor	mg dm^{-3}	1	6	Increased
Sulfur	mg dm^{-3}	7	-	Not detected
Boron	mg dm^{-3}	0.10	0.02	Decreased
Copper	mg dm^{-3}	0.1	0.2	Increased
Iron	mg dm^{-3}	4	2	Decreased
Manganese	mg dm^{-3}	1.8	1.2	Increased
Zinc	mg dm^{-3}	0.1	0.3	Increased
Sum of bases	mg dm^{-3}	8	75.6	Increased
Cation exchange capacity	mg dm^{-3}	41	83.6	Increased
Base saturation	%	20	90	Increased
Aluminum saturation	%	61	0	Decreased

Increased—the value in the soil was higher in 70 DAS (day after the sowing) than the initial time (0 DAS); Decreased—the value in the soil was lower in 70 DAS than the initial time (0 DAS); Not detected—there was no comparison between 0 and 70 DAS due to the minimum concentration of the parameter not detected by the analysis.

The herbicide used was Combine® 500SC (Batch: 041-14-2000) from Dow AgroSciences Industrial Ltda. (São Paulo, Brazil), a commercially available formulation of tebuthiuron (TBT).

To augment the soil microbial community and enhance phytoremediation, microbial inoculants were obtained from Microgreen® Ltda. (http://microgreen.agr.br/, Piracicaba, Brazil), a company specializing in soil microbial reclamation. Two types of inoculants were utilized: a bacterial inoculant (BACT) rich in actinomycetes, *Bacillus* spp., and lactic acid bacteria and a fungal inoculant (FUNG) containing *Trichoderma* spp., *Purpureocillium* spp., and *Beauveria* spp., whose application is for the restoration of microbiota in agricultural soils.

2.2. Plant Species: Phytoremediator Species, Indicator Plant, and Test Organism

Mucuna pruriens (MP) and *Canavalia ensiformis* (CE) were selected for their well-documented phytoremediation capabilities, especially in soils contaminated with tebuthiuron [6–8]. These leguminous species form symbiotic relationships with nitrogen-fixing bacteria, enhancing nutrient availability and improving soil fertility.

Sunn hemp (*Crotalaria juncea*) was chosen as a bioindicator plant due to its known sensitivity to tebuthiuron [15]. Acting as a sentinel species, *C. juncea* aids in assessing soil contamination levels and the efficacy of phytoremediation efforts. Seeds of *C. juncea*, *C.*

ensiformis, and *M. pruriens* were obtained from BR SEEDS® (Araçatuba, Brazil), ensuring uniformity and reliability of the experimental material.

For ecotoxicological bioassays, commercially available seeds of *Lactuca sativa* L. (variety Butterhead) were procured from Feltrin Sementes® (Caxias do Sul, Brazil).

2.3. Experimental Setup

The experiment was conducted using a completely randomized design with a 2 × 3 × 3 factorial scheme, comprising seven replicates and five analysis times, totaling 630 pots. The factors studied were tebuthiuron concentration (presence or absence), microbial inoculant type (bacterial, fungal, or none), and plant species (*C. ensiformis*, *M. pruriens*, or none). This comprehensive design allowed for a thorough investigation of the independent and combined effects of these variables on the study parameters (Figure 1). Randomization minimized bias and ensured equal representation of treatment groups across experimental units.

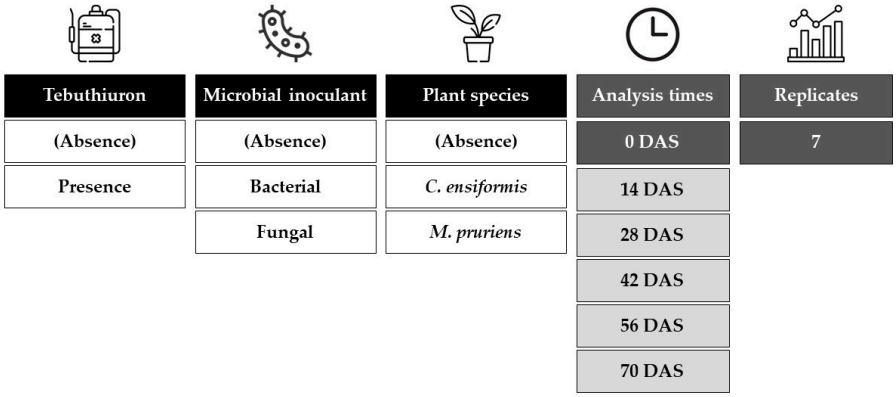

Figure 1. Summary of the experimental design with the three factors (tebuthiuron, microbial inoculant, and plant species), the five analysis times (0, 14, 28, 42, 56, and 70 DAS—days after sowing), and the number of replicates.

Prior to the experiment, the soil underwent a preparatory phase to adjust its acidity and fertility, following the procedures of Ferreira et al. [7] and Frias et al. [8]. For every 504 kg of soil, amendments were meticulously applied as follows: 454 g (1.8 t ha^{-1}) of limestone to regulate pH, 10 g (40 kg ha^{-1}) of urea as a nitrogen source, 56 g (222 kg ha^{-1}) of single superphosphate for phosphorus supplementation, and 13 g (52 kg ha^{-1}) of potassium chloride to ensure adequate potassium levels for optimal plant growth. After thorough mixing through uniform distribution, the soil was used to fill pots with a capacity of approximately 4.0 L each (19 cm × 15 cm × 19 cm).

The microbial inoculants were incorporated into the soil according to their respective treatment groups. The fungal inoculant (FUNG) was applied at a rate of 0.36 g (180 t ha^{-1}) per pot, while the bacterial inoculant (BACT) was added at a volume of 50 mL (25 m^3 ha^{-1}) per pot. Three days after inoculant incorporation, the herbicide Combine® was applied at the recommended dosage for sandy soils of 2.0 L ha^{-1} (1000 g active ingredient ha^{-1}). The application was performed using a laboratory sprayer equipped with four XR 11002 flat-fan nozzles (Jacto®, Pompéia, Brazil), operating at a pressure of 2 bar and a flow rate of 0.65 L· min^{-1}. Following the manufacturer's guidelines, the sprayer was calibrated to a speed of 5 km·h^{-1}, with the spray boom positioned 0.75 m above the pots, delivering an application volume of 156 L ha^{-1}. Environmental conditions during spraying were monitored, with a temperature of 27.2 °C and relative humidity of 63%. The control treatments without tebuthiuron received an equivalent volume of water to maintain consistency.

Seven days following herbicide application, three seeds of either *C. ensiformis* or *M. pruriens* were sown in each pot, according to the treatment design. Although three seeds were initially sown per pot, thinning was performed to retain only one plant per pot to

ensure uniform growth conditions. These plants were cultivated for 70 days after sowing (DAS). Three days after harvesting the leguminous plants, three seeds of sunn hemp (*C. juncea*) were sown in each pot and cultivated for an additional 70 days, following the same thinning procedure.

All plants were cultivated in a greenhouse equipped with an automated irrigation system to maintain optimal growth conditions. Irrigation was performed daily to maintain the soil at 60% of its field capacity. The system was programmed via a digital timer to execute up to four irrigation cycles per day, each lasting approximately 40 ± 10 min. A micro-sprinkler located at the top of the greenhouse provided irrigation at a flow rate of approximately 80 L h^{-1} under a pressure of 2 bar, as indicated by the irrigation manometer. This controlled environment facilitated the investigation of the tolerance and phytoremediation capabilities of *C. ensiformis* and *M. pruriens*, as well as the influence of microbial inoculants in tebuthiuron-contaminated soil.

2.4. Plant Growth and Development Evaluation

Plant growth was monitored weekly by recording plant height in centimeters. At 70 DAS, the plants were harvested, the roots were gently cleaned to remove soil, and the samples were dried in a forced-air oven at 65 °C for 72 h. Dry biomass was then weighed and recorded in grams.

2.5. Ecotoxicological Bioassays

The ecotoxicological potential of the treatments was assessed at specific time points: 0, 20, 40, 60, 70 DAS (end of *M. pruriens* and *C. ensiformis* cultivation), and 140 DAS (end of *C. juncea* cultivation). Bioassays were conducted following the methodologies described in NBR 10006 [16] and Sobrero and Ronco [17].

For each treatment, five replicates of aqueous soil extracts were prepared. Superficial soil samples (approximately 2 cm deep) were collected from the edges of different pots within each treatment group. A 25 g sample of soil was mixed with 100 mL of deionized water in a 250 mL Erlenmeyer flask. The flasks were sealed with PVC plastic film, shaken at 120 rpm for five minutes, and then incubated in a Biochemical Oxygen Demand (BOD) chamber without lighting at 20 ± 2 °C for 7 days.

