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CHARACTERIZATION OF THE ANTAGONIST MICROBIOTA IN BIOFERTILIZERS AND THEIR ROLE IN MULTITROPHIC INTERACTIONS FOR THE MANAGEMENT OF *Meloidogyne exigua* IN ARABICA COFFEE UNDER A RANDOMIZED BLOCK DESIGN WITH A 9×2 FACTORIAL SCHEME

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Abstract

Root-knot nematodes are among the main pathogens of Arabica coffee, with emphasis on Meloidogyne exigua. Among the management alternatives, the use of biofertilizers of microbial origin stands out, but this is commonly limited, due to the lack of information about which species make up the product. This information is essential for the management and association of biofertilizers with chemical nematicides, for example. Therefore, the objective of this study was to assess the genetic diversity of microorganisms present in two commercial biofertilizers used for the management of M. exigua and to evaluate their effectiveness in reducing the population of M. exigua in Arabica coffee crops when associated with two chemical nematicides. To this end, the microorganisms present in biofertilizers Bio1 and Bio2 were isolated in different culture media and morphologically and genetically characterized. The effectiveness of biofertilizers alone and associated with chemical nematicides in controlling the M. exigua population was evaluated in an Arabica coffee plantation cv. Catuaí 44. For this, 218 bacteria distributed in 61 genetic groups were isolated. Higher Relative Efficiency values were observed in plants treated with Bio1+Bio2+Fluensulfone, Bio2, Bio1+Bio2, and Bio1, respectively. The reduction of root nematodes was greater in the Fluensulfone treatment, followed by Bio1 and Bio2. For the number of soil nematodes, the greatest reduction occurred in the Bio1+Bio2 and Bio2 treatments. The results demonstrate the effectiveness of biofertilizers containing microorganisms that act to promote growth and bioprotection, when used for reducing the population of M. exigua in Arabica coffee crops.

Keywords: Biological control agentes. Coffea. Microorganisms. Nematode. Pests.

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1. Introduction

The cultivation of coffee (*Coffea arabica* L. and *C. canephora* Pierre ex Froehner) is stimulated and promoted, due to ongoing growing consumption of coffee as a drink, and it is considered the second most traded commodity worldwide (Czarniecka-Skubina et al. 2021). Brazil has an area dedicated to coffee cultivation (arabica and conilon) of 2.26 million hectares, ranking first in world coffee production. In this ranking, Minas Gerais, Espírito Santo and São Paulo stand out as the states with the highest production, in that order (Companhia Nacional de Abastecimento – CONAB, 2023).

Among the main factors that limit coffee production in Brazil, root-knot nematodes stand out, especially the species *Meloidogyne exigua Göldi,* 1887 (Afridi et al. 2024), which inflicts damage by inducing rounded galls on the roots, resulting in economic losses (Machado et al. 2023). *Meloidogyne exigua* normally infects coffee plants from the seedling stage to the adult stage, and the nematode's attack on the plant's root system makes the plant inefficient in absorbing water and nutrients, resulting in less vigorous plants that are unable to grow (Zinger et al. 2021). According to the level of initial infestation, and the spatial distribution of nematodes, among other factors, plants grow to unequal sizes, forming clods in the crop, chlorotic leaves, similar to symptoms of nutritional deficiency and decreased production, making economic exploitation unfeasible (Al Sadek et al. 2023).

The management strategies used to reduce the *Meloidogyne* spp. population include the application of biofertilizers and low-toxicity nematicides, aiming for sustainable control (Alves et al. 2021; Nawaz et al. 2023).

However, despite the recommendation for the use of biofertilizers, the effects of these products are not fully explained by manufacturers. This lack of information limits the understanding of these products in the management of *M. exigua*, since in addition to their protective potential, biofertilizers also act to promote plant development, due to the probable association of microorganisms with the multitrophic communities of the rhizosphere (Sousa et al. 2022).

