

In vitro efficacy of cyclobutrifluram and biocontrol microorganisms against banana plant-parasitic nematodes

Eficácia *in vitro* do ciclobutrifluram e de microrganismos de biocontrole contra fitonematoides da bananeira

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ABSTRACT - Banana production is affected by plant-health challenges caused by infestations of plant-parasitic nematodes such as *Meloidogyne incognita*, *Meloidogyne enterolobii*, and *Radopholus similis*, which impair root development, yield, and plantation longevity. The efficacy of cyclobutrifluram and its physiological compatibility with biocontrol microorganisms were evaluated under in vitro conditions using a sequential and integrated approach. In Experiment I, the nematocidal activity of cyclobutrifluram was evaluated at different concentrations; in Experiment II, the effects of *Bacillus siamensis* (CNPMF1009), *B. pumilus* (BR12158), *B. methylotrophicus*, *B. velezensis* (BR12157), and *Bacillus* sp. (BSF66) strains on plant-parasitic nematodes were determined; in Experiment III, physiological compatibility between cyclobutrifluram and beneficial microorganisms (*B. siamensis* (CNPMF1009), *B. velezensis* (BR12157), and *Trichoderma asperellum* (CNPMF 1007)) was analyzed; and in Experiment IV, biofilm formation by *Bacillus* spp. was evaluated as an indicator of microbial adaptation and survival. Cyclobutrifluram showed nematocidal activity and efficacy against *M. incognita* and *M. enterolobii*, with mortality greater than 90%, as well as activity against *R. similis*. The *Bacillus siamensis* and *B. velezensis* strains also showed nematocidal activity and maintained physiological compatibility with the product at the tested concentrations, whereas *T. asperellum* growth was reduced at specific product concentrations, indicating greater sensitivity than that of the bacterial strains. Most bacterial strains showed biofilm-forming capacity, indicating potential for adaptation and survival under in vitro conditions. The results showed nematocidal activity and physiological compatibility among cyclobutrifluram, *B. siamensis* (CNPMF1009), and *B. velezensis* (BR12157).

Keywords: *Meloidogyne* spp., *Radopholus similis*, *Bacillus siamensis*, *Trichoderma asperellum*, integrated management.

RESUMO - A produção de banana é afetada por desafios fitossanitários causados por infestações de fitonematoides, como *Meloidogyne incognita*, *Meloidogyne enterolobii* e *Radopholus similis*, que prejudicam o desenvolvimento radicular, a produtividade e a longevidade dos plantios. A eficácia do ciclobutrifluram e sua compatibilidade fisiológica com microrganismos de biocontrole foram avaliadas em condições in vitro, por meio de uma abordagem sequencial e integrada. No Experimento I, a atividade nematocida do ciclobutrifluram foi avaliada em diferentes concentrações; no Experimento II, foram determinados os efeitos das cepas *Bacillus siamensis* (CNPMF1009), *B. pumilus* (BR12158), *B. methylotrophicus*, *B. velezensis* (BR12157) e *Bacillus* sp. (BSF66) sobre fitonematoides; no Experimento III, foi analisada a compatibilidade fisiológica entre o ciclobutrifluram e microrganismos benéficos (*B. siamensis* (CNPMF1009), *B. velezensis* (BR12157) e *Trichoderma asperellum* (CNPMF 1007)); e, no Experimento IV, a formação de biofilme por *Bacillus* spp. foi avaliada como indicador de adaptação e sobrevivência microbiana. O ciclobutrifluram apresentou atividade nematocida e eficácia contra *M. incognita* e *M. enterolobii*, com mortalidade superior a 90%, além de atividade contra *R. similis*. As cepas *Bacillus siamensis* e *B. velezensis* também apresentaram atividade nematocida e mantiveram compatibilidade fisiológica com o produto nas concentrações testadas, enquanto o crescimento de *T. asperellum* foi reduzido em concentrações específicas do produto, indicando maior sensibilidade em comparação às cepas bacterianas. A maioria das cepas bacterianas apresentou capacidade de formação de biofilme, indicando potencial de adaptação e sobrevivência em condições in vitro. Os resultados evidenciaram atividade nematocida e compatibilidade fisiológica entre o ciclobutrifluram, *B. siamensis* (CNPMF1009) e *B. velezensis* (BR12157).

Palavras-chave: *Meloidogyne* spp.; *Radopholus similis*; *Bacillus siamensis*; *Trichoderma asperellum*; manejo integrado.

Conflict of interest: The authors declare no conflict of interest related to the publication of this manuscript.



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INTRODUCTION

Banana production is one of the main agricultural activities in tropical and subtropical regions and is essential for food security and income generation in countries of Latin America, Africa, and Asia. Globally, banana (*Musa* spp.) is among the crops of greatest dietary and economic importance (SOUSA et al., 2024).

In Brazil, banana occupies a prominent position among fruit crops, with production close to 7 million Mg in 2024 and broad territorial distribution, particularly in the states of São Paulo, Minas Gerais, Bahia, Santa Catarina, and Pernambuco (EMBRAPA, 2024). Despite its importance, productivity is limited by plant-parasitic nematodes such as *Meloidogyne incognita*, *M. enterolobii*, and *Radopholus similis*, which impair the root system, reduce water and nutrient absorption, and affect plant development (LUQUINI et al., 2019; GUZMÁN-PIEDRAHITA et al., 2023).

Chemical control has been the primary management method for these

pathogens. Innovative molecules with selective modes of action and lower ecotoxicological effects have therefore been developed. Cyclobutrifluram, the active ingredient of Tymirium® (Syngenta, Basel, Switzerland), belongs to the succinate dehydrogenase inhibitor (SDHI) group, which targets Complex II of the mitochondrial respiratory chain and impairs energy metabolism in target organisms (LIU et al., 2024; MAIENFISCH; KOERBER, 2024). Nematicidal activity of this compound against *M. enterolobii* has been reported (LIU et al., 2024; MAIENFISCH; KOERBER, 2024; THOMPSON et al., 2024; CHEN et al., 2024). However, studies involving other economically important species, such as *M. incognita* and *R. similis*, remain scarce, indicating the need to expand knowledge of its spectrum of activity.

Biological control has also advanced as a sustainable strategy for plant-parasitic nematode management. Among promising microorganisms, *Bacillus* spp. have potential to inhibit nematode populations, produce bioactive metabolites, and promote plant growth. Recent assays have confirmed that isolates of this genus can reduce *Meloidogyne* populations, supporting their use as biocontrol agents (ZHANG et al., 2023; VASANTHA-SRINIVASAN et al., 2025).

The use of *Trichoderma* species, including *T. asperellum*, has been reported for plant-parasitic nematode management and is associated with mechanisms such as egg parasitism, metabolite production, and resistance induction in the host plant (POVEDA; ABRIL-URIAS; ESCOBAR, 2020). However, these mechanisms depend on environmental colonization and may not be fully expressed in short-term in vitro bioassays. The efficacy of integrated management depends on compatibility between nematicides and biological control agents, because exposure to certain compounds can affect microbial viability. Therefore, these interactions must be understood to validate combined management strategies.

The objectives of this study were to evaluate the nematicidal activity of cyclobutrifluram, the potential of biocontrol microorganisms to suppress plant-parasitic nematodes, and the physiological compatibility between cyclobutrifluram and selected biocontrol agents under in vitro conditions. The study was conducted at the Brazilian Agricultural Research Corporation (Embrapa Cassava and Tropical Fruits), in Cruz das Almas, Bahia, Brazil, and was structured into four complementary experiments: (i) efficacy of cyclobutrifluram on plant-parasitic nematode mortality; (ii) potential of biocontrol microorganisms to suppress plant-parasitic nematodes; (iii) compatibility between the nematicide and the microorganisms; and (iv) biofilm formation by *Bacillus* spp.

MATERIALS AND METHODS

Experiment I - Efficacy of cyclobutrifluram on plant-parasitic nematode mortality in vitro

The nematicidal effect of cyclobutrifluram (Tymirium®, Syngenta, Basel, Switzerland; 450 g a.i. L⁻¹), corresponding to the experimental product A22011 (sample RET 121422), was evaluated against *Meloidogyne incognita*, *M. enterolobii*, and *Radopholus similis*. The nematodes were obtained from the plant-parasitic nematode collection of the National Cassava and Tropical Fruits Research Center (CNPMPF), Brazilian Agricultural Research Corporation

(Empresa Brasileira de Pesquisa Agropecuária – Embrapa), Cruz das Almas, Bahia, Brazil. The nematodes were extracted according to Coolen and D’Herde (1972) and exposed to five final active ingredient concentrations (0.01, 0.1, 1, 10, and 100 mg L⁻¹), in addition to the negative control (water filtered through a 500-mesh sieve).

The bioassay was conducted in 96-well microplates containing 50 µL of nematode suspension (≈30 juveniles) and 50 µL of treatment solution. The plates were sealed and maintained at room temperature (25 ± 2 °C), protected from direct light, according to He et al. (2020). The experiment was conducted in a completely randomized design, with six treatments (concentrations) and three replicates (microplates); each replicate consisted of ten sample units per treatment, totaling 180 experimental units per evaluation period and species.