After incubation, 2.0 mL of the solubilized soil extract was applied to Petri dishes lined with filter paper and containing 10 seeds of *L. sativa*. The dishes were sealed with PVC plastic film to prevent moisture loss and incubated in a BOD chamber at 20 ± 2 °C with a 12 h photoperiod for 5 days. The positive control (CP) and negative control (CN) treatments were prepared using 0.05 M zinc sulfate solution and deionized water, respectively, to test seed sensitivity.

During the bioassays, seed germination, hypocotyl elongation, and root elongation were measured. These parameters were used to calculate the germination index (GI) of *L. sativa* seeds, as described in Equation (1) [18]:

$$GI = \frac{(G\% \times R\%)}{100} \quad (1)$$

This equation includes the following:
GI represents the germination index;
G% denotes the percentage of seed germination;
R% indicates the percentage of root elongation.

2.6. Statistical Data Analysis

Prior to conducting statistical analyses, the data were evaluated to ensure compliance with the assumptions of homogeneity of variances and normality. Homoscedasticity was assessed using Bartlett's test, while the Shapiro–Wilk test was employed to verify the normal distribution of residuals. Once these assumptions were confirmed, an Analysis of Variance (ANOVA) was performed at a 5% significance level ($p < 0.05$) to identify

significant differences among treatment groups. When the ANOVA indicated significant effects, Tukey's Honest Significant Difference (HSD) post hoc test was utilized for pairwise comparisons to determine which specific means differed.

To model the growth dynamics of plant height and the germination index over time, we applied the Gompertz sigmoid function (Equation (2)). This nonlinear model is particularly suitable for describing sigmoidal growth patterns and has been widely used in ecological and biological studies due to its flexibility and interpretability [12,19]. The Gompertz function allowed us to estimate key growth parameters, such as the maximum attainable value (asymptote), growth rate constant, and inflection point, providing insights into the developmental processes under different treatment conditions. By fitting the Gompertz model to our data, we could predict growth trends beyond the observed time frames of 70 and 140 days after sowing (DAS), offering a more comprehensive understanding of the long-term effects of the treatments.

$$f_x = \alpha e^{-\beta e^{-kx}} \quad (2)$$

This equation includes the following:

fx represents the plant height in centimeters or the germination index;

x denotes the time in days after sowing (DAS);

α represents the upper asymptote or the maximum height development and germination index that the plants can reach;

β indicates the inflection point, which corresponds to the time when the growth rate starts to decrease;

k represents the exponential decay of the specific growth rate, indicating how quickly the growth rate decreases over time;

e represents Euler's constant, a mathematical constant approximately equal to 2.71828.

$$\gamma = \alpha + \beta + \sum((t_i \times f_{i1}) + (t_i \times f_{i2})) + \varepsilon \quad (3)$$

This equation includes the following:

γ represents the dependent variable (e.g., plant height or germination index);

α denotes the global intercept of the model;

β represents the random effect (tebuthiuron);

ti indicates the time in days after sowing (DAS);

f_{i1} represents the fixed effect 1 (green manure);

f_{i2} represents the fixed effect 2 (inoculant);

ε represents the residual term.

In cases where time was a significant variable, we extended the model to include time as a continuous covariate or as repeated measures, depending on the data structure. This allowed us to capture temporal trends and assess how the treatments influenced growth trajectories over time.

All statistical analyses were conducted using R software 4.2.2. for its advanced statistical capabilities and flexibility. The nlme package was utilized for fitting mixed-effects models (Equation (3)), while the nls function facilitated nonlinear regression for the Gompertz function. GraphPad Prism 9 and Microsoft Excel® 2019 were employed for data visualization, preliminary analyses, and to generate graphical representations of the results. These tools collectively enabled efficient data management, rigorous statistical testing, and clear presentation of findings.

2.6.1. Model Validation and Goodness-of-Fit and Consideration of Multiple Comparisons

Model adequacy was evaluated through diagnostic plots and goodness-of-fit statistics. For the Gompertz function, the coefficient of determination (R^2) and residual analysis were used to assess how well the model described the observed data. The Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) were also considered for model selection and comparison. In the mixed-effects models, the significance of fixed effects

was tested using likelihood ratio tests, and random effects were assessed through variance component analysis. Residuals were examined for homoscedasticity and normality to validate model assumptions. Given the multiple treatment groups and comparisons, we employed the Tukey–Kramer method in the post hoc analysis to control for Type I error rates associated with multiple testing. This approach ensures that the overall family wise error rate remains at the desired significance level, enhancing the reliability of the statistical conclusions.

2.6.2. Statistical Significance and Reporting

All statistical findings were interpreted in the context of the biological and ecological implications for phytoremediation practices; therefore, the results were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Soil's Properties: Enhancing Fertility

As discussed in the preceding section, the soil used in this study was classified as a Dystrophic Red-Yellow Oxisol, a common soil type in tropical regions characterized by low natural fertility, acidic pH, and high levels of iron and aluminum oxides, which can influence nutrient and contaminant retention [20]. These properties make it an ideal substrate for simulating real-world scenarios of pesticide-contaminated agricultural soils.

Therefore, according to Table 1, significant improvements in the soil's chemical attributes were observed following amendments with lime and fertilizers, as well as the cultivation of *M. pruriens* and *C. ensiformis*. Various nutritional parameters increased numerically, indicating enhanced soil fertility. Notably, the concentration of exchangeable aluminum (Al^{3+}) did not increase, which is advantageous since high levels of aluminum can inhibit root growth and impair plant development [21]. The application of lime and fertilizers contributed to balancing soil pH and improving nutrient availability, thereby enhancing overall soil conditions.

Implementing appropriate agricultural practices, such as soil chemical correction and the cultivation of leguminous plants, can have multiple positive effects on soil health. Additionally, the presence of plants and soil microorganisms, combined with soil amendments, can lead to the release of organic acids. These organic acids aid in the solubilization of essential nutrients like phosphorus and potassium, making them more available for plant uptake. Furthermore, they contribute to an increase in the soil's cation exchange capacity, which helps reduce toxic levels of aluminum, ultimately benefiting plant growth and development [22].

Enhancing soil fertility through these practices creates a more conducive environment for plant growth, which is essential for successful phytoremediation efforts. Improved soil conditions can enhance plant vigor, allowing for more effective uptake and degradation of contaminants such as tebuthiuron.

3.2. Production of Leguminous Plants with Microbial Inoculation: Unveiling the Biocatalytic Phytoremediation

3.2.1. Growth Dynamics

The growth dynamics of *C. ensiformis* and *M. pruriens* exhibited distinct patterns (Figure 2). *M. pruriens* demonstrated a faster growth rate, indicated by a steeper growth curve compared to *C. ensiformis*. Table 2 presents the parameters of the growth curves for each treatment, including the maximum height (α), the inflection point (β), and the specific growth rate (k). A higher k value signifies a faster growth rate; both *M. pruriens* (k = 0.113) and *C. ensiformis* (k = 0.075) showed considerable growth rates.