Microorganisms potentially protect and promote the development of economically important crops. Given the global demand for coffee production (arabica and conilon) and the significant losses caused by *M. exigua* in the field, efficient control strategies are needed (Afridi et al. 2024). The morphological and genetic characterization of the microorganisms present in biofertilizers aims to optimize their application and to modulate the composition of the effective microbial community.

Therefore, the objective of this research was to identify the morphological and genetic diversity of cultivated microorganisms present in commercial organic fertilizers and to evaluate the effectiveness of the combined application of chemical nematicides and biofertilizers in the management of *M. exigua* in Arabica coffee crops in Espírito Santo state (Brazil).

2. Material and Methods

Isolation and morphological characterization of microorganisms

The commercial biofertilizers Bio[1] (composed of strains of *Bacillus subtilis Cohn*, *B. licheniformis Weigmann Chester* and *Lactobacillus* spp.) and Bio[2] (100% organic commercial fluid rich in organic matter containing humic and fulvic acids) were used for bacterial isolation on Nutrient Agar (NA), Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA), Eosin Methylene Blue Agar (EMB), Lactose Broth (LB) and Kado medium. After three days of incubation in a bacterial growth chamber at 28°C, the plates were analyzed with the aid of a Leica model S8AP0 magnifying glass and photographed for morphological characterization of the bacteria.

Detection of siderophore production by bacterial isolates

For the detection of the production of siderophores by *Pseudomonas* spp. from the fluorescent group, a Spot test was carried out with King B culture medium, which allows the production of these molecules to be identified through the fluorescence property after exposure to ultraviolet light. Bacterial suspension (5µL - OD 570nm= 0.52) of 28 isolates was inoculated into Petri dishes containing King B.

Pseudomonas syringae Van Hall pv. tomato was used as a positive control for siderophore production and *Ralstonia solanacearum* (Smith) (Yabuuchi et al. 1995) as a negative control. The plates were incubated in a bacterial growth chamber for a period of three days at 28 °C. After the incubation period, analysis was carried out for fluorescence detection by exposing the bacterial colonies to UV light from the Loccus L-Pix EX photodocumenter.

Molecular characterization of bacterial isolates presents in biofertilizers

Extraction of genomic DNA

The bacteria present were previously grown in solid Kado culture medium for 48-72h and used to extract genomic DNA (Narayan et al. 2016) with adaptations. Therefore, bacterial cultures were homogenized in 500 µl of Suspension Buffer (S Buffer) (10 % Sucrose, 10 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 50 nM NaCl) until a pellet of approximately 20 to 40 mg was obtained. Then, 20 µl of lysozyme (10 mg/ml) was added to the cell suspension and incubated in a water bath at 37° C for 45 min, with vigorous inversions being carried out at 15-min intervals, followed by the addition of 5 µl of Proteinase K (20 mg/ml) and incubated again at 37 °C for 45 min. Subsequently, 50 µl of sodium dodecyl sulfate (SDS) (20% w/v) was added and taken to a water bath at 65 °C for 45 min. The lysate was centrifuged at 11,000xg for 3 min at 20 °C with the purpose of sedimenting cellular debris at the bottom of the microtubes and then collecting and transferring the supernatant to a new 1.5 ml microtube containing 20 µl of RNAse A (5 mg/ml) and incubated again in a water bath at 37 °C for 45 min. To promote the precipitation of cellular proteins and other cellular residues, the equivalent of 0.35X of 2.5 M potassium acetate (pH 8.0) was added, and 15 to 20 gentle inversions were performed to return to centrifuge at low speed (6,500xg) and high (8,000xg) at 20 °C for 3 min in order to promote sedimentation of the precipitate. The supernatant was collected again and transferred to a new 1.5 ml microtube, and the same volume of isopropanol-collected supernatant was added; gentle inversions were performed and incubated for 5 min at room temperature.