Live (motile) and dead (immotile) nematodes were counted after 24 and 48 h under an optical microscope (EVOSTM XL Core Imaging System, Thermo Fisher Scientific, USA). Nematode mortality was confirmed after the addition of 10 µL of sodium hydroxide (1 N NaOH), according to Chen and Dickson (2000).

Mortality data were analyzed with generalized linear mixed models (GLMM) using the glmmTMB package, assuming a beta-binomial distribution and logit link function, with replicates considered a random effect. Significance was evaluated by deviance analysis (likelihood ratio test; $p \leq 0.05$), and means were compared using estimated marginal means (emmeans) with Sidak adjustment. Dose-response curves were fitted as a function of log-transformed dose to estimate effective doses (ED₅₀, ED₇₀, ED₉₀, and ED₉₅), corresponding to the concentrations required to cause 50%, 70%, 90%, and 95% mortality, respectively, with 95% confidence intervals. The analyses were performed in the R environment (R CORE TEAM, 2025).

Experiment II - Evaluation of plant-parasitic nematode inhibition by biocontrol microorganisms

The nematicidal activity of biocontrol microorganisms was evaluated based on plant-parasitic nematode juvenile mortality. Five *Bacillus* spp. strains obtained from the CNPMF microorganism collection, *B. siamensis* (CNPMPF1009), *B. pumilus* (BR12158), *B. methylotrophicus* (currently recognized as a later heterotypic synonym of *Bacillus velezensis*), *B. velezensis* (BR12157) and *Bacillus* sp. (BSF66). The strains were tested against *M. incognita*, *M. enterolobii*, and *R. similis*.

The strains were reactivated on nutrient agar (NA) at 28 ± 1 °C for 24 h under a 12-h photoperiod and subsequently cultured in nutrient broth (NB) under orbital shaking (100 rpm) at 28 °C for 48 h. After centrifugation (5,000 rpm for 5 min), the supernatant was discarded, and the cell pellet was resuspended in sterile saline solution (0.85% NaCl) (ARYA et al., 2025). This standardization was adopted after it was observed that the culture medium influenced plant-parasitic nematode mortality independently of bacterial activity.

Bacterial suspensions were adjusted to an optical density of 0.5 measured at 540 nm (OD₅₄₀). Nematode suspension (10 µL; ≈15 juveniles) and bacterial suspension (100 µL) were added to each well of 96-well microplates. Two controls were used: distilled water and saline solution

(0.85% NaCl). The plates were sealed with plastic film and maintained at room temperature ($25 \pm 2^\circ\text{C}$).

The experiment was conducted in a completely randomized design, with three independent replicates (microplates). Each replicate included seven treatments (five bacterial isolates and two controls), with eight sample units (wells) per treatment, totaling 168 experimental units per evaluation time and species. Evaluations were performed after 24 and 48 h, and juvenile mortality was used as the response variable according to the protocol described in Experiment I.

Juvenile mortality was analyzed in the R environment (R DEVELOPMENT CORE TEAM, 2025) using GLMM, assuming a binomial or beta-binomial distribution according to data fit and residual dispersion diagnostics and using a logit link function. Treatments were considered fixed effects, and independent replicates (microplates) were considered random effects. Comparisons among treatments were performed using estimated marginal means (emmeans), with Sidak adjustment ($p \leq 0.05$). Model adequacy was evaluated by residual simulation in the DHARMA package, including overdispersion and zero-inflation tests.

Experiment III - Physiological compatibility between cyclobutrifluram and biocontrol microorganisms

Physiological compatibility between cyclobutrifluram and beneficial microorganisms was evaluated by analyzing the effect of the nematicide on microbial growth at different concentrations. *Bacillus* spp. isolates previously evaluated in Experiment II were used, along with *T. asperellum*, which was included as a microorganism widely used in biological control strategies.

The *B. siamensis* (CNPMF1009) and *B. velezensis* (BR12157) strains were cultured in nutrient broth (NB) under orbital shaking (100 rpm, 28°C , 48 h) and subsequently used directly in the compatibility assays. *T. asperellum* (CNPMF 1007) was cultured on potato dextrose agar (PDA), and the conidial suspension was prepared in sterile distilled water. The suspensions were exposed to five cyclobutrifluram concentrations (0.01, 0.1, 1, 10, and 100 mg L^{-1}), in addition to two controls (medium with and without microorganisms).

The assay was conducted in 96-well microplates containing 100 μL of the appropriate medium (nutrient broth for *Bacillus* spp. and potato dextrose broth for *T. asperellum*), 10 μL of microbial suspension, and 10 μL of cyclobutrifluram solution, totaling 120 μL per well. Potato dextrose broth was prepared according to Sibounnavong et al. (2009).

The plates were sealed with plastic film and incubated in a biochemical oxygen demand (BOD) incubator at 28°C . Absorbance (OD_{540}) was measured using a microplate reader (Loccus LMR-96) at 0, 24, 48, 72, and 96 h, with independent microplates for each evaluation period. The incubation period was extended to 96 h because static cultivation in microplates without medium renewal may delay microbial growth and reveal progressive inhibitory effects. Absorbance was used as an indirect estimate of microbial growth, according to the methods described by Kurokawa and Ying (2017). The plates were discarded after each analysis.

The experiment was conducted in a completely randomized design and consisted of three independent microplates (replicates) for each microorganism evaluated.

Each microplate included seven treatments, with eight sample units (wells) per treatment, totaling 168 experimental units per microorganism at each evaluation time.

The area under the microbial growth curve (AUMGC) was calculated according to the method proposed by Shaner and Finney (1977), using corrected absorbance values (OD_{540}) relative to the initial time (D_0) and the culture medium control. AUMGC was used as the response variable to estimate microbial growth over time as a function of the cyclobutrifluram concentrations. The data met the assumptions of normality (Shapiro–Wilk) and homogeneity of variances (Levene) and were subjected to analysis of variance (ANOVA), followed by Tukey’s mean comparison test ($p \leq 0.05$). The analyses were performed in the R environment (R CORE TEAM, 2025).

Experiment IV - Biofilm formation by *Bacillus* spp. using the crystal violet (CV) assay

Biofilm formation by *Bacillus* spp. strains (*B. siamensis* (CNPMF1009), *B. pumilus* (BR12158), *B. methylotrophicus*, *B. velezensis* (BR12157) and *Bacillus* sp. (BSF66)) was evaluated by the crystal violet colorimetric method in 96-well microplates, according to Stepanović et al. (2007). The strains were reactivated on nutrient agar (NA) and incubated at 30°C for 48 h. Isolated colonies were transferred to tryptic soy broth (TSB) medium and incubated under shaking (100 rpm, $28 \pm 1^\circ\text{C}$, 48 h). Cell density was adjusted to an absorbance of 0.5 at 540 nm.

TSB supplemented with 1% glucose (180 μL) and bacterial suspension (20 μL) were added to each well, with TSB alone used as the negative control. The plates were incubated at 30°C for 48 h under static conditions. After washing with phosphate-buffered saline (pH 7.2), the wells were dried, stained with 0.1% crystal violet, and washed again to remove excess dye. The adhered crystal violet was solubilized with 95% ethanol, and absorbance was subsequently measured with a microplate reader (Loccus LMR-96) at 570 nm.

The experiment was conducted in a completely randomized design and consisted of three independent microplates (replicates), each containing six treatments and eight sample units, totaling 144 experimental units. The intensity of biofilm formation was interpreted according to Stepanović et al. (2007) by comparing treatment absorbance values with the negative control.

The optical density cutoff value (OD_c) was initially calculated as the mean absorbance of the negative control plus three standard deviations. The strains were classified as non-biofilm-forming ($\text{OD} \leq \text{OD}_c$), weak biofilm-forming ($\text{OD}_c < \text{OD} \leq 2 \times \text{OD}_c$), moderate biofilm-forming ($2 \times \text{OD}_c < \text{OD} \leq 4 \times \text{OD}_c$), or strong biofilm-forming ($\text{OD} > 4 \times \text{OD}_c$), according to Stepanović et al. (2007).

Absorbance data (OD_{570}) were subjected to the Shapiro–Wilk normality test and Levene’s test for homogeneity of variances. Because the parametric assumptions were not met, the Kruskal–Wallis test was applied and was followed by Dunn’s test with Holm adjustment ($p \leq 0.05$). The analyses were performed in the R environment (R DEVELOPMENT CORE TEAM, 2025).

RESULTS AND DISCUSSION

Efficacy of cyclobutrifluram on plant-parasitic nematode mortality in vitro

Cyclobutrifluram increased mortality in a dose-dependent manner in all three species (Figures 1, 2, and 3), with significant differences among treatments, according to

beta-binomial generalized linear mixed models (GLMM; $p < 0.001$). The distribution plots and dose-response curves for *M. incognita* (Figure 1) showed that mortality ranged from 2.7% in the control to 95.8% at 100 mg L⁻¹ after 24 h and remained high after 48 h. The effective doses estimated to cause 50% (ED₅₀) and 70% (ED₇₀) mortality were 0.0039 and 0.0665 mg L⁻¹ after 24 h, respectively, and 0.0137 and 0.133 mg L⁻¹ after 48 h.