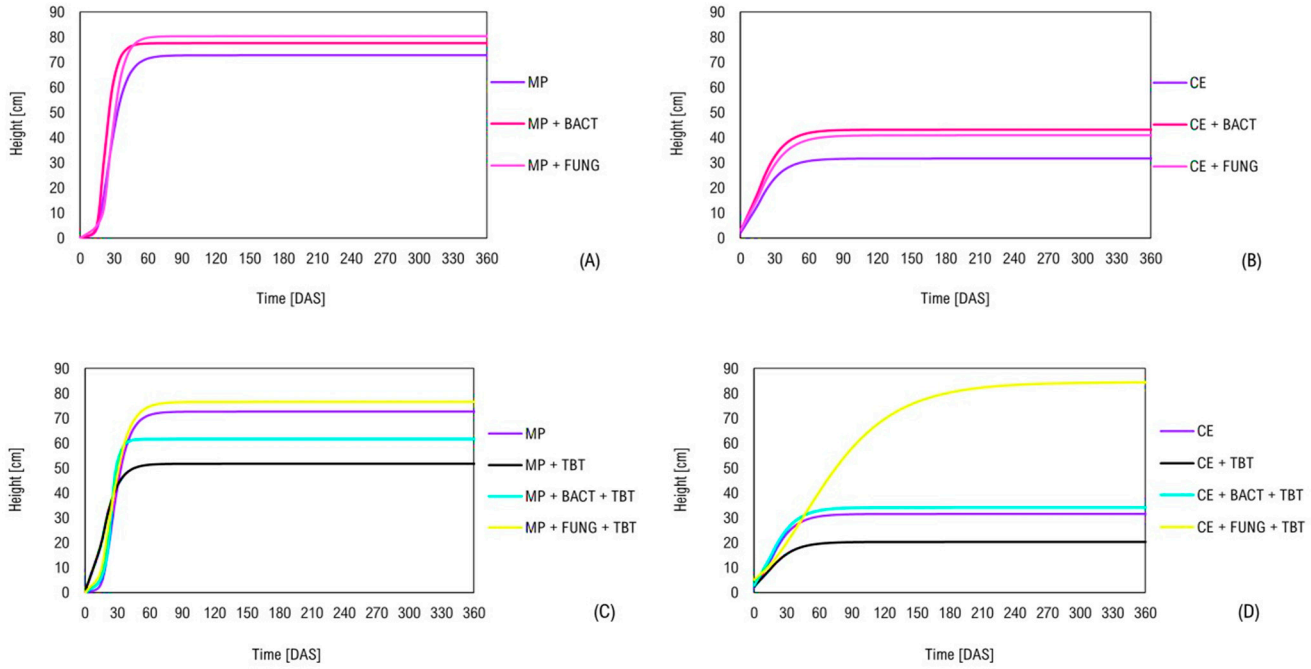

Figure 2. Kinetic growth of *M. pruriens* (MP) and *C. ensiformis* (CE) as potential phytoremediators of the herbicide tebuthiuron (TBT) in soil with fungal (FUNG) or bacterial (BACT) inoculants from the Gompertz model. (**A**,**B**) Treatments without TBT. (**C**,**D**) Treatments with TBT.

Table 2. Parameters of Gompertz kinetic models for the height of *M. pruriens* (MP) and *C. ensiformis* (CE) as potential phytoremediators of tebuthiuron (TBT) with fungal (FUNG) or bacterial (BACT) inoculants.

Treatments	Complexity			Adequacy		
	α	β	k	R^2	AIC	BIC
MP	72.72	15.49	0.1127	0.72	63.96	65.17
MP + TBT	51.69	4.76	0.1087	0.94	63.96	65.17
MP + BACT	77.66	32.71	0.1695	0.97	69.10	70.31
MP + FUNG	80.31	38.14	0.1425	0.99	56.33	57.54
MP + BACT + TBT	61.58	144.19	0.2272	0.89	78.80	80.01
MP + FUNG + TBT	76.63	9.15	0.0997	0.93	75.82	77.04
CE	31.64	2.72	0.0748	0.97	46.29	47.50
CE + TBT	20.27	2.13	0.0686	0.93	44.71	45.92
CE + BACT	43.10	2.73	0.0751	0.97	53.67	54.88
CE + FUNG	40.90	2.62	0.0679	0.97	50.41	51.62
CE + BACT + TBT	34.26	2.51	0.0707	0.96	49.03	50.24
CE + FUNG + TBT	84.52	2.81	0.0224	0.94	60.08	61.29

Parameters α, β, and k denote the superior asymmetry, the inflection point, and the exponential decay of the specific growth rate, respectively; β = 1 keeps the relative decrease with time constant; β > 1 accelerates the relative decrease with time; β < 1 slows the relative decrease with time; R^2: coefficient of determination; AIC: Akaike Information Criterion; BIC: Bayesian Information Criterion.

The application of microbial inoculants influenced the growth dynamics of both species. Within the *M. pruriens* group, the fungal inoculant treatment (MP + FUNG) resulted in faster growth compared to the bacterial inoculant (MP + BACT) and the control (MP alone). Similarly, for *C. ensiformis*, the bacterial inoculant (CE + BACT) promoted faster growth than the fungal inoculant (CE + FUNG) and the control (CE alone). These results suggest that the type of microbial inoculant can differentially affect plant growth, potentially due to specific interactions between the inoculant microorganisms and the plant species.

The introduction of tebuthiuron had a noticeable impact on plant growth, reducing plant height compared to the control treatments without the herbicide. As shown in Table 2, the CE + TBT treatment exhibited a greater reduction in height-related parameters, including maximum height (α) and specific growth rate (k), compared to MP + TBT. This indicates that *C. ensiformis* is more sensitive to tebuthiuron than *M. pruriens*. Previous studies by Ferreira et al. [7] and Belo et al. [23] also reported the sensitivity of *C. ensiformis* to tebuthiuron, suggesting that this species may not be ideal for the phytoremediation of soils contaminated with this herbicide.

Conversely, Mendes et al. [6] demonstrated that *C. ensiformis* can tolerate and even degrade tebuthiuron, potentially due to specific microorganisms present in its rhizosphere. This highlights the importance of considering plant–microbe interactions when selecting species for phytoremediation. For *M. pruriens*, Ferreira et al. [7] observed a 15% decrease in plant height when exposed to tebuthiuron. However, they found that the phytotoxicity of tebuthiuron was mitigated by the addition of vinasse (an industrial by-product) at 150 m^3 ha^{-1}, allowing the plant to develop even at high herbicide concentrations (2 L ha^{-1}).

Discrepancies between our results and previous studies may be attributed to factors such as differences in experimental conditions, soil types, moisture levels, environmental temperatures, and light exposure. These variables can directly influence phytoremediation efficiency and should be carefully considered when selecting plants for remediating tebuthiuron-contaminated soils [24].

An important finding in our study was the mitigating effect of microbial inoculants on plant sensitivity to tebuthiuron in contaminated soil. The inoculants appeared to enhance plant growth and increase height, particularly in *C. ensiformis*. For instance, the CE + BACT + TBT treatment showed a higher specific growth rate (k = 0.071) compared to CE + TBT without inoculants, although only the CE + FUNG + TBT treatment achieved the maximum height (α = 84.52). This suggests that fungal inoculants may be more effective in promoting growth under herbicide stress in *C. ensiformis*.

In *M. pruriens*, the MP + FUNG + TBT treatment displayed a steeper growth curve than MP + BACT + TBT, reaching a greater maximum height (α = 76.63). Although the bacterial inoculant (MP + BACT + TBT) exhibited a higher specific growth rate (k = 0.227 vs. k = 0.100), the overall growth performance was better with the fungal inoculant. These results could be explained by several factors: (a) Fungal inoculants may enhance plant resilience to herbicide-induced stress by improving nutrient uptake or producing growth-promoting substances, allowing plants to maintain or increase growth under adverse conditions. (b) The fungal inoculant may have the ability to degrade or metabolize tebuthiuron, reducing its toxicity in the soil environment. (c) The inoculants may stimulate beneficial soil microorganisms, creating a more favorable rhizosphere environment for plant growth.

Previous studies have demonstrated the effectiveness of such microbial combinations. Zhang et al. [25] reported successful remediation of soils contaminated with pentachloronitrobenzene using a fungal–bacterial inoculum in association with *Panax notoginseng*. Similarly, Madariaga-Navarrete et al. [26] observed substantial atrazine removal from soil within 40 days using *Trichoderma* sp. combined with *Phaseolus vulgaris*. These findings support the potential of microbial inoculants in enhancing phytoremediation efficiency.

3.2.2. Phytomass Accumulation

The application of microbial inoculants in the absence of tebuthiuron led to a significant increase in biomass production in both *M. pruriens* and *C. ensiformis* compared to the control treatments (Figure 3). This positive impact underscores the efficacy of microbial inoculants in promoting plant growth and enhancing phytomass accumulation, which is pivotal for the plants' capacity to tolerate and effectively remediate contaminated soils.

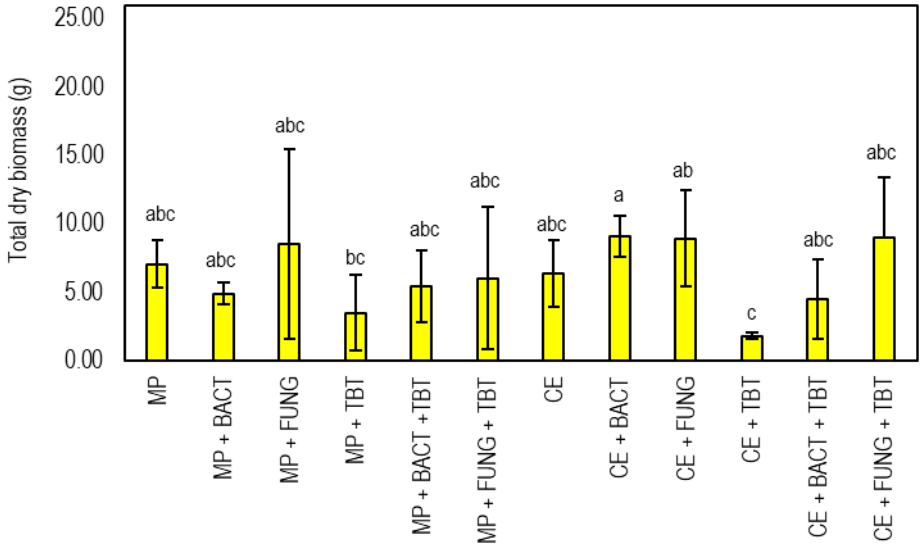

Figure 3. Production of total dry biomass of *M. pruriens* (MP) and *C. ensiformis* (CE) in soil associated or not with tebuthiuron (TBT) and/or fungal (FUNG) or bacterial (BACT) inoculants after 70 DAS. Different lowercase letters indicate statistical differences by the Tukey test ($p < 0.05$).