After incubation, the solution was centrifuged at 11,000 xg at 4 °C for 20 min to promote DNA sedimentation and discard the supernatant. The DNA pellets were washed twice with 200 µl 70 % ethanol and, after each addition, the microtubes were taken to the centrifuge at 11,000xg at 4 °C for 2 min, and the supernatant was subsequently discarded. A new centrifugation at 11,000 xg at 4 °C for 2 min was carried out in order to promote the descent of the remaining 70% ethanol present on the walls of the microtubes, which was removed with the aid of 10µl tips. After this procedure, the microtubes were left open to complete drying in a fume hood for 10 min and resuspended in 50 µl of TE (pH 8.0) and stored at -20 ° C. After quantification, the integrity of the genomic DNA was analyzed on a 1% agarose gel in 1X TAE buffer, under electrophoresis at 80 V for 1 hour. Based on the gel, it was possible to observe that the selected samples presented a good concentration of genetic material and good integrity for carrying out Rep-PCR.

Genomic fingerprint using Rep-PCR

PCR reactions were performed as proposed by Silva and Valicente (2013). For reactions using the ERIC and BOX primers, amplifications were conducted using 1µl of genomic DNA (30 ng / µl), 0.5 µl of primers (10 µM / 35 µl) and 12.5 µl of Taq Polymerase – Master mix (2X) Green (Cellco Biotec) and volume of ultrapure water until 25 µl of reaction was completed. Primer sequences were designed by Versalovic (1994): ERIC-1: 5’ - ATG TAA GCT CCT GGG GAT TCA C - 3’; ERIC-2: 5’ - AAG TAA GTG ACT GGG GTG AGC G - 3’; BOX A1R: 5' - CTA CGG CAA GGC GAC GCT GAC G - 3’. Amplifications were performed in a Loccus TC-9639 thermocycler using the following program: 5 min at 94 °C, followed by 40 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min. An extension step at 72 ° C for 7 min was added. The amplified fragments were separated on a 1.2 % agarose gel in TAE buffer (0.5 M EDTA; 1M TRIS, pH 8.0; acetic acid), under electrophoresis at 80V for 1 hour and 30 min, and before the run, 1 µl of GelRed 10,000X was added to the samples. After the run, the agarose gel was taken to the Loccus L-Pix EX photodocumenter to be analyzed and photographed. For reactions using REP primers, amplifications were conducted using 2µl of genomic DNA (30 ng/ µl), 0.5 µl of primers (10µM/ µl) and 12.5 µl of Taq Polymerase – Master mix (2X) Green (Cellco

Biotec) and volume of ultrapure water until 25 µL of reaction was completed. Primer sequences were designed by Versalovic et al. (1994): REP1R - 5' - III ICG ICG ICA TCI GGC -3'. REP2I - 5' - ICG ICT TAT CIG GCC TAC -3'. Amplifications were performed in a Loccus TC-9639 thermocycler using the following program: 5 min at 94 °C, followed by 41 cycles at 94 °C for 1 min, 45 °C for 1 min and 72 °C for 2 min. An extension step at 72 °C for 7 min was added. The amplified fragments were separated on a 1.5% agarose gel in TAE buffer (0.5 M EDTA; 1M TRIS, pH 8.0; acetic acid), under electrophoresis at 80V for 1 hour and 30 min, and then 2 µl of GelRed 10,000x was added to the samples. After the run, the agarose gel was taken to the Loccus L-Pix EX photodocumenter to be analyzed and photographed. The fingerprinting results were analyzed using the GelJ software package (Heras et al. 2015). The similarity between the digitized profiles was calculated using the Jaccard similarity coefficient (CSJ), and, based on the dissimilarity estimates of the isolates, these profiles were grouped using the hierarchical UPGMA method (Method of Average Linkage Between Groups).

Efficacy of biofertilizers and chemical nematicides in the management of *M. exigua* in Arabica coffee.