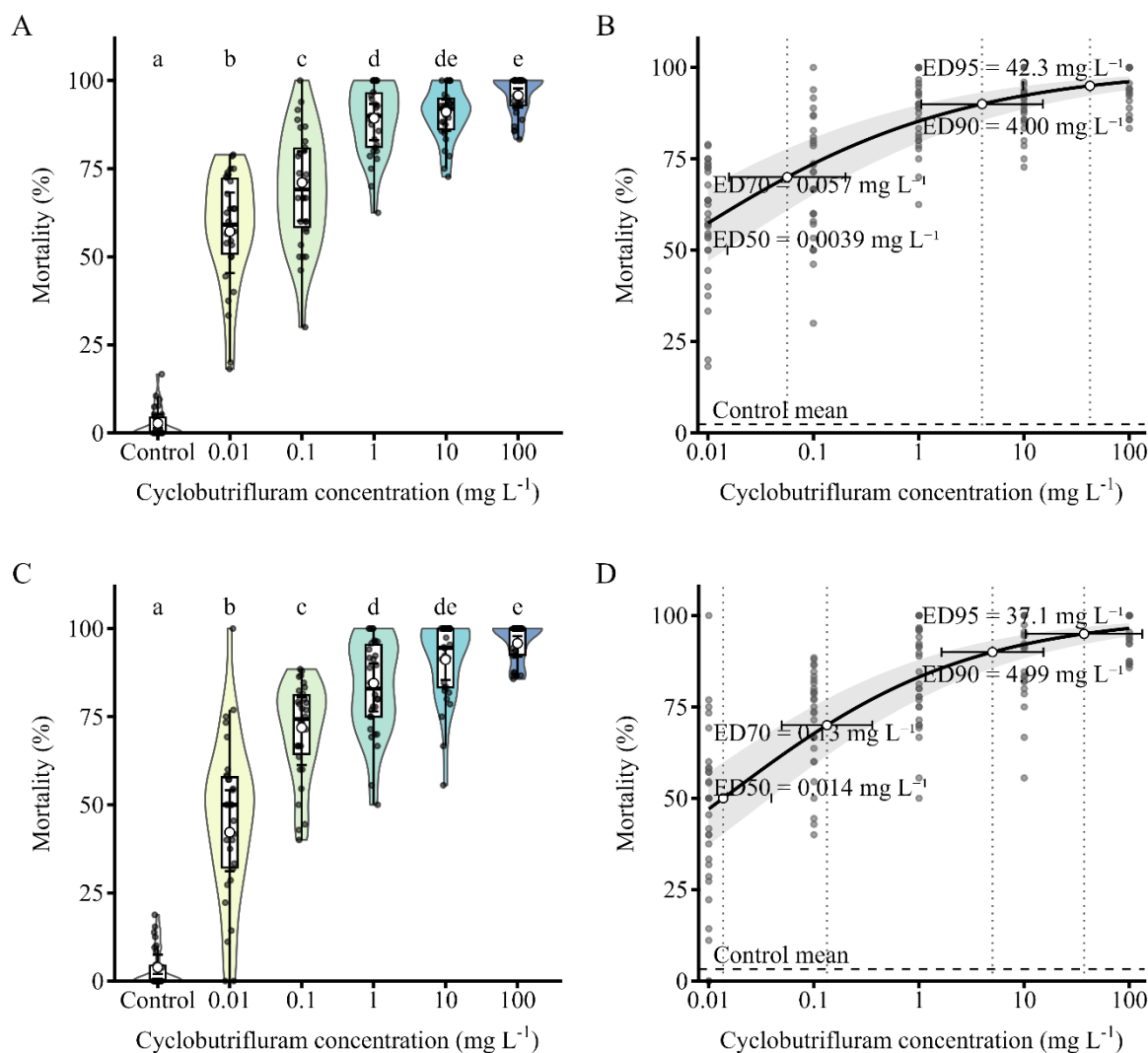


Figure 1. Mortality of *Meloidogyne incognita* in response to cyclobutrifluram concentrations (0.01–100 mg L⁻¹) after 24 and 48 h. Violin plots (A, C) show model-adjusted means from the beta-binomial generalized linear mixed models (GLMM) and 95% confidence intervals (CI). Dose-response curves (B, D) indicate the estimated effective doses (ED₅₀–ED₉₅).

The high mortality observed even at low concentrations suggests high sensitivity of *M. incognita* to cyclobutrifluram, indicating early effects on essential physiological processes in juveniles. Wram and Zasada (2019) also reported significant reductions in the viability and motility of nematodes exposed to nematicides, supporting the high sensitivity observed in this study.

The distribution plots and dose-response curves for *M.*

enterolobii (Figure 2) showed that mortality increased from 2.9% in the control to 97.9% at 100 mg L⁻¹ after 24 h and remained high after 48 h, with a tendency toward stabilization at the highest concentrations. The estimated effective doses were ED₅₀ = 0.024 mg L⁻¹ and ED₇₀ = 0.137 mg L⁻¹ after 24 h, and ED₅₀ = 0.021 mg L⁻¹ and ED₇₀ = 0.088 mg L⁻¹ after 48 h.

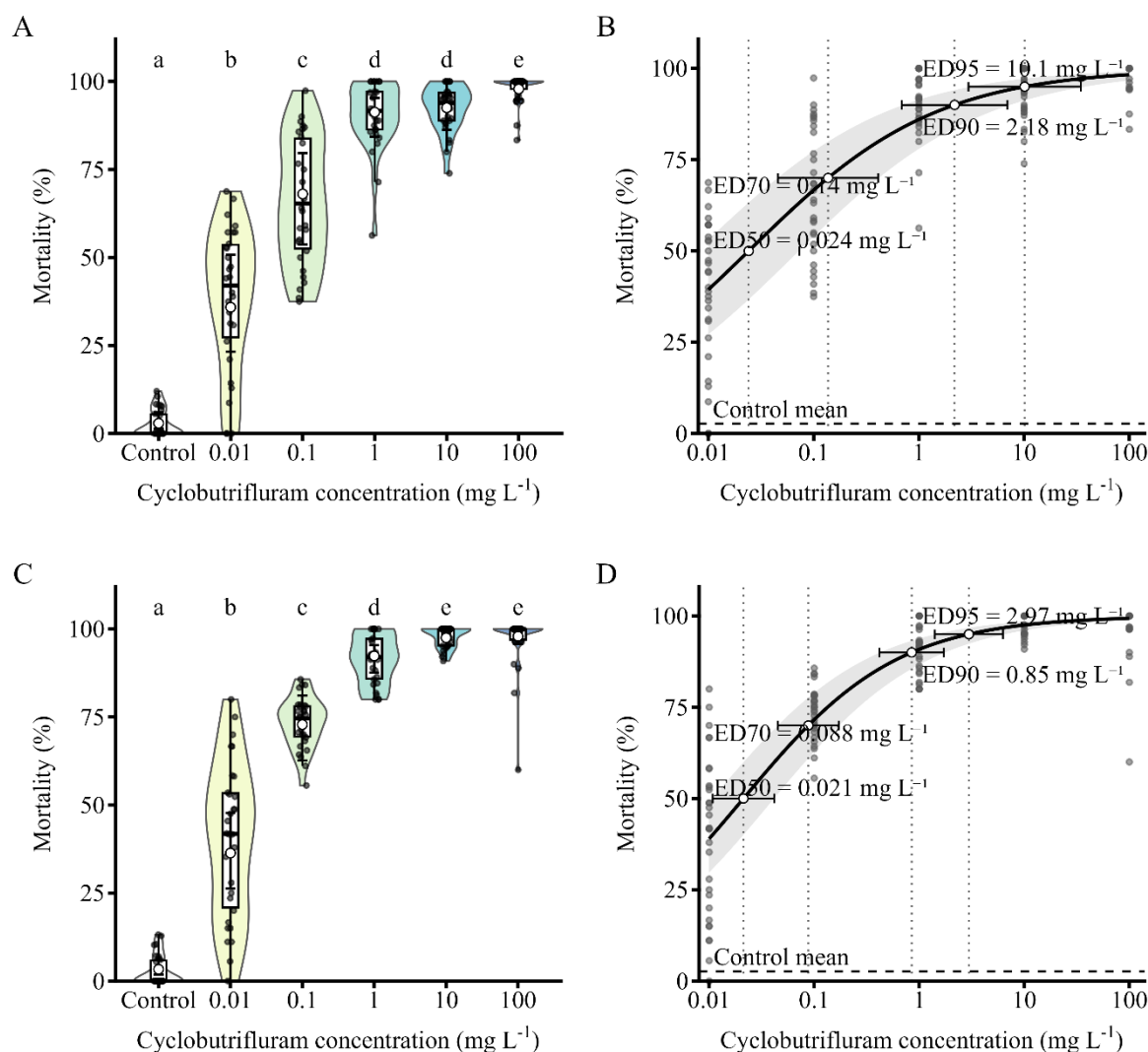


Figure 2. Mortality of *Meloidogyne enterolobii* as a function of increasing cyclobutrifluram concentrations (0.01–100 mg L⁻¹) after 24 and 48 h. Violin/box plots (A, C) show adjusted means and 95% CI; dose–response curves (B, D) were fitted using beta-binomial generalized linear mixed models (GLMM), with ED₅₀–ED₉₅ indicated.