In contrast, the introduction of tebuthiuron had an adverse effect on biomass production in both species. The most pronounced reduction was observed in the MP + TBT treatment, indicating that *M. pruriens* was particularly affected by the herbicide. Tebuthiuron's negative influence on photosynthesis during plant development can impair biomass production and compromise the overall efficiency of phytoremediation processes [27], even though *M. pruriens* and *C. ensiformis* are not target plants for this herbicide in agroecosystems. Efficient biomass production is crucial for facilitating the transformation of pollutants into less toxic substances, a process optimized when plants grow without intense stress [28].

Further examination revealed that, in the presence of tebuthiuron, the bacterial inoculant did not significantly improve biomass production in *M. pruriens*. For *C. ensiformis*, microbial inoculation did not result in a substantial increase in phytomass when tebuthiuron was present, despite contributing to increased plant height. These observations emphasize the limitations of relying solely on variables like height and biomass accumulation to evaluate phytoremediation efficiency.

Moreover, the rhizospheric interactions between plants and microorganisms play a key role in the degradation of soil contaminants. Root exudates from leguminous plants like *M. pruriens* and *C. ensiformis* can enhance microbial activity by providing essential nutrients and signaling molecules that stimulate the growth and metabolic functions of degradative microbes [29]. These exudates may increase the bioavailability of tebuthiuron by altering soil pH and releasing chelating agents, thereby facilitating its uptake and degradation. Understanding the synergistic relationships within the rhizosphere is crucial, as it can lead to optimized phytoremediation strategies that harness both plant and microbial capabilities for more efficient contaminant removal.

Multiple uncontrolled factors can influence the bioavailability and environmental behavior of the herbicide, including soil properties, microbial community dynamics, and environmental conditions [30]. Therefore, relying exclusively on plant growth parameters may not provide a comprehensive assessment of phytoremediation effectiveness. Complementary approaches, such as cultivating bioindicator plants and implementing ecotoxicological bioassays, are indispensable for thoroughly evaluating environmental reclamation efforts [31].

In summary, our findings highlight the critical aspect of biomass accumulation in the context of phytoremediation. While microbial inoculants positively influenced biomass production in the absence of tebuthiuron, the presence of the herbicide negated these benefits.

This underscores the significance of considering multiple parameters and employing complementary assessment methods to accurately evaluate the efficiency of phytoremediation practices, thereby facilitating sustainable and effective soil remediation strategies.

3.3. Production of C. juncea: Evaluating Ecotoxicity and Phytoremediation Efficiency

3.3.1. Growth Dynamics

The cultivation of *C. juncea* as a bioindicator provided valuable insights into the residual phytotoxicity of tebuthiuron and the effectiveness of the phytoremediation treatments. In the reference treatment (Ref), which involved soil without prior plant cultivation (*CE* or *MP*), no inoculants (BACT or FUNG), and no herbicide (TBT), *C. juncea* exhibited slow height development (Figure 4). This baseline serves as a control for comparing the effects of various treatments.

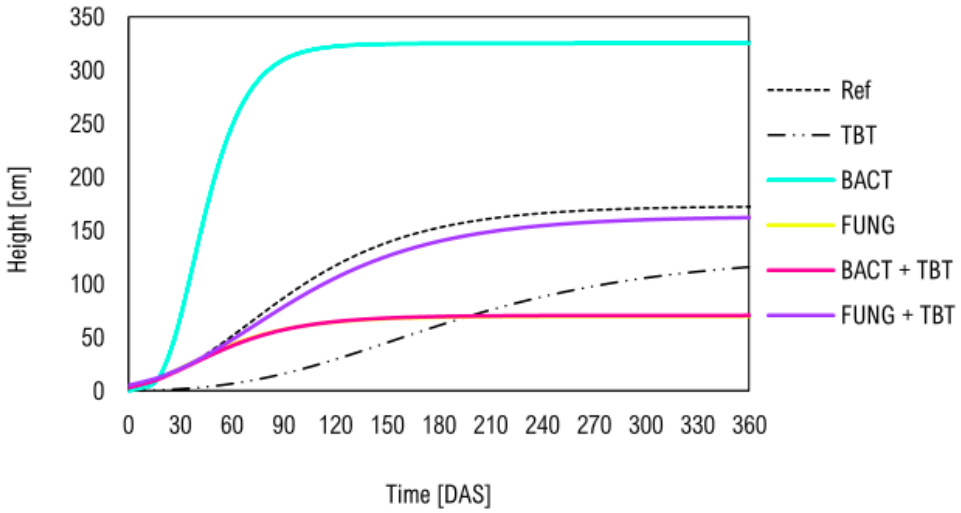

Figure 4. Kinetic growth of *C. juncea* height as bioindicator species in soil with tebuthiuron (TBT) and fungal (FUNG) or bacterial (BACT) inoculants from the Gompertz model. Ref—reference control soil without leguminous plants, inoculants, and tebuthiuron.

Interestingly, soil treated with the bacterial inoculant (BACT) without prior leguminous cultivation or herbicide application showed a faster increase in *C. juncea* height, with a higher specific growth rate (k = 0.0582) and greater maximum height (α = 324.99) around 60 DAS (Table 3). In contrast, the fungal inoculant (FUNG) had a minimal contribution to *C. juncea*'s height development, indicated by a lower growth rate (k = 0.0302) and lower maximum height (α = 69.95). This suggests that the bacterial inoculant may promote the growth of *C. juncea* in uncontaminated soils more effectively than the fungal inoculant.

The presence of tebuthiuron in the soil demonstrated a significant phytotoxic effect on *C. juncea*, evidenced by a lower specific growth rate (k = 0.0115) (Table 3 and Figure 5). Despite the slower growth rate, *C. juncea* in soil with tebuthiuron alone (without inoculants or prior cultivation) reached a maximum height exceeding 100 cm, which was higher than in treatments with FUNG or BACT + TBT. This indicates that while tebuthiuron adversely affects growth, *C. juncea* can still attain considerable height in its presence.

Remarkably, the phytotoxic effect of tebuthiuron was mitigated by the fungal inoculant in certain treatments, suggesting the involvement of microorganisms in bioremediation. Previous studies have associated microbial genera such as *Methylobacterium*, *Microbacterium*, *Paenibacillus*, and *Streptomyces* with tebuthiuron degradation [32,33]. The long-term presence of the fungal inoculant may contribute to the dissipation of tebuthiuron, reducing its toxicity to subsequent plantings.

Table 3. Parameters of the Gompertz kinetic models for the height of *C. juncea* as a bioindicator species in tebuthiuron (TBT) soil with *M. pruriens* (MP) and *C. ensiformis* (CE) and bacterial (BACT) or fungal (FUNG) inoculants. Ref—reference control soil without leguminous plants, inoculants, and tebuthiuron.

Treatments	Complexity			Adequacy		
	α	β	k	R^2	AIC	BIC
MP	156.35	5.94	0.01061	0.98	49.51	50.30
MP + TBT	86.91	3.29	0.03295	0.98	45.17	45.96
MP + BACT	181.96	3.76	0.01741	0.98	45.39	46.18
MP + FUNG	42.01	3.23	0.05290	0.98	41.15	41.94
MP + BACT + TBT	147.18	3.75	0.02018	0.99	41.67	42.46
MP + FUNG + TBT	248.45	8.52	0.06591	0.98	54.98	55.77
CE	178.13	3.82	0.01486	0.96	47.42	48.20
CE + TBT	115.67	3.34	0.02142	0.98	46.18	45.39
CE + BACT	78.74	3.00	0.02500	0.97	45.81	45.02
CE + FUNG	66.01	2.91	0.02667	0.96	47.20	47.98
CE + BACT + TBT	115.76	5.60	0.09303	0.97	47.70	48.49
CE + FUNG + TBT	199.88	4.11	0.01602	0.98	43.88	44.67
Ref	172.93	3.78	0.01897	0.98	44.72	45.51
TBT	324.99	8.91	0.05819	0.98	51.98	52.77
BACT	69.95	2.95	0.03017	0.97	48.00	47.21
FUNG	127.47	5.76	0.01148	0.99	44.82	45.61
BACT + TBT	70.50	3.08	0.02991	0.98	42.20	42.99
FUNG + TBT	163.15	3.47	0.01731	0.97	50.26	51.05

Parameters α, β, and k denote the superior asymmetry, the inflection point, and the exponential decay of the specific growth rate, respectively; β = 1 keeps the relative decrease with time constant; β > 1 accelerates the relative decrease with time; β < 1 slows the relative decrease with time; R^2: coefficient of determination; AIC: Akaike Information Criterion; BIC: Bayesian Information Criterion.