The experiment was carried out in a commercial Arabica cv. Catuai 44 coffee plantation on plants aged 23 years old, cultivated in 3.0 x 1.5 spacing, located near the Bonsucesso river in Iúna, Espirito Santo state, latitude 20°17'38.2" South, longitude 41°30'02.9" West and altitude of 435 m, with the soil classified as a dystrophic Red-Yellow Oxisol with a clayey texture with natural occurrence of *M. exigua*. The two biofertilizers were purchased from a local market and used in this experiment: Bio[1]: organic product containing strains of *Bacillus subtilis*, *B. licheniformis* and *Lactobacillus* spp.; Bio[2]: 100% organic commercial fluid rich in organic matter containing humic and fulvic acids.

The experiment was carried out in a randomized block design in a 9 x 2 factorial scheme, in which the efficacies of nine products were studied: (T1 - negative control); T2 - Cadusafos (15 L/ha: Positive control); T3 - Fluensulfone (1.0 L/ha); T4 - Bio[1] (10 L/ha); T5 – Bio[2] (20 L/ha); T6 - Bio[1] 10 L/ha + Fluensulfone (1.0 L/ha); T7 – Bio[2] 20 L/ha + Fluensulfone 1.0 L/ha; T8 - Bio[1]10 L/ha + Bio[2] 20 L/ha and T9 - Bio[1] 10 L/ha + Bio[2] 120 L/ha + Fluensulfone 1.0 L/ha); in two evaluation periods: before applying the products (initial population) and 45 days after applying the products (45 DAA). The experiment consisted of four blocks in a total of 72 experimental plots, each consisting of eight plants, with the six central plants being analyzed.

The application of the products was carried out with the aid of a backpack applicator, with a spray volume of 16 L / ha, applying 50 cm from the projection of the crown at the top of the planting line, a drench of 100 ml per plant in a half-moon.

Before setting up the experiment, soil and roots were collected to quantify the initial population of *M. exigua*. Subsequently, 45 days after application of the products (DAA), soil and root samples were taken from 0 cm to 30 cm deep in the rhizosphere region, totaling 1kg of soil and 100g of root from each plant. These samples were identified and packaged in plastic bags and taken to the Laboratory for processing, extraction and quantification of second-stage juveniles (J_2) of *M. exigua*. The extraction of soil nematodes was carried out according to Jenkins (1964) and root nematodes were carried out according to Boneti & Ferraz (1981).

The percentage of *M. exigua* population reduction was calculated considering the population at 45 DAA with the respective initial nematode population for each treatment. This calculation was made both for the total number of nematodes /10g of root and for the total number of nematodes / 100 cc of soil. Control efficiency was estimated for each treatment, in accordance with Paul et al. (2008):

$$L^{sev}=\ln\left(\frac{Sev_{Trt}}{Sev_{Test}}\right)$$

The ratio of the natural log response of severity (L^{sev}) is calculated from the average severity of the nematicide treatment (Sev_{Trt}) and the average severity in the control (Sev_{Test}).

The effects of treatments and time on *M. exigua* were evaluated by fitting generalized linear mixed effects models (GLMM). All models were fit using the *lme4* package Bates et al. (2015) in R 4.3.2 (Komelj 2023). Pairwise comparisons between factor levels were based on the Tukey test at 5% significance.

3. Results

Morphological and genomic profile of bacteria associated with two biofertilizers used in the management of *M. exigua*

A total of 218 colonies were grown from the two biofertilizers, 166 isolated from Bio[1] and 52 from Bio[2] (Table 1). The morphological characteristics of the colonies varied in relation to size, shape, elevation, surface, color, brightness and edge, and these were used to group the isolates found into 61 groups, with two isolates found in Bio[2], 38 in Bio[1] and 21 present in both biofertilizers. This diversity of colonies is demonstrated in Figure 1. Among the culture media used for isolation, no colonies were detected on the Eosin Methylene Blue Agar (EMB) medium, suggesting the absence of representatives of the Enterobacteriaceae family. The presence of fungi was also not found in the media indicated for this group of microorganisms (PDA/SDA) (Table 1).