The reduction in effective doses over time indicates a progressive increase in juvenile sensitivity to cyclobutrifluram. Chen et al. (2024) reported an EC₅₀ of 0.423 mg L⁻¹ for cyclobutrifluram against *M. enterolobii*, supporting the high efficacy of the active ingredient and the positive association between exposure time and mortality.

Lower mortality was observed for *R. similis* during the first 24 h (Figure 3), and higher concentrations were required to achieve high efficacy, with 86% mortality at 100 mg L⁻¹. After 48 h, mortality reached 93.3%, with a marked reduction

in the dose required to cause 70% mortality (ED₇₀ from 1.93 to 0.234 mg L⁻¹), indicating greater initial tolerance and a progressive increase in sensitivity to the nematicide over the exposure period.

These results show that the observed dose–time pattern is consistent with the mode of action of succinate dehydrogenase inhibitors (SDHI), which inhibit mitochondrial Complex II and impair juvenile energy metabolism (LIU et al., 2024; MAIENFISCH; KOERBER, 2024).

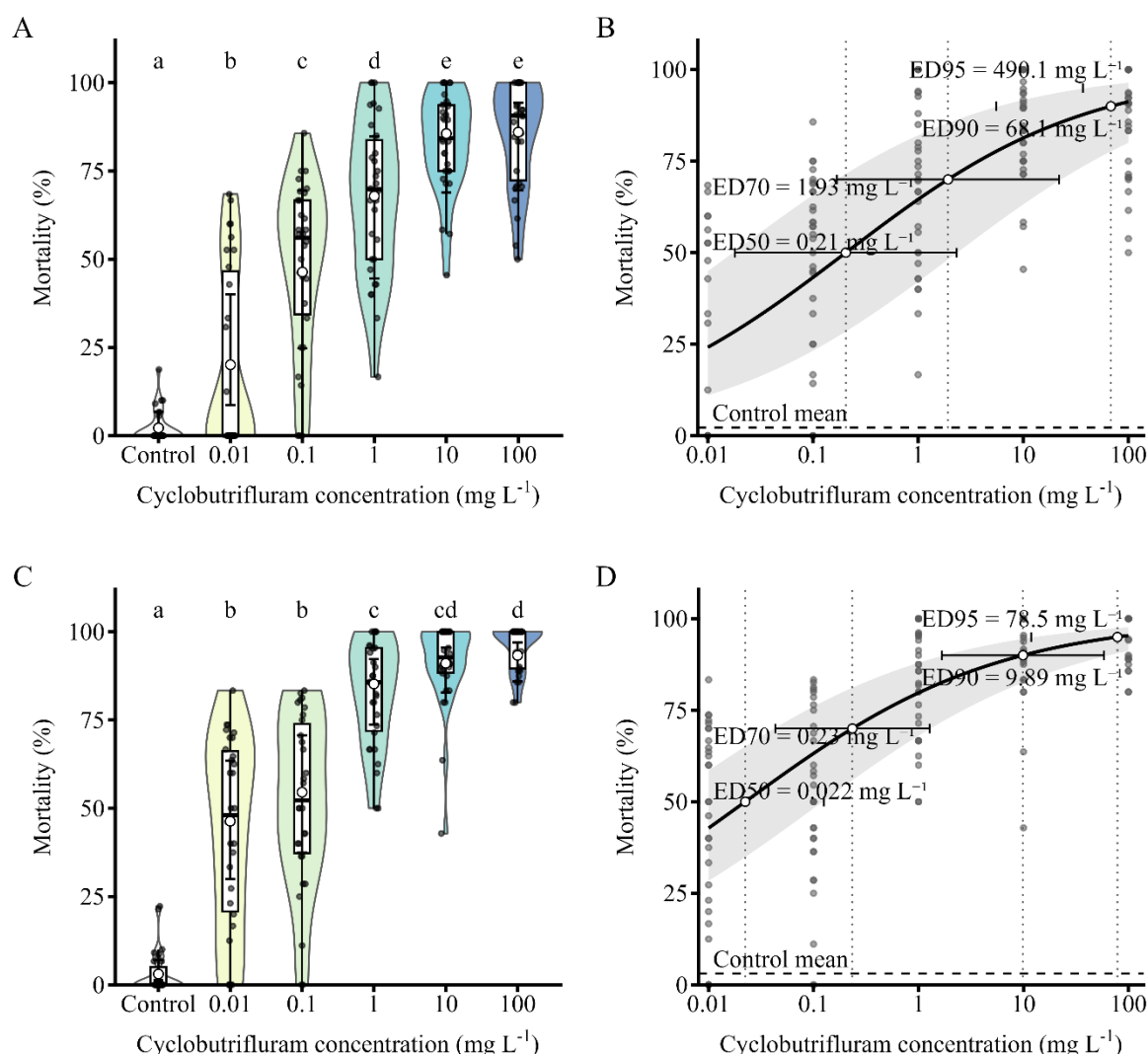


Figure 3. Mortality of *R. similis* exposed to cyclobutrifluram concentrations (0.01–100 mg L⁻¹) after 24 and 48 h. Violin plots (A, C) show adjusted means from beta-binomial generalized linear mixed models (GLMM) and 95% confidence intervals (CI); dose-response curves (B, D) show ED₅₀–ED₉₅ estimates.

Evaluation of plant-parasitic nematode inhibition by biocontrol microorganisms

Mortality of *M. incognita* varied among treatments in both evaluation periods according to beta-binomial generalized linear mixed models (GLMM; 24 h: $\chi^2 = 190.44$; 48 h: $\chi^2 = 175.60$; both df = 6 and $p < 0.0001$). All bacterial isolates resulted in significantly higher mortality than the controls, with mortality ranging from 36 to 46% at 24 h and from 33 to 44% at 48 h. Control mortality remained below 5% (Table 1; Figures 4A, 4B).

A similar pattern was observed for *M. enterolobii* (GLMM; 24 h: $\chi^2 = 124.57$; 48 h: $\chi^2 = 151.16$; both df = 6 and $p < 0.0001$). At 24 h, all bacterial strains formed a single

statistical group, with mortality ranging from 47% to 51%, whereas control mortality remained below 10%. At 48 h, *B. pumilus* (BR12158) resulted in 66.9% mortality and differed significantly from the other isolates (Table 1; Figures 5A, 5B).

These results are consistent with those of Engelbrecht et al. (2022), who reported high nematicidal activity of *Bacillus* spp. against *Meloidogyne* juveniles, supporting the mortality observed in the bacterial suspensions evaluated in the present study. Additional studies, such as that of Vasantha-Srinivasan et al. (2025), indicate that *Bacillus* spp. can affect plant-parasitic nematodes through multiple biological mechanisms, including the production of hydrolytic enzymes associated with reduced juvenile viability.

Table 1. Adjusted mortality percentage (AM%) and 95% confidence interval (CI95%) for *Meloidogyne incognita*, *M. enterolobii*, and *Radopholus similis* after 24 h and 48 h of exposure to different *Bacillus* spp. strains. Values were estimated using generalized linear mixed models (GLMM) with binomial or beta-binomial error distributions.

Treatment	AM% 24 h	CI95% 24 h	AM% 48 h	CI95% 48 h
<i>Meloidogyne incognita</i>				
Control (water)	4.15	2.25–7.54	3.06	1.60–5.78
Control (NaCl)	2.86	1.63–4.96	1.62	0.81–3.21
<i>B. siamensis</i> (CNPMF1009)	36.61	30.92–42.71	39.71	34.06–45.64
<i>B. pumilus</i> (BR12158)	43.24	37.71–48.95	34.44	29.29–39.97
<i>B. methylotrophicus</i>	44.44	38.72–50.32	33.21	27.94–38.94
<i>B. velezensis</i> (BR12157)	45.96	40.12–51.91	43.80	37.68–50.12
<i>Bacillus</i> sp. (BSF66)	38.11	32.46–44.11	35.52	30.22–41.20
<i>Meloidogyne enterolobii</i>				
Control (water)	1.86	0.60–5.61	3.08	1.29–7.20
Control (NaCl)	9.57	6.11–14.68	1.80	0.58–5.45
<i>B. siamensis</i> (CNPMF1009)	50.89	45.95–55.82	52.87	47.77–57.90
<i>B. pumilus</i> (BR12158)	51.08	43.12–58.99	66.95	61.75–71.78
<i>B. methylotrophicus</i>	48.73	44.06–53.43	48.37	43.60–53.18
<i>B. velezensis</i> (BR12157)	47.63	42.65–52.66	51.15	46.18–56.09
<i>Bacillus</i> sp. (BSF66)	50.47	45.71–55.22	43.91	39.22–48.72
<i>Radopholus similis</i>				
Control (water)	5.05	2.84–8.84	3.69	1.75–7.60
Control (NaCl)	9.42	6.30–13.88	1.63	0.52–4.97
<i>B. siamensis</i> (CNPMF1009)	36.78	30.31–43.78	34.13	27.58–41.34
<i>B. pumilus</i> (BR12158)	33.41	27.17–40.29	39.41	32.76–46.48
<i>B. methylotrophicus</i>	47.28	39.86–54.81	44.51	37.40–51.85
<i>B. velezensis</i> (BR12157)	43.19	36.76–49.87	49.58	42.44–56.74
<i>Bacillus</i> sp. (BSF66)	30.98	25.33–37.26	34.65	28.29–41.61