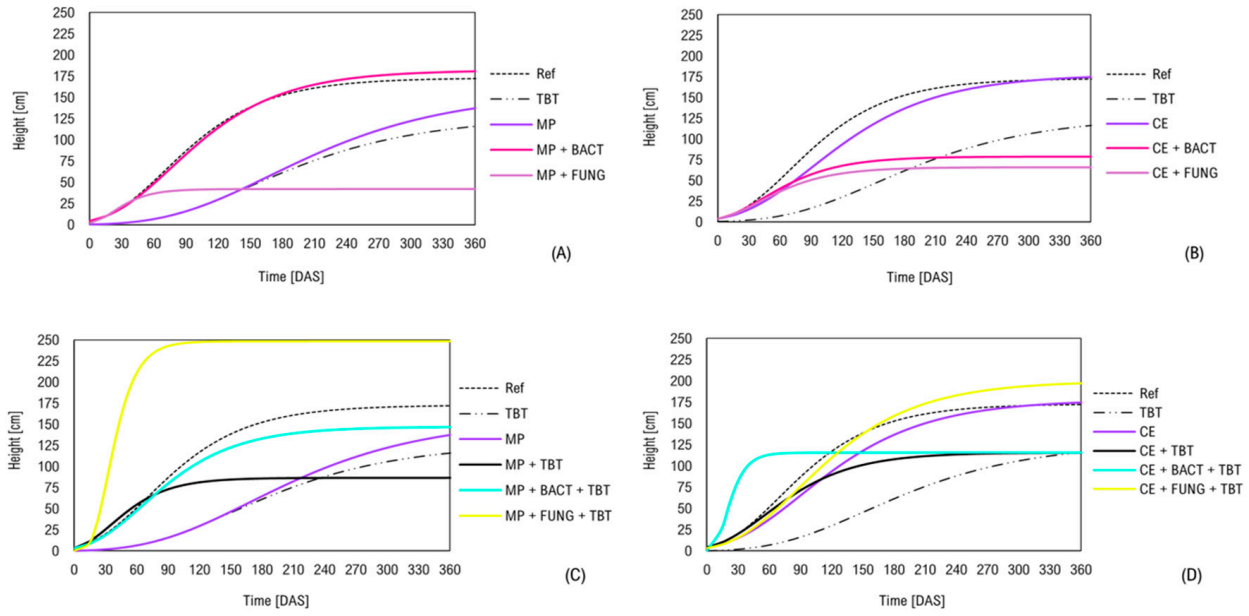

Figure 5. Kinetic growth of *C. juncea* height as bioindicator species in tebuthiuron (TBT) soil with *M. pruriens* (MP) and *C. ensiformis* (CE) and fungal (FUNG) or bacterial (BACT) inoculants. Ref—reference control soil without leguminous plants, inoculants, and tebuthiuron. (**A**,**B**) Treatments without TBT. (**C**,**D**) Treatments with TBT.

Prior cultivation of *M. pruriens* and *C. ensiformis* significantly influenced the growth of *C. juncea*. Soil previously cultivated with *C. ensiformis* had a more positive effect on *C. juncea*'s height compared to soil with *M. pruriens*. As shown in Table 3, the parameters

α and k for *C. ensiformis* were greater than those for *M. pruriens* (α = 156.35 vs. 78.13; k = 0.0149 vs. 0.0106). While *M. pruriens* is beneficial for soil health due to nitrogen fixation and nutrient cycling, it may exhibit allelopathic effects that inhibit the growth of nearby plants through the production of bioactive compounds [34].

Interactions between the green manure species and microbial inoculants yielded distinct results in *C. juncea*'s growth. For instance, the negative effect of *M. pruriens* on *C. juncea* was alleviated when associated with the bacterial inoculant (MP + BACT), resulting in a higher specific growth rate (k = 0.0174) and maximum height (α = 181.96 cm) compared to MP alone. Conversely, the association of *C. ensiformis* with the bacterial inoculant (CE + BACT) had an antagonistic effect on *C. juncea*'s growth, yielding a smaller growth curve compared to soil with *CE* alone. Similarly, the association of fungal inoculants with either phytoremediation species was generally detrimental to *C. juncea*, particularly in the MP + FUNG treatment, where the maximum height was the lowest (α = 42.01 cm), despite a higher growth rate (k = 0.0529).

When analyzing treatments involving tebuthiuron and prior cultivation with *M. pruriens* or *C. ensiformis*, high phytotoxicity and severe limitations in *C. juncea* height were observed compared to the control treatments without the herbicide. Nevertheless, the height growth rates after prior cultivation with the herbicide remained higher than those in the control tests, ranging from k = 0.0330 for MP + TBT to k = 0.0214 for CE + TBT. The presence of phytotoxic compounds in the soil and the natural senescence of the phytoremediator and sentinel species may contribute to these adverse effects [7].

Notably, the presence of the fungal inoculant reduced the phytotoxic effect of tebuthiuron in certain treatments. In the MP + FUNG + TBT treatment, *C. juncea* exhibited a rapid height increase (k = 0.0659), resulting in a steep growth curve and an impressive maximum height (α = 248.45 cm). Similar results were observed in the CE + FUNG + TBT treatment, where the maximum height (α = 199.88 cm) was higher compared to other treatments with the same species. These findings indicate the potential of the fungal inoculant in mitigating tebuthiuron toxicity, possibly through microbial degradation of the herbicide.

The bacterial inoculant also exhibited a mitigating effect, though it was generally less pronounced than that of the fungal inoculant. In the CE + BACT + TBT treatment, *C. juncea*'s growth rate was higher (k = 0.0930), and in the MP + BACT + TBT treatment, the maximum height (α = 147.18 cm) was higher compared to other treatments with the same species. This suggests that the bacterial inoculant can aid in tebuthiuron remediation in soil, albeit with some limitations [35].

Overall, these findings provide a detailed assessment of the growth dynamics of *C. juncea* as a bioindicator plant. The results elucidate the impact of microbial inoculants and the herbicide tebuthiuron on the development of the bioindicator, highlighting the potential of certain inoculants—particularly the fungal inoculant—in mitigating the herbicide's phytotoxic effects. Additionally, the efficacy of fungal inoculants in mitigating tebuthiuron toxicity may be attributed to their robust enzymatic systems capable of degrading complex organic pollutants. Fungi such as *Trichoderma* spp. produce a variety of extracellular enzymes, including laccases and peroxidases, which can oxidize and break down persistent herbicides [36]. These enzymes facilitate the cleavage of chemical bonds within the tebuthiuron molecule, transforming it into less toxic metabolites that are more amenable to further microbial degradation or assimilation by plants. The deployment of such fungi in bioaugmentation strategies not only enhances the degradation of recalcitrant compounds but also improves soil health by suppressing pathogenic microorganisms and promoting plant growth [37]. This contributes valuable knowledge to the field of phytoremediation, informing strategies for effectively addressing tebuthiuron-contaminated soils.

3.3.2. Phytomass Accumulation

The accumulation of dry biomass in *Crotalaria juncea* varied significantly across treatments, revealing critical insights into the interplay between microbial inoculants, herbicide presence, and phytoremediation efficacy (Figure 6). Notably, the treatment combining

tebuthiuron with bacterial inoculants (TBT + BACT) resulted in the highest dry biomass production, reaching approximately 18 g. Despite being higher, there was no statistical significance compared to the other treatments, which suggested that the addition of specific bacterial strains effectively mitigated the herbicide's phytotoxic effects on *C. juncea*. The bacteria likely facilitated enhanced degradation or transformation of tebuthiuron, reducing its toxicity and promoting plant growth. This finding underscores the potential of bacterial inoculants as a pivotal component in phytoremediation strategies for tebuthiuron-contaminated soils.