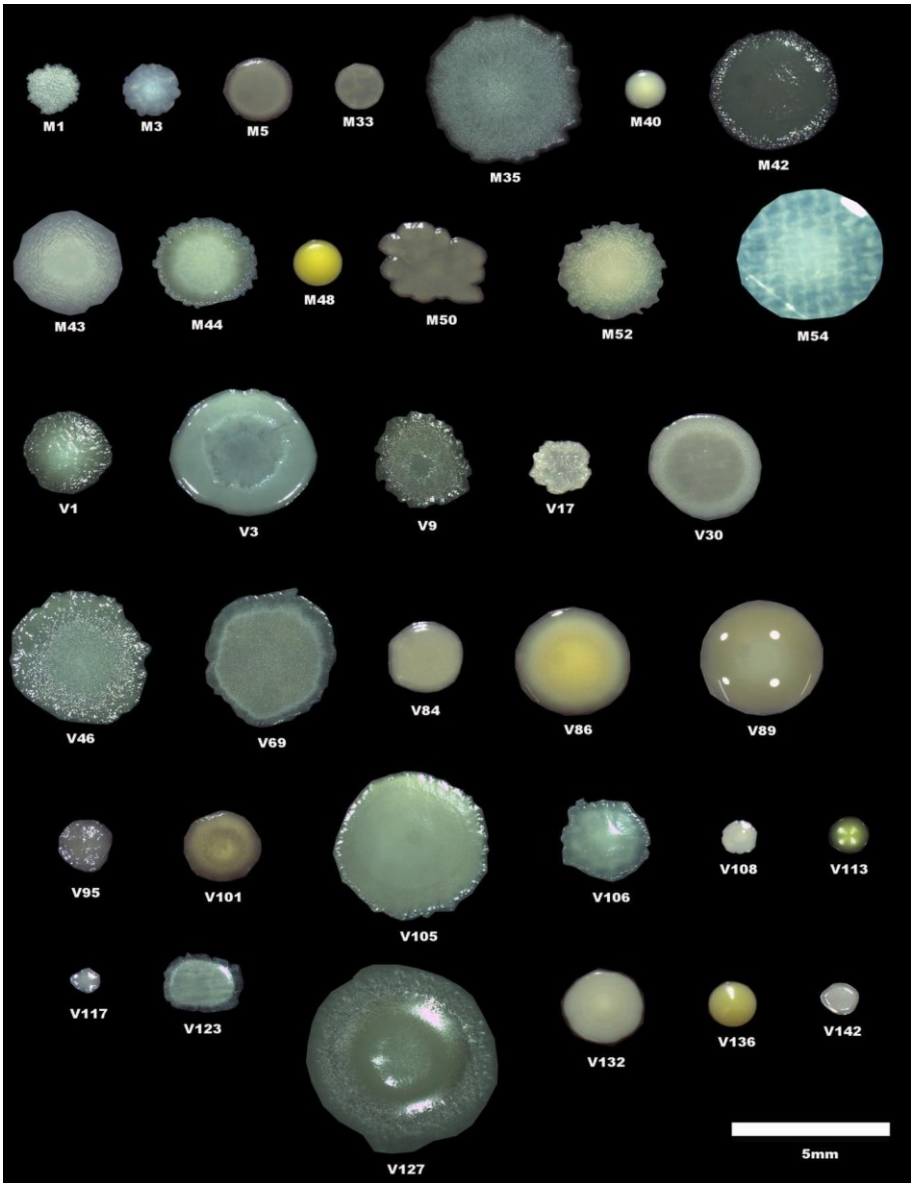

Figure 1. Colonies of some bacterial groups isolated in different culture media from two biofertilizers; one of them (Bio[1] (V)) is an organic product containing strains of *Bacillus subtilis*, *B. licheniformis* and *Lactobacillus* spp.; and the other (Bio[2] (M)) a 100% organic commercial fluid rich in organic matter containing humic and fulvic acids, which were purchased from the local market.