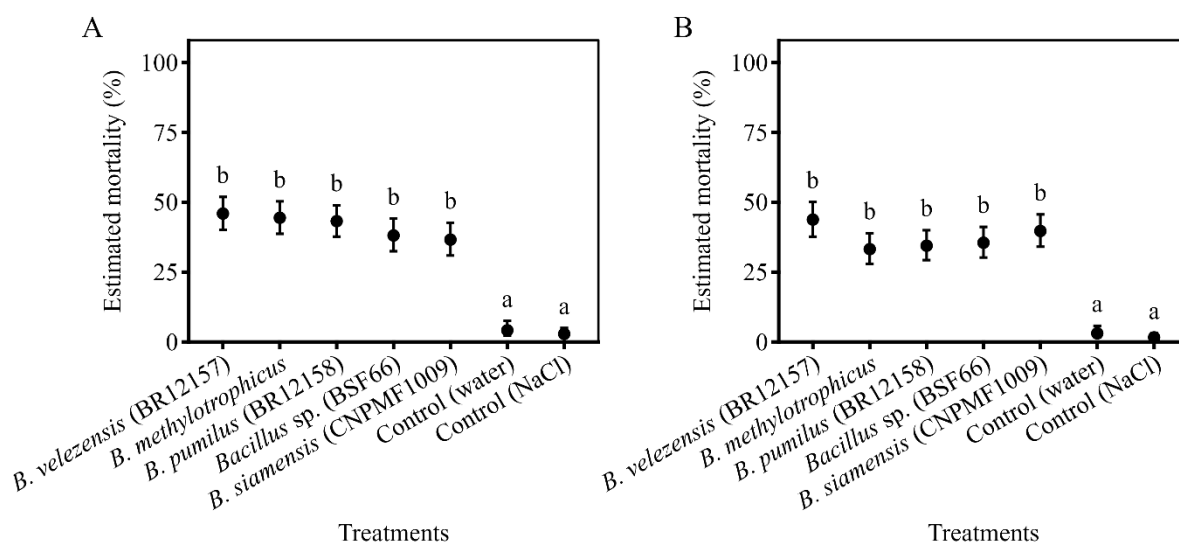


Figure 4. Adjusted mortality (%) of *Meloidogyne incognita* after 24 h (A) and 48 h (B) of exposure to different *Bacillus* spp. strains. Values were estimated using binomial generalized linear mixed models (GLMM). Different letters indicate significant differences (Sidak, $p \leq 0.05$). Points and error bars represent adjusted means and 95% CI.

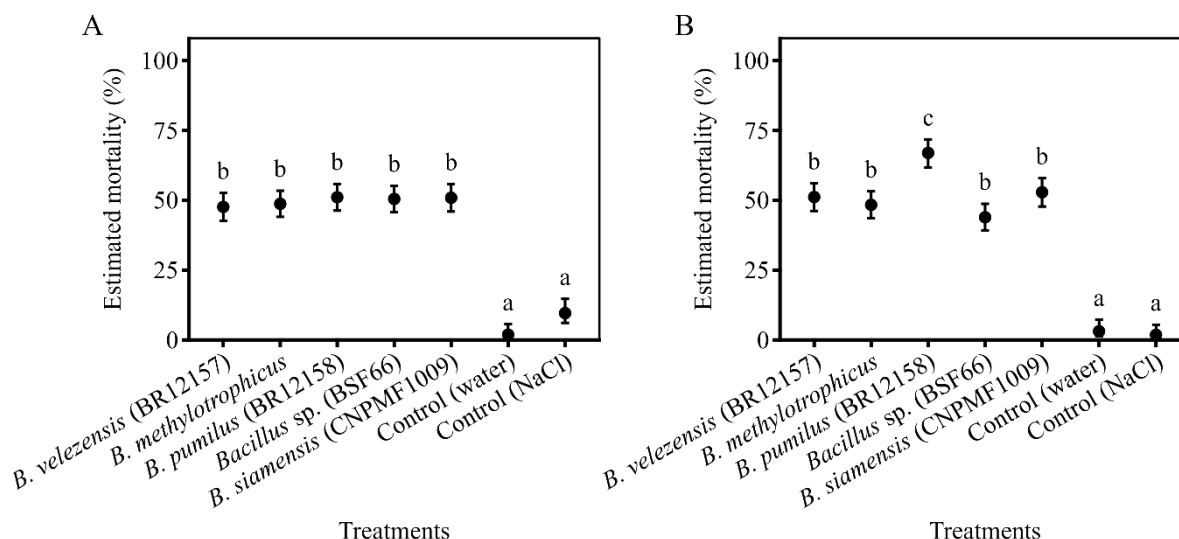


Figure 5. Adjusted mortality (%) of *Meloidogyne enterolobii* after 24 h (A) and 48 h (B) of exposure to different *Bacillus* spp. strains. Values were estimated using binomial generalized linear mixed models (GLMM). Different letters indicate significant differences (Sidak, $p \leq 0.05$). Points and error bars represent adjusted means and 95% CI.

Mortality of *R. similis* was significantly affected by treatment (GLMM; 24 h: $\chi^2 = 123.03$; 48 h: $\chi^2 = 102.63$; both $df = 6$ and $p < 0.0001$). At 24 h, *B. methylotrophicus* resulted in the highest mortality (47.3%), whereas BSF66 resulted in

the lowest mortality (31.0%). At 48 h, *B. velezensis* (BR12157) resulted in 49.6% mortality, followed by *B. methylotrophicus* (44.5%). Control mortality remained below 10% (Table 1; Figures 6A, 6B).

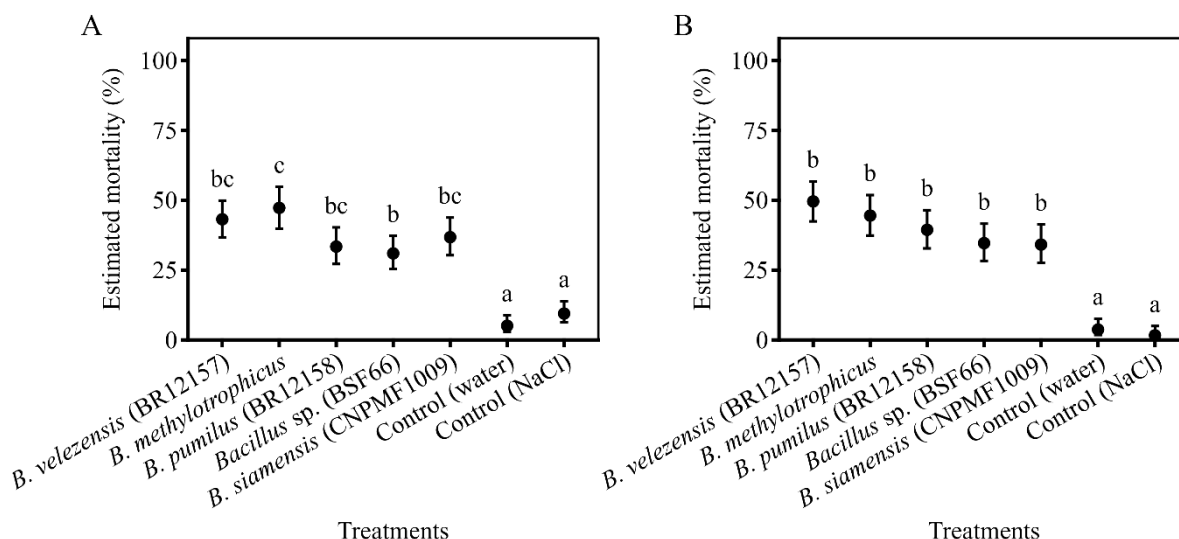


Figure 6. Adjusted mortality (%) of *Radopholus similis* after 24 h (A) and 48 h (B) of exposure to different *Bacillus* spp. strains. Values were estimated using beta-binomial generalized linear mixed models (GLMM). Different letters indicate significant differences (Sidak, $p \leq 0.05$). Points and error bars represent adjusted means and 95% CI.

This pattern indicates that *R. similis* was also susceptible to the bacterial suspensions evaluated in this study. Mendoza and Sikora (2009) reported marked reductions in *R. similis* in banana plants treated with *B. firmus*, whereas Chaves et al. (2009) observed lower root invasion, reduced

final pathogen populations, and stimulated plant growth. Similarly, Thammaiah et al. (2019) demonstrated that *B. subtilis* significantly reduced *R. similis* population density in soil. These findings support the potential of *Bacillus* spp. for *R. similis* management and indicate nematicidal activity

associated with the bacterial strains evaluated in vitro.