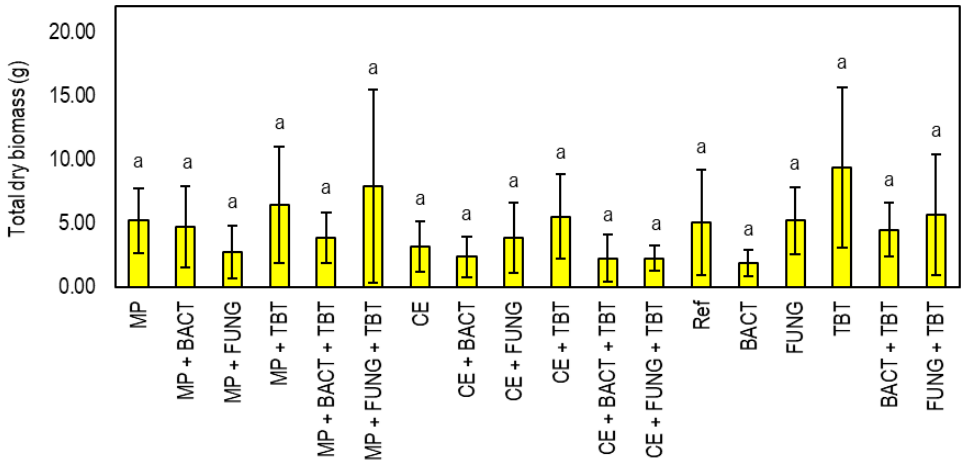

Figure 6. Production of total dry biomass of *C. juncea* in soil associated or not with tebuthiuron (TBT), bacterial (BACT), or fungal (FUNG) inoculants and/or the different plants *M. pruriens* (MP) and *C. ensiformis* (CE) after 70 DAS. Ref—reference control soil without leguminous plants, inoculants, and tebuthiuron. Same lowercase letters did not indicate statistical differences by the Tukey test ($p < 0.05$).

In stark contrast, the treatment with tebuthiuron alone (TBT) without any inoculants resulted in significantly lower biomass accumulation, around 7 g. This substantial reduction reflects the negative impact of the herbicide on the growth of *C. juncea*, confirming its phytotoxicity in the absence of bioremediation interventions. The persistence and toxicity of tebuthiuron in this treatment could be attributed to its chemical stability and the substantial organic matter content in the soil, which may enhance herbicide adsorption and reduce its bioavailability for degradation [30].

Fungal inoculants (FUNG), when applied in combination with tebuthiuron (TBT + FUNG), were less effective than bacterial inoculants in mitigating the herbicide's phytotoxicity, resulting in dry biomass of approximately 9 g. While fungi are known to play roles in biodegradation and plant growth promotion, their efficacy in this context was inferior to that of bacteria. This suggests that the specific fungal species used may not have possessed the necessary metabolic pathways to effectively degrade tebuthiuron or may have had less synergistic interactions with *C. juncea* compared to the bacterial strains.

The combinations involving prior cultivation of *M. pruriens* (MP) or *C. ensiformis* (CE) with microbial inoculants and tebuthiuron yielded variable biomass outcomes. Generally, these treatments did not achieve biomass values as high as the TBT + BACT treatment. Biomass production in these groups ranged between 5 and 12 g, indicating that the interactions between the leguminous plants, microbial inoculants, and tebuthiuron are complex. Possible antagonistic effects, such as competition for nutrients, allelopathic interactions, or microbial community shifts, may have limited the growth of *C. juncea* in these scenarios [7,34].

These findings align with previous studies emphasizing the critical role of microorganisms in enhancing plant tolerance to soil contaminants. The superior efficacy observed with bacterial inoculants may be attributed to their ability to metabolize toxic compounds, produce plant growth-promoting substances, or enhance nutrient availability, thereby creat-

ing more favorable conditions for plant development [10,32]. In contrast, the lower efficacy of fungal inoculants could be due to less efficient degradation pathways for tebuthiuron or weaker interactions with the phytoremediator and bioindicator plants.

Overall, the results highlight the importance of selecting appropriate microbial inoculants in phytoremediation strategies. The significant increase in biomass with bacterial inoculation demonstrates its potential application in agricultural practices to mitigate tebuthiuron contamination. The enhanced biomass production not only indicates improved plant health but also suggests a greater capacity for phytoremediation, as higher biomass is often correlated with increased pollutant uptake and degradation [27,28].

3.4. Bioassays with L. sativa: Validating Ecotoxicity and Phytoremediation Efficiency

Ecotoxicity testing with *L. sativa* is a pivotal tool for evaluating soil quality, particularly in environments potentially affected by herbicides like tebuthiuron. This indirect method not only detects the presence of herbicide but also verifies reductions in soil toxicity. Multiple researchers have conducted ecotoxicity tests using *L. sativa* following biological pesticide remediation experiments [7,19].

The germination index (GI) of *Lactuca sativa* served as a sensitive indicator of soil ecotoxicity and the effectiveness of phytoremediation treatments over time. In the uncultivated treatments (Figure 7), the reference soil (Ref) initially exhibited a significantly higher GI compared to other treatments without tebuthiuron from 0 to 40 DAS. However, by the end of the experimental period, the bacterial inoculant treatment (BACT) surpassed both the Ref and fungal inoculant (FUNG) treatments in final GI values (0.95 > 0.92 > 0.89, respectively) (Table 4). The higher growth rate (k value) observed for BACT indicates its positive influence on the germination and early development of *L. sativa* seeds, possibly through enhanced nutrient availability or the production of growth-promoting substances.

Table 4. Parameters of the Gompertz kinetic models for the germination index of *L. sativa* in ecotoxicity bioassays in tebuthiuron (TBT) soil with *M. pruriens* (MP) and *C. ensiformis* (CE) and bacterial (BACT) or fungal (FUNG) inoculants. Ref—reference control soil without leguminous plants, inoculants, and tebuthiuron.

Treatments	Complexity			Adequacy		
	α	β	k	R^2	AIC	BIC
MP	1.00	6.98	0.04774	0.98	−11.64	−12.47
MP + TBT	0.97	10.09	0.06319	0.98	−13.20	−14.04
MP + BACT	1.00	7.06	0.04656	0.98	−13.15	−13.99
MP + FUNG	0.97	10.09	0.06319	0.98	−13.20	−14.04
MP + BACT + TBT	1.00	6.81	0.04618	0.98	−11.94	−12.77
MP + FUNG + TBT	1.00	7.49	0.05091	0.98	−11.67	−12.51
CE	1.00	5.94	0.04001	0.98	−11.13	−11.97
CE + TBT	1.00	7.25	0.03861	0.99	−21.18	−22.02
CE + BACT	1.00	6.96	0.04861	0.98	−10.72	−11.56
CE + FUNG	1.00	7.03	0.04880	0.98	−10.94	−11.77
CE + BACT + TBT	1.00	6.46	0.04224	0.98	−12.94	−13.77
CE + FUNG + TBT	1.00	7.06	0.04797	0.98	−11.94	−12.77
Ref	1.00	3.27	0.02642	0.99	−19.10	−19.93
TBT	1.00	3.65	0.03053	0.99	−24.09	−24.92
BACT	1.00	2.84	0.02250	0.96	−8.90	−9.73
FUNG	1.00	3.01	0.03141	0.99	−15.20	−16.03
BACT + TBT	1.00	3.28	0.03499	0.99	−18.46	−19.29
FUNG + TBT	1.00	3.33	0.02641	0.99	−19.68	−20.51

Parameters α, β, and k denote the superior asymmetry, the inflection point, and the exponential decay of the specific growth rate, respectively; $\beta = 1$ keeps the relative decrease with time constant; $\beta > 1$ accelerates the relative decrease with time; $\beta < 1$ slows the relative decrease with time; R^2: coefficient of determination; AIC: Akaike Information Criterion; BIC: Bayesian Information Criterion.

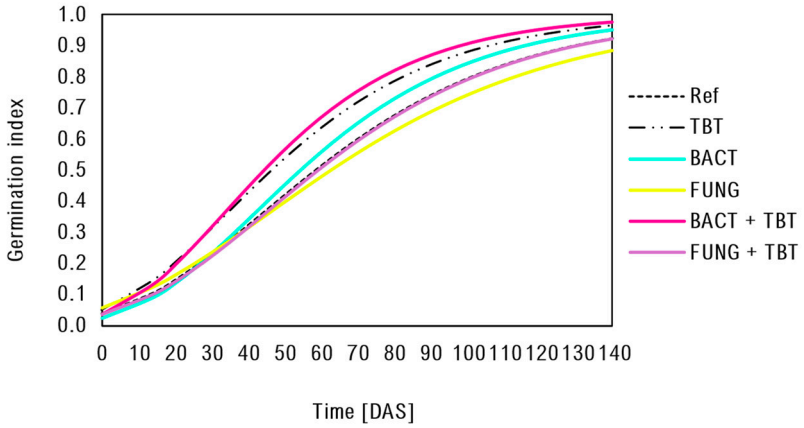

Figure 7. Kinetic evolution from the Gompertz model of the germination index of *L. sativa* in ecotoxicity bioassays in soil with tebuthiuron (TBT) and fungal (FUNG) or bacterial (BACT) inoculants from the Gompertz model. Ref—reference control soil without leguminous plants, inoculants, and tebuthiuron.