Table 1. Relationship of the number of bacteria isolated in different culture media from biofertilizers, Bio[1], an organic product containing strains of *Bacillus subtilis*, *B. licheniformis* and *Lactobacillus* spp., and Bio[2], a 100% organic commercial fluid rich in organic matter containing humic and fulvic acids.

Culture media	Bio[1]	Bio[2]	Total
Nutrient Agar (NA)	7	3	10
Potato Dextrose Agar (PDA)	39	9	48
Sabouraud Dextrose Agar (SDA)	6	0	6
Eosin Methylene Blue Ágar (EMB)	0	0	0
Lactose Broth (LB)	50	8	58
Kado Growing Medium	64	32	96
Total	166	52	218

Detection of siderophore production by bacterial isolates present in Bio[1] and Bio[2] biofertilizers

The results obtained by the spot test in King B medium showed that none of the 218 isolated bacteria showed fluorescent pigments (Figure 2A and 2B). This result was evidenced when we generated a 3D image that highlights the excitation peak for bacterial isolates in the presence of UV light, when the production of siderophores occurs in the culture medium (Figure 2C). The main objective of the test on King B medium is to detect the presence of *Pseudomanas* spp. of the fluorescent group, which are easily distinguishable from other rhizobacteria, when cultivated under these nutritional conditions.

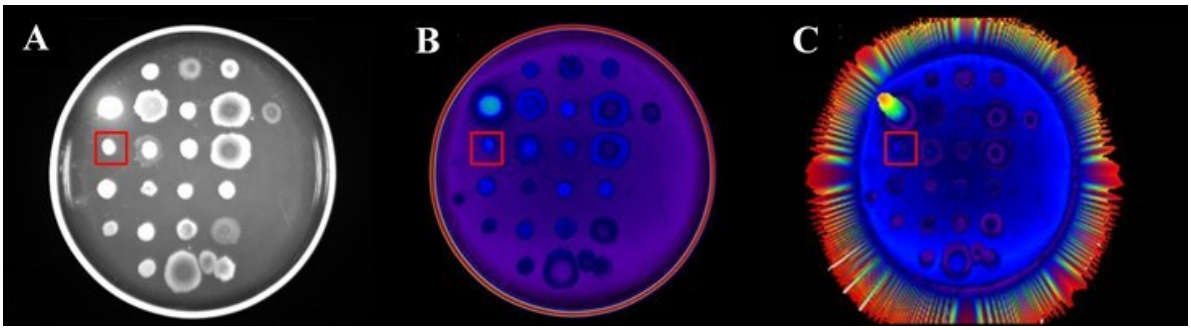

Figure 2. Test on King B medium: A) in the presence of UV light; B) in the presence of saturated UV light to highlight fluorescence and C) 3D image showing the excitation peak of the siderophores generated in the positive control (*Pseudomonas syringae* pv. tomato). Photos taken on the Loccus L-Pix EX photodocumenter. Red marking indicates *Ralstonia solanacearum* negative control.

Molecular characterization of bacterial isolates presents in Bio[1] and Bio[2] biofertilizers

Representatives of each bacterial group were chosen according to the morphological characteristics mentioned above. After extracting the genomic DNA, it was possible to classify the groups according to the band patterns observed in the electrophoresis gel. The number of fragments generated using the REP primers generated 2 to 5 fragments that ranged from 7,000 to 200 bp (Figure 3A). The ERIC primers generated 2 to 8 fragments per isolate, varying in size from 3,000 to 75 bp (Figure 3B). The BOX primer varied from 1 to 7 per isolate with a size between 4,000 and 300 bp (Figure 3C).

Efficacy of biofertilizers and chemical nematicides in reducing the population of *Meloidogyne exigua* in Arabica coffee

All treatments reduced the population of *M. exigua* both in the root system and in the soil (Table 2 and Figure 4). For the number of nematodes in the roots, the treatments that reduced the most were Fluensulfone (T3) (54.31%) followed by Bio[1] (T4) (49.67%) and Bio[2] (T5) (48.45%). The best results in reducing the number of soil nematodes were Bio[1] + Bio[2] (T8) (44.79%) and Bio[2] (T5) (44.47%). The chemical nematicides Cadusafos (T2) and Fluensulfone (T3), compared to the other treatments, reduced the *M. exigua* population to 45 DAA, with Cadusafos (T2) providing a 32.27% reduction in roots and 37.88% in the soil while Fluensulfone (T3) reduced it by 54.31% in the root system and 24.14% in the soil.