In summary, the *Bacillus* strains exhibited consistent nematocidal activity against the three species evaluated, indicating their potential as complementary tools for the management of plant-parasitic nematodes. These findings are consistent with recent reviews that identify *Bacillus* spp. among the most promising biological agents for the management of plant-parasitic nematodes due to their multiple modes of action and broad spectrum of activity against different nematode species (PIRES et al., 2022). The results obtained support the next stage of this study, which focused on the physiological compatibility between *Bacillus* spp. and cyclobutrifluram to evaluate the feasibility of their combined use for the management of plant-parasitic nematodes.

Physiological compatibility between cyclobutrifluram and biocontrol microorganisms

B. siamensis (CNPMF1009) showed consistent growth at all concentrations evaluated ($0.01\text{--}100\text{ mg L}^{-1}$), with growth curves overlapping the control and maintaining an upward trend throughout 96 h of incubation (Figure 7). The area under the microbial growth curve (AUMGC) values indicated no inhibition by cyclobutrifluram, with means ranging from 21.4 to 24.5, higher than the control value of 20.0. The statistical assumptions were met, and ANOVA followed by Tukey's test ($\alpha = 0.05$) detected no significant differences among doses ($p > 0.05$; Figure 8).

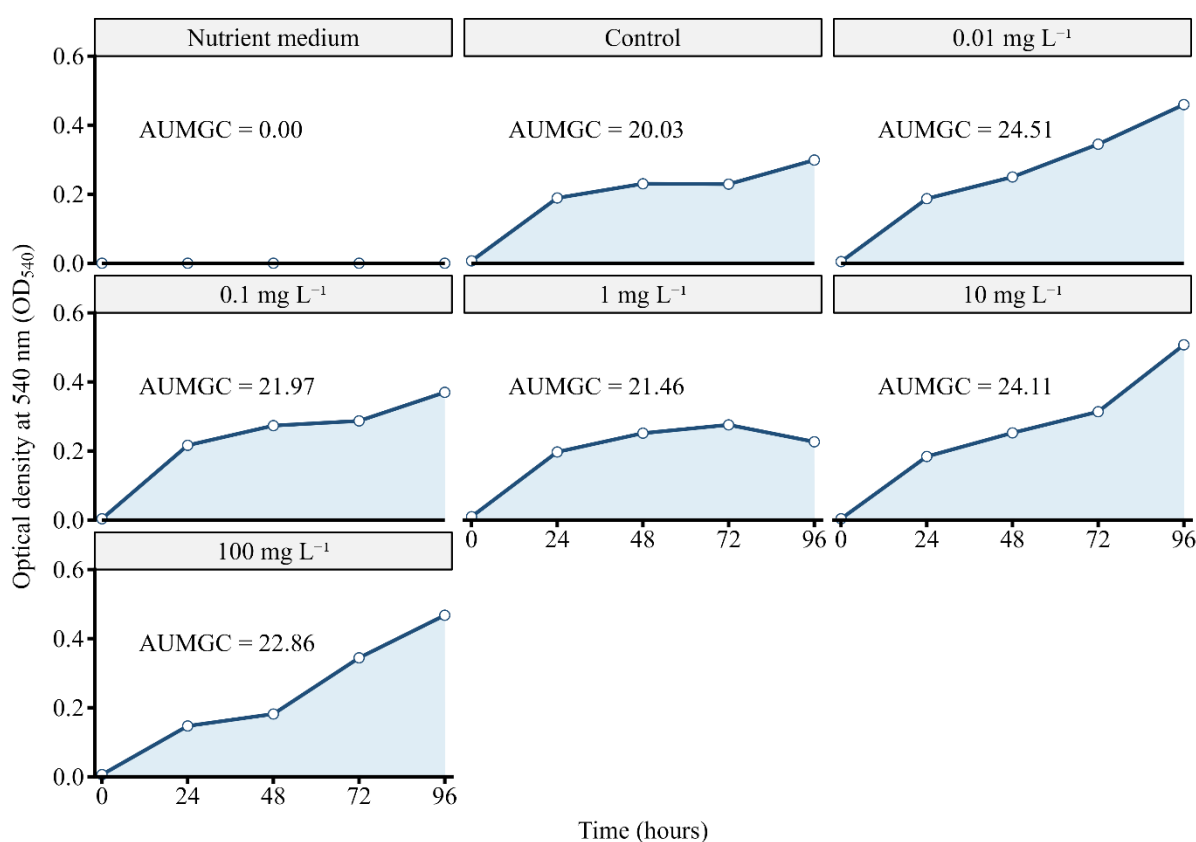


Figure 7. Growth curves of *Bacillus siamensis* (CNPMF1009) at different cyclobutrifluram concentrations ($0.01\text{--}100\text{ mg L}^{-1}$) during 96 h of incubation. Shaded areas represent the accumulated area under the microbial growth curve (AUMGC). Readings were performed every 24 h in 96-well microplates.

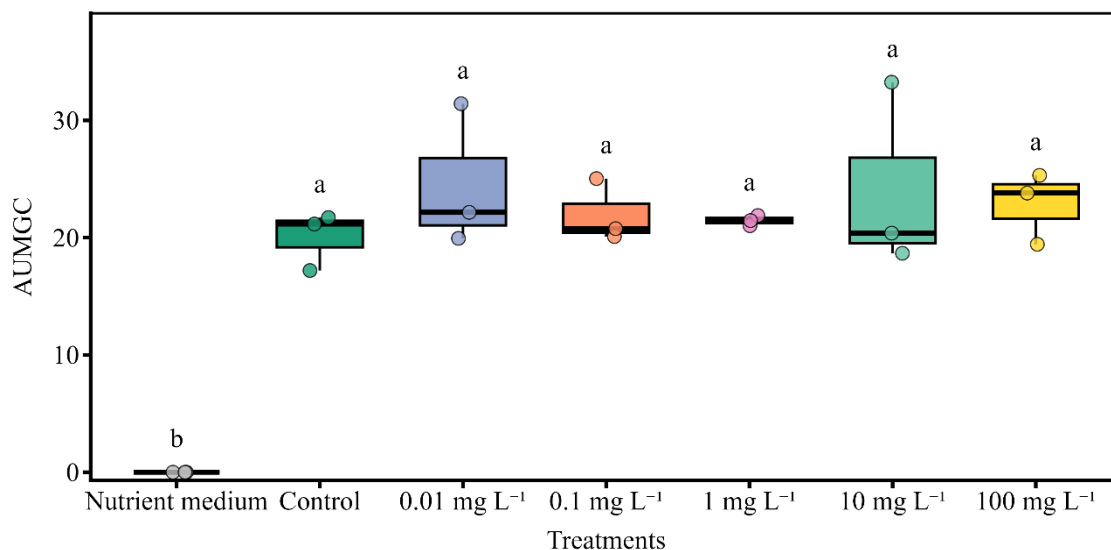


Figure 8. Distribution of area under the microbial growth curve (AUMGC) values for *Bacillus siamensis* (CNPMF1009) at different cyclobutrifluram concentrations over 96 h of incubation. Equal letters indicate no significant differences by Tukey's test ($\alpha = 0.05$).

Similarly, *B. velezensis* (BR12157) maintained consistent growth in the presence of the nematicide (Figure 9), with AUMGC values ranging from 18.7 to 25.2, equal to or higher than the control value of 16.2, and no significant

differences among doses ($p > 0.05$; Figure 10). Even the highest concentrations did not substantially reduce growth, indicating metabolic stability of the strain in response to the compound.