Intriguingly, the treatment containing only tebuthiuron (TBT) without any inoculants showed a higher final GI (0.96) and faster growth rate (k = 0.0314) compared to the Ref. This unexpected result suggests that natural attenuation processes were at play, whereby indigenous soil microorganisms gradually degraded the herbicide over time, reducing its phytotoxicity [38,39]. As tebuthiuron concentrations decreased, the inhibitory effects on seed germination diminished, allowing *L. sativa* to achieve higher GI values.

The impact of microbial inoculants in tebuthiuron-contaminated soil differed between bacteria and fungi. The BACT + TBT treatment exhibited the highest final GI (0.98) and growth rate (k = 0.0350), indicating that bacterial inoculants effectively enhanced the dissipation of tebuthiuron's toxic effects, facilitating seed germination and growth. In contrast, the FUNG + TBT treatment had a lower final GI (0.92) and growth rate (k = 0.0264), suggesting that the fungal inoculant was less effective in mitigating the herbicide's ecotoxicity.

The prior cultivation of *M. pruriens* (MP) and *C. ensiformis* (CE) also influenced the GI of *L. sativa* (Figure 8A,C). Initially, the GI in these treatments was lower than the Ref between 0 and 40 DAS, possibly due to residual allelopathic compounds from the leguminous plants or incomplete degradation of tebuthiuron. Over time, however, the GI increased, reaching 0.99 in the MP treatment (Table 4), indicating a reduction in soil toxicity. The higher growth rate (k = 0.0477) compared to the Ref (k = 0.0264) suggests that the prior cultivation of *M. pruriens* improved soil conditions, possibly through enhanced microbial activity, nutrient cycling, and the degradation of residual herbicides.

The combination of microbial inoculants with leguminous plants further affected the GI. In the MP + BACT treatment without tebuthiuron, the growth rate increased (k = 0.0632) compared to MP alone, although the final GI was slightly lower (0.97 < 0.99). This indicates that while bacterial inoculants can accelerate the reduction in ecotoxicity, they may also introduce competitive dynamics that slightly affect germination rates. In treatments with tebuthiuron, microbial inoculation appeared to reduce the negative ecotoxicological impact, as evidenced by higher GI values compared to treatments without inoculants.

Overall, the GI of *L. sativa* provided valuable insights into the temporal dynamics of soil ecotoxicity and the effectiveness of phytoremediation strategies. The results highlight the potential of combining leguminous plants with specific microbial inoculants to enhance the degradation of tebuthiuron and reduce its phytotoxic effects. However, the complexity of interactions among plants, microorganisms, and contaminants underscores the need for careful selection and optimization of phytoremediation components to achieve effective soil remediation [38,39].

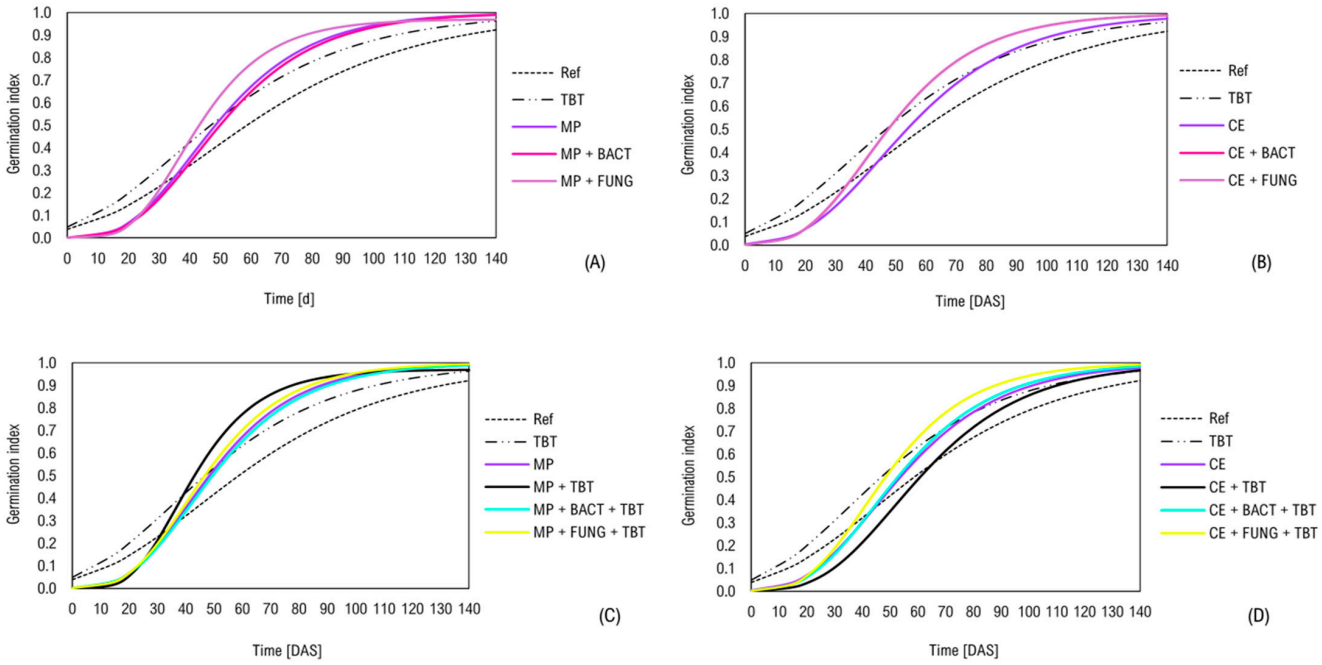

Figure 8. Kinetic evolution from the Gompertz model of the germination index of *L. sativa* in ecotoxicity bioassays in tebuthiuron (TBT) soil with *M. pruriens* (MP) and *C. ensiformis* (CE) and bacterial (BACT) or fungal (FUNG) inoculants. Ref—reference control soil without leguminous plants, inoculants, and tebuthiuron. (**A**,**B**) Treatments without TBT. (**C**,**D**) Treatments with TBT.

Furthermore, the physicochemical properties of the soil, such as pH, organic matter content, and cation exchange capacity, significantly influence the bioavailability and persistence of tebuthiuron [40]. Soils with high organic matter can adsorb greater amounts of herbicides, potentially reducing their immediate bioavailability to plants and microbes but also prolonging their environmental persistence [41]. Adjusting soil conditions through amendments like compost or biochar can enhance microbial activity and modify sorption characteristics, thereby improving degradation rates [42]. Tailoring phytoremediation strategies to account for these soil properties is essential for optimizing contaminant removal and ensuring the sustainability of remediation efforts [43].

3.5. The Impact of the Soil–Herbicide–Plant–Microbe Nexus on L. sativa's GI: A Deeper Understanding of Bioremediation

A multivariate response analysis using a mixed linear model (Figure 9) from 0 to 140 DAS elucidated the intricate interactions among tebuthiuron, leguminous plants, and microbial inoculants on the germination index of *L. sativa*. This analysis revealed that the combined effects of these factors played a crucial role in determining seed germination rates and seedling development.

When leguminous plants and microbial inoculants were combined, the treatments displayed a less steep slope in the model, indicating a strong interdependence between these factors. This synergistic interaction resulted in higher germination rates of *L. sativa*, even in the presence of tebuthiuron (accounted for as a random effect in the model). The combination of potential phytoremediators and bioaugmentation appeared to mitigate the phytotoxic effects of the herbicide more effectively than either factor alone.

In contrast, the individual effects of leguminous plants or microbial inoculants exhibited steeper slopes, suggesting a lower impact on reducing tebuthiuron toxicity when applied independently. This implies that microbial inoculants alone may not sufficiently alleviate the herbicide's phytotoxicity, and similarly, the cultivation of leguminous species without microbial augmentation may have limited efficacy.

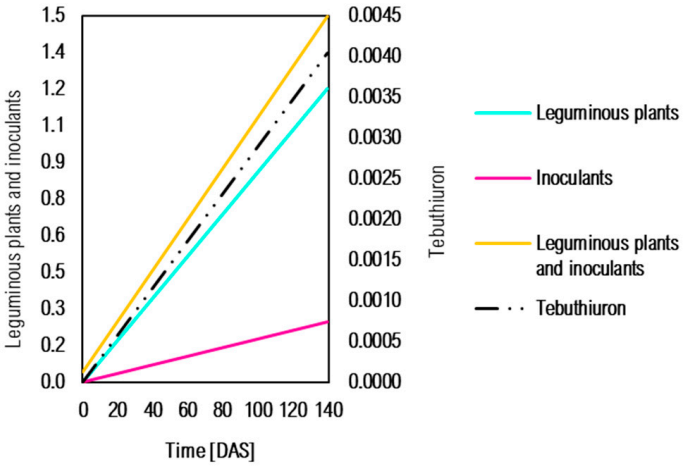

Figure 9. Dynamics of random (tebuthiuron) and fixed (leguminous plants—*M. pruriens* and *C. ensiformis*; and microbial inoculants—fungal and bacterial) effects on the specific rate of the germination index (GI) of *L. sativa* in ecotoxicity bioassays.