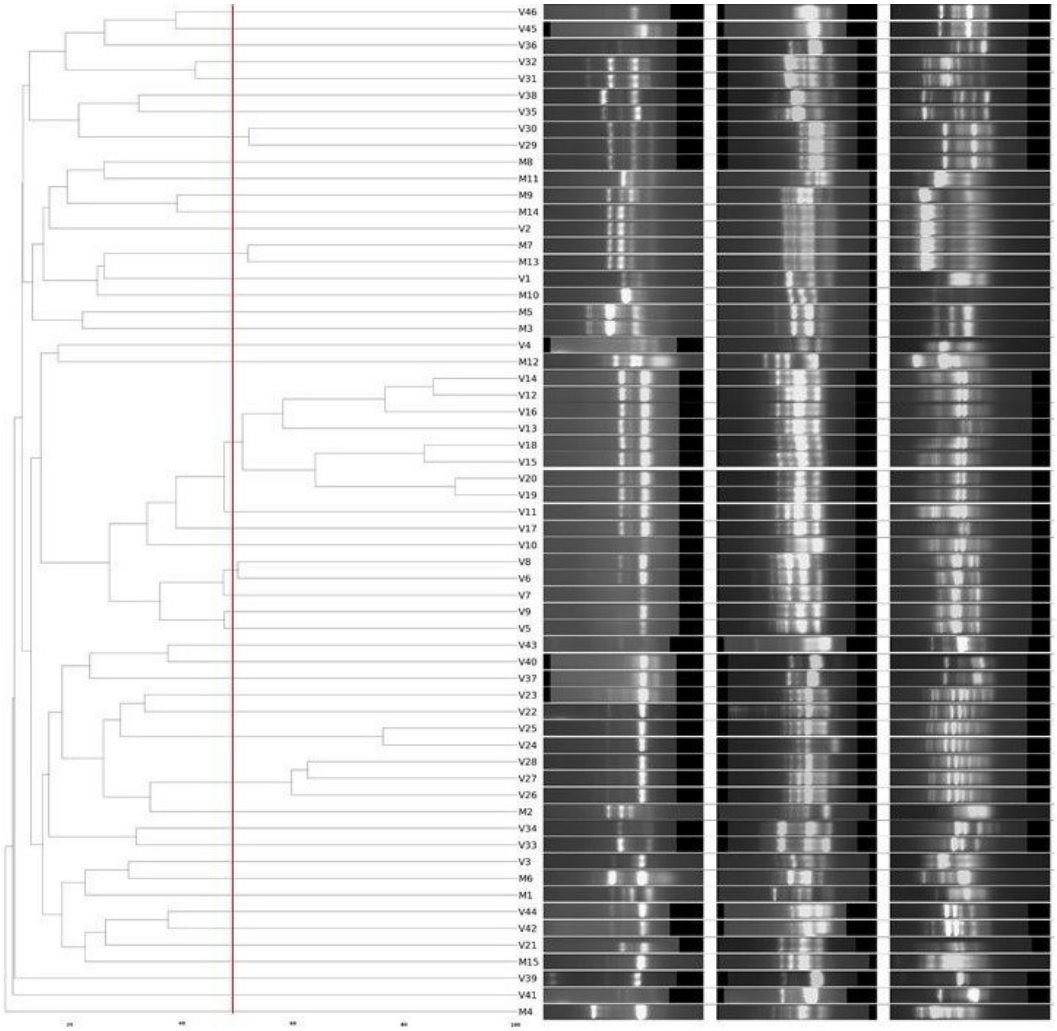

Figure 3. Dendrogram of the gene diversity of isolates found in biofertilizers Bio[1] (V) and Bio[2] (M) constructed based on the similarity matrix by complementing the Jaccard Coefficient, with data generated by the primers REP (A), ERIC (B) and BOX (C) respectively, using the average distance method (UPGMA). Red line represents the cutoff point (70%).