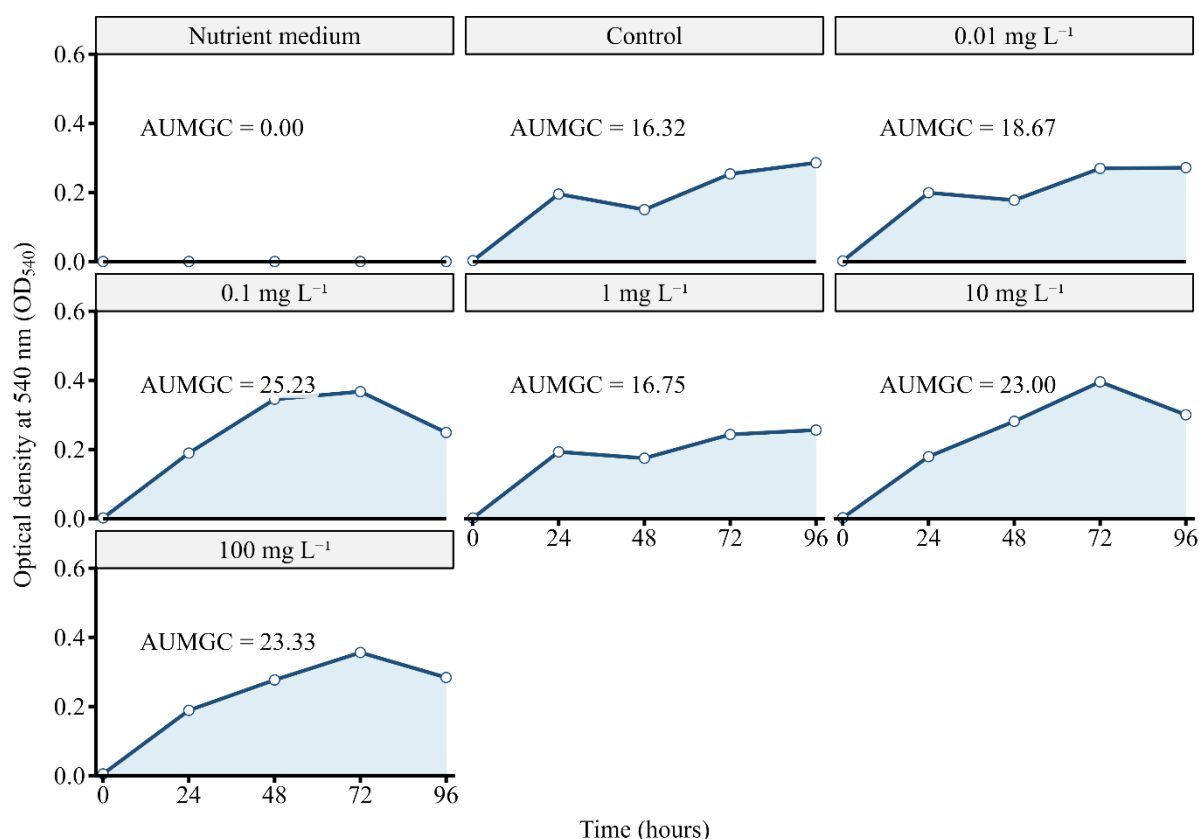


Figure 9. Growth curves of *Bacillus velezensis* (BR12157) at different cyclobutrifluram concentrations, with the shaded area representing the area under the microbial growth curve (AUMGC) during 96 h of incubation.

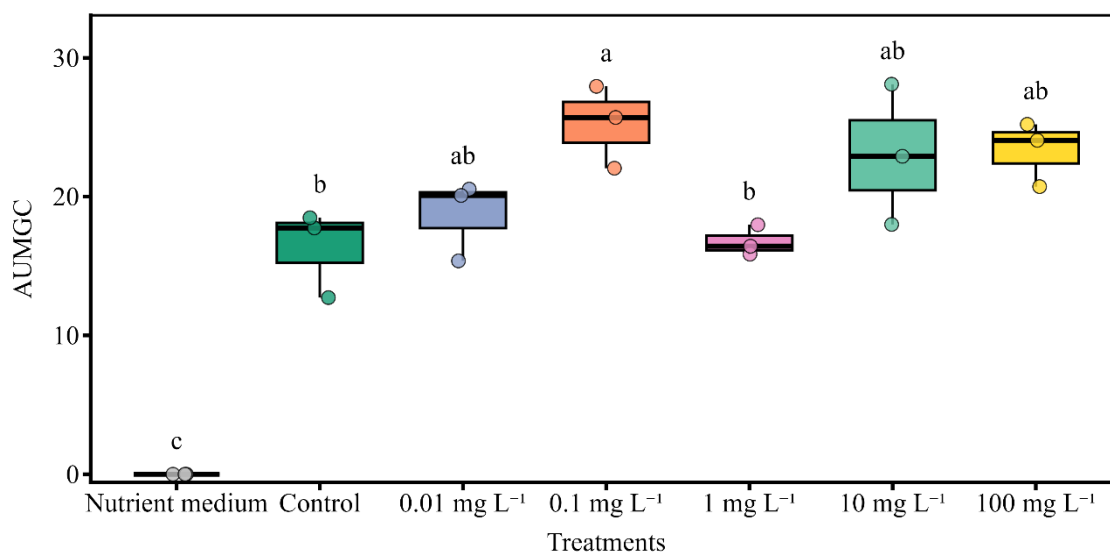


Figure 10. Distribution of area under the microbial growth curve (AUMGC) values for *Bacillus velezensis* (BR12157) at different cyclobutrifluram concentrations over 96 h of incubation. Equal letters indicate no significant differences by Tukey's test ($\alpha = 0.05$).

These results indicate physiological compatibility among *B. siamensis* (CNPMF1009), *B. velezensis* (BR12157), and cyclobutrifluram, which is consistent with the mode of action of the compound on mitochondrial succinate dehydrogenase, a target absent in prokaryotes (LIU et al., 2024; MAIENFISCH; KOERBER, 2024). The slight numerical increase in growth at some concentrations may reflect experimental variability or effects of formulation components on the physicochemical properties of the medium, which affect nutrient absorption and cellular metabolism.

Sublethal concentrations may also induce adaptive stress responses by activating genes related to heat shock proteins, enzymes, and secondary metabolites, as previously reported for *Bacillus* under chemical exposure (JIANG et al., 2022). This response is consistent with the physiological profile of the genus, which is recognized for adaptive capacity under biotic and abiotic stresses (LAHLALI et al., 2022). Taken together, the results indicate that *B. siamensis* (CNPMF1009) and *B. velezensis* (BR12157) are compatible with cyclobutrifluram at the concentrations evaluated, supporting the potential for combined use of these agents in integrated plant-parasitic nematode management strategies.

The control had the highest AUMGC for *T. asperellum* (CNPMF 1007) (81.9), whereas concentrations of 0.01, 0.1,

and 10 mg L⁻¹ resulted in intermediate values (34.1–40.5). In contrast, 1 mg L⁻¹ markedly reduced AUMGC (10.2), followed by 100 mg L⁻¹ (24.4), indicating greater sensitivity at specific product concentrations (Figure 11). The box plot (Figure 12) supported these differences, with distinct significance letters based on Tukey's test ($\alpha = 0.05$).

This pattern indicates partial compatibility, with maintained viability but a significant reduction in fungal growth at specific concentrations. The observed sensitivity is consistent with the cyclobutrifluram mode of action on succinate dehydrogenase (SDH) in mitochondrial Complex II, which impairs energy metabolism in eukaryotic organisms (LIU et al., 2024; MAIENFISCH; KOERBER, 2024). Unlike *Bacillus*, *T. asperellum* depends on the mitochondrial respiratory chain, which explains its greater susceptibility. However, the absence of complete inhibition suggests partial tolerance, possibly associated with antioxidant mechanisms and compensatory metabolic pathways.

Therefore, simultaneous use of *T. asperellum* and cyclobutrifluram should be approached with caution. Sequential application with a time interval between treatments may be preferable to preserve biocontrol agent efficacy in soil, consistent with chemical–biological compatibility principles in integrated management (ABD-ELGAWAD; ASKARY, 2018).

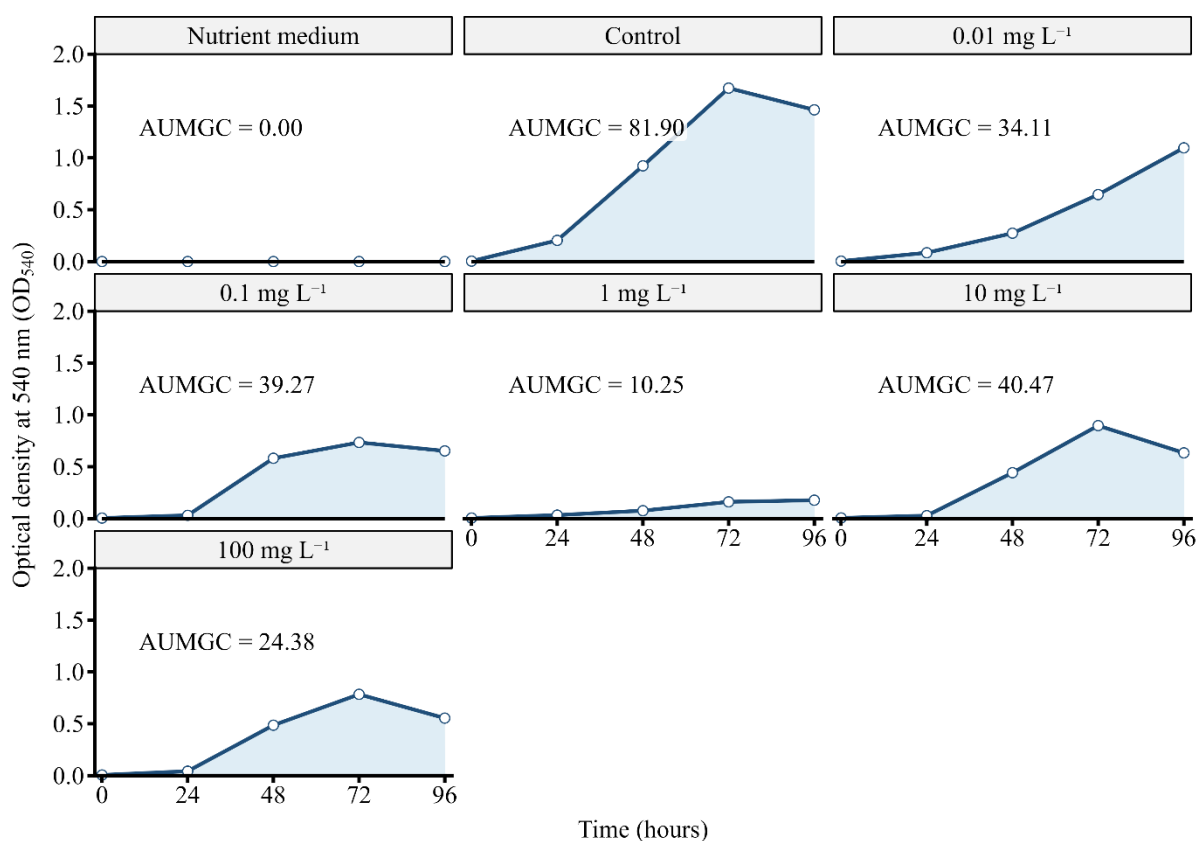


Figure 11. Growth curves of *Trichoderma asperellum* after exposure to different cyclobutrifluram concentrations (0.01–100 mg L⁻¹) over 96 h of incubation, highlighting the area under the microbial growth curve (AUMGC).