The enhanced performance of the combined treatments can be attributed to several mechanisms. The leguminous plants likely improved soil health by increasing organic matter content, enhancing nutrient availability through nitrogen fixation, and stimulating microbial activity [21,22]. The introduced microbial inoculants may have possessed specific degradative capabilities for tebuthiuron or facilitated the proliferation of indigenous degrader populations, leading to accelerated herbicide dissipation [9,10].

These findings underscore the importance of integrated phytoremediation strategies that leverage synergistic interactions between plants and microorganisms. By combining phytoremediators with bioaugmentation, it is possible to enhance the degradation of persistent contaminants like tebuthiuron, improve soil quality, and reduce ecotoxicity more effectively than with single-factor approaches.

3.6. Limitations and Directions to Improve the Credibility and Practicality of Phytoremediation

While this study demonstrates the potential of combining leguminous plants and microbial inoculants for the phytoremediation of tebuthiuron-contaminated soils, several limitations warrant consideration for future research and practical application.

The experiments were conducted under controlled greenhouse conditions, which may not fully capture the complexities of field environments. Factors such as soil heterogeneity, climatic variations, and ecological interactions can significantly influence phytoremediation outcomes. Field trials are essential to validate the effectiveness of the proposed strategies under real-world conditions, accounting for spatial and temporal variability.

Assessing the long-term sustainability of phytoremediation efforts is crucial. Continuous monitoring of contaminant levels, soil health indicators, and ecological impacts over extended periods will provide insights into the persistence of remediation effects and potential rebound of contaminant concentrations. In addition, agricultural soils are often contaminated with a mixture of pesticides and other pollutants. Future studies should investigate the efficacy of phytoremediation strategies in the context of multiple contaminants to develop comprehensive remediation approaches. Additionally, understanding how soil properties such as texture, organic matter content, and microbial diversity influence remediation processes will enable more tailored interventions. A deeper understanding of the microbial community dynamics is also essential. Metagenomic and metatranscriptomic analyses can identify key microbial taxa involved in contaminant degradation and elucidate functional pathways. This knowledge can inform the selection or engineering of more effective microbial consortia for bioaugmentation.

Economic analyses comparing phytoremediation to conventional remediation methods are necessary to assess cost-effectiveness. Factors such as the cost of microbial in-

oculants, plant cultivation, timeframes for remediation, and potential economic benefits from biomass utilization should be considered. Developing scalable and economically viable phytoremediation models will facilitate broader adoption in agricultural practices. The effective implementation of phytoremediation strategies requires supportive policies, regulatory frameworks, and stakeholder engagement. Educating farmers, land managers, and policymakers about the benefits and limitations of phytoremediation will promote its integration into sustainable land management practices.

3.7. Future Perspectives

Advancements in phytoremediation research hold promise for enhancing the efficiency and applicability of this eco-friendly remediation method. Future directions include (a) plant–microbe interactions, exploring novel symbiotic relationships, and co-cultivation techniques can optimize contaminant degradation. Genetic studies may reveal plant traits that enhance microbial colonization and activity; (b) genetic engineering, developing transgenic plants with enhanced metabolic capabilities to degrade specific contaminants offers potential, though ecological risks and ethical considerations must be addressed; (c) integrated remediation strategies, combining phytoremediation with other remediation technologies, such as biostimulation, chemical oxidation, or nanotechnology, may overcome limitations associated with single-method approaches; (d) urban and aquatic applications, extending phytoremediation to urban settings and aquatic environments requires adaptation to unique challenges, such as space constraints and pollutant dispersion dynamics; and (e) policy development and public awareness, establishing clear guidelines, safety protocols, and public education initiatives will facilitate acceptance and trust in phytoremediation practices.

As environmental concerns escalate globally, the practical application of phytoremediation on a larger scale becomes increasingly significant. By addressing the current limitations and capitalizing on emerging research, phytoremediation can evolve into a more robust and versatile tool for mitigating environmental pollution, promoting ecosystem health, and supporting sustainable agricultural practices.

4. Conclusions

This study has demonstrated that integrating leguminous plants with microbial inoculants offers a promising and sustainable strategy for the phytoremediation of tebuthiuron-contaminated agricultural soils. *M. pruriens* exhibited a faster growth rate than *C. ensiformis*, but both species experienced growth inhibition due to the herbicide's phytotoxic effects. The application of microbial inoculants significantly mitigated these negative impacts, with fungal inoculants particularly enhancing plant performance in *M. pruriens*. This suggests that specific fungi may possess metabolic pathways capable of degrading or transforming tebuthiuron into less toxic compounds, thereby promoting plant growth even in contaminated conditions. The use of *C. juncea* as a bioindicator validated the effectiveness of the remediation process. Treatments combining tebuthiuron with bacterial inoculants resulted in the highest biomass production of *C. juncea* and a marked reduction in herbicide toxicity, highlighting the potential of bacterial strains in enhancing tebuthiuron degradation. Ecotoxicological bioassays with *L. sativa* further confirmed that the combination of leguminous plants with microbial inoculants accelerated soil detoxification, achieving higher germination indices and growth rates compared to treatments without inoculants or prior cultivation. These findings accentuate the synergistic effects of plant–microbe interactions in enhancing the degradation of persistent herbicides, improving soil health, and reducing environmental toxicity.

While the results are promising, it is important to recognize that the experiments were conducted under controlled greenhouse conditions, which may not fully replicate the complexities of field environments. Field trials are essential to validate these phytoremediation strategies in real-world settings and to assess their long-term sustainability and effectiveness under diverse environmental conditions. Future research should focus on

scaling up these approaches, analyzing microbial community dynamics to identify key degraders of tebuthiuron, optimizing plant–microbe combinations for specific soils and climates, evaluating economic feasibility, and developing supportive policies to facilitate the integration of phytoremediation into sustainable agricultural practices. In conclusion, the integration of leguminous plants with specific microbial inoculants presents a viable and eco-friendly approach for remediating tebuthiuron-contaminated soils. This strategy not only contributes to the restoration of soil quality and protection of environmental health but also advances sustainable land management practices, promoting healthier agricultural ecosystems and benefiting society as a whole.

Author Contributions: Conceptualization: V.H.C., V.L.d.S. and P.R.M.L.; methodology: V.H.C., E.B.d.M., L.G.d.V., E.P.P., R.N.M. and P.R.M.L.; investigation: V.H.C., T.S.V. and Y.A.F.; data curation: B.R.d.A.M., R.N.M. and P.R.M.L.; formal analysis: V.H.C. and B.R.d.A.M.; writing original draft: V.H.C., B.R.d.A.M., YAF, L.T., E.P.P. and P.R.M.L.; review and editing: V.H.C., V.L.d.S., E.B.d.M., L.G.d.V., L.T., E.P.P., R.N.M. and P.R.M.L.; funding acquisition, project administration, resources, supervision, and validation: P.R.M.L. All authors have read and agreed to the published version of the manuscript.

Funding: São Paulo Research Foundation (FAPESP-Brazil, 2021/01884-6); National Council for Scientific and Technological Development (CNPq-Brazil, 313530/2021-1), Fundação Agrisus—Brazil (PA 3740/24), Coordination for Improvement of Higher Education Personnel (CAPES-Brazil, financing code n. 001); and Pro-Rectory of Graduate Studies (Pró-Reitoria de Pós-Graduação) of São Paulo State University (Unesp-Brazil).

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available in an online repository (UNESP-Brazil) and on request from the corresponding author (PRML).

Acknowledgments: The authors acknowledge the São Paulo Research Foundation (FAPESP-Brazil, 2021/01884-6); the National Council for Scientific and Technological Development (CNPq-Brazil, 313530/2021-1), the Fundação Agrisus—Brazil (PA 3740/24), the Coordination for Improvement of Higher Education Personnel (CAPES-Brazil, financing code n. 001); and Pro-Rectory of Graduate Studies (Pró-Reitoria de Pós-Graduação) of São Paulo State University (Unesp-Brazil). We would also like to thank the NETA group (FCAT/Unesp-Brazil), led by Evandro Pereira Prado, for helping with the application of pesticides.

Conflicts of Interest: The authors declare no conflicts of interest.

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