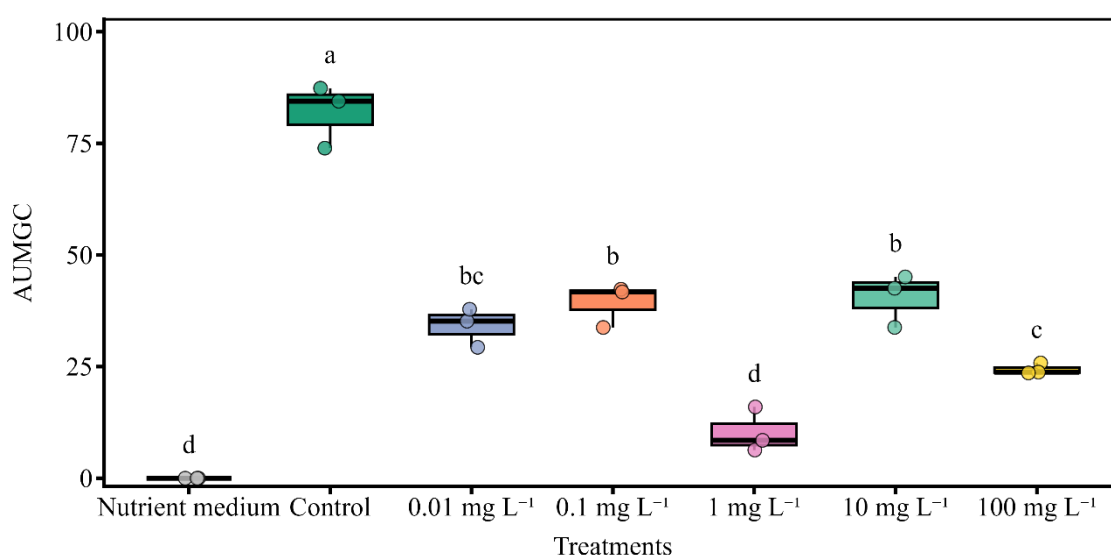


Figure 12. Distribution of area under the microbial growth curve (AUMGC) values for *Trichoderma asperellum* (CNPMF 1007) at different cyclobutrifluram concentrations (0.01–100 mg L⁻¹) over 96 h of incubation. Equal letters indicate no significant differences by Tukey's test ($\alpha = 0.05$).

Biofilm formation by *Bacillus* spp. using the crystal violet (CV) assay

Biofilm formation varied significantly among the strains evaluated ($\chi^2 = 77.94$; $df = 4$; $p = 0.001$). The box plot (Figure 13A) and statistical analysis (Table 2) showed marked differences in adhesion capacity and extracellular matrix production among the isolates.

The results indicated that biofilm formation varied among the evaluated strains, with most strains exhibiting biofilm-forming capacity at different intensities. *B. velezensis* (BR12157), *B. methylotrophicus*, and *Bacillus* sp. (BSF-66) were predominantly classified as strong biofilm formers, whereas *B. siamensis* (CNPMF1009) was predominantly classified as a weak biofilm former. In contrast, *B. pumilus* (BR12158) was predominantly classified as a non-biofilm-forming strain, although weak and strong biofilm-forming classifications were also observed among individual observations (Figure 13B). Nevertheless, *B. siamensis* (CNPMF1009) and *B. velezensis* (BR12157) remain the strains of greatest agronomic relevance because of their compatibility with cyclobutrifluram and the nematicidal activity demonstrated in previous experiments.

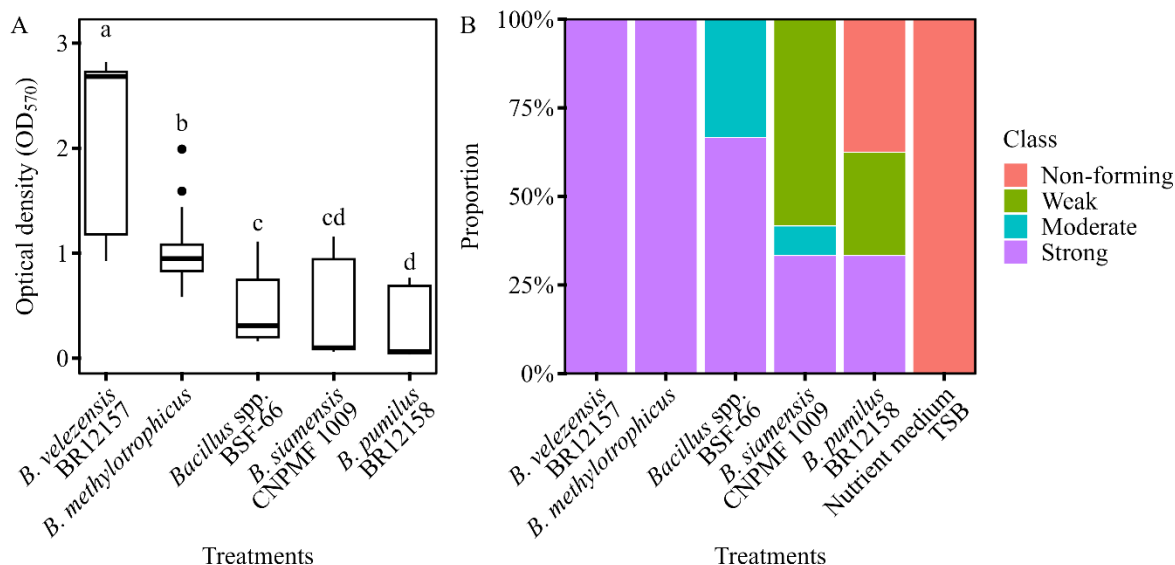


Figure 13. Biofilm formation by *Bacillus* spp. strains. (A) Box plot of biofilm formation. (B) Distribution of biofilm classes, expressed as the proportion of readings per treatment. Different letters indicate significant differences according to the Kruskal–Wallis test followed by Dunn’s test with Holm adjustment ($p \leq 0.05$).

Table 2. Descriptive statistics for biofilm formation by *Bacillus* spp. strains, including mean optical density at 570 nm (mean OD₅₇₀), standard deviation (SD), standard error (SE), 95% confidence interval (95% CI), and predominant class according to the criteria of Stepanović et al. (2007).

Treatment	Mean OD ₅₇₀	SD	SE	CI95% lower	CI95% upper	Predominant class
<i>B. velezensis</i> (BR12157)	2.12	0.76	0.16	1.80	2.44	Strong
<i>B. methylotrophicus</i>	1.01	0.31	0.06	0.87	1.14	Strong
<i>Bacillus</i> sp. (BSF66)	0.48	0.33	0.07	0.34	0.61	Strong
<i>B. siamensis</i> (CNPMF1009)	0.39	0.44	0.09	0.21	0.58	Weak
<i>B. pumilus</i> (BR12158)	0.27	0.32	0.06	0.14	0.41	Non-forming

The results suggest that the nematicidal activity of strains CNPMF 1009 and BR12157 may involve mechanisms other than biofilm formation, potentially including the production of antimicrobial metabolites and other physiological processes. Although biofilm may increase microbial persistence in some environments (FESSIA et al., 2022), *Bacillus* uses multiple biocontrol strategies, such as secretion of antimicrobial compounds and induction of plant

defenses (ZHANG et al., 2023).

In summary, although biofilm formation is advantageous for microbial colonization and persistence, the results suggest that strains CNPMF1009 and BR12157 may retain nematicidal activity despite limited surface adherence. These strains are therefore promising candidates for combined use with cyclobutrifluram in integrated plant-parasitic nematode management programs.

CONCLUSION

Cyclobutrifluram showed high nematocidal activity against *Meloidogyne incognita*, *M. enterolobii*, and *Radopholus similis* under in vitro conditions. The strains *Bacillus siamensis* (CNPMF1009), *B. pumilus* (BR12158), *B. methylotrophicus*, *B. velezensis* (BR12157) and *Bacillus* sp. (BSF66) showed nematocidal activity. In addition, *B. siamensis* (CNPMF1009) and *B. velezensis* (BR12157) maintained growth compatible with cyclobutrifluram at the evaluated concentrations. Reduced growth was observed for *Trichoderma asperellum* at specific product concentrations, indicating differences in sensitivity among the evaluated microorganisms.

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