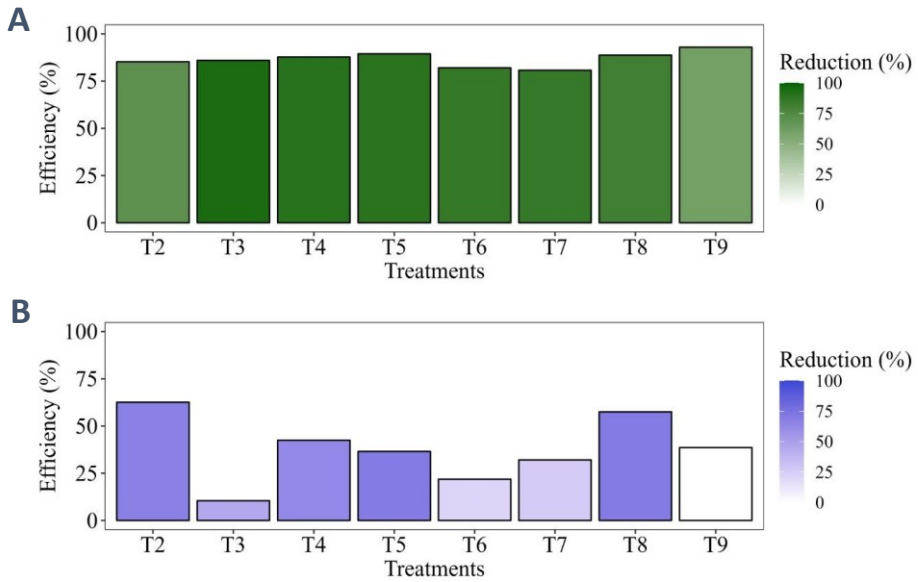

Figure 4. Relative Efficiency (RE) (%) of two biofertilizers and two chemical products used alone or in combination to reduce the population of Meloidogyne exigua in roots (A) and soil (B) of coffee plants cv. Catuaí 44 in a cultivation field in the municipality of Iúna, ES. Legend: T1 - 120 L/ha + Fluensulfone 1.0 L/ha Negative control; T2 - Cadusafos (15 L/ha: Positive control); T3 - Fluensulfone (1.0 L/ha); T4 - Bio1 (10 L/ha); T5 – Bio[2] (20 L/ha); T6 - Bio[1] 10 L/ha + Fluensulfone (1.0 L/ha); T7 – Bio[2] 20 L/ha + Fluensulfone 1.0 L/ha; T8 - Bio110 L/ha + Bio[2] 20 L/ha; T9 - Bio[1] 10 L/ha + Bio[2].

Table 2. Efficacy of chemical nematicides (Cadusafos and Fluensulfone 40EC) and two biofertilizers (Bio[1] and Bio[2] *) applied alone or in combination in reducing the population of *Meloidogyne exigua* parasitizing coffee plants cv. Catuaí 44 in a cultivation field in the municipality of Iúna, ES.

	Total number of nematodes/ 10 root[g]			**Total number of nematodes/ 100 of soil[g]**		
Treatments **	Initial Population ***	45 DAA ***	Reduction (%)	Initial Population ***	45 DAA ***	Reduction (%)
T1	42.29^{bA}	42.36^{aA}	-	13.52^{aA}	12.22^{aA}	-
T2	32.51^{cA}	22.02^{cB}	32.27	11.59^{bA}	7.20^{bB}	37.88
T3	58.66^{aA}	26.80^{bB}	54.31	14.46^{aA}	10.97^{aB}	24.14
T4	41.05^{bA}	20.66^{cB}	49.67	14.50^{aA}	9.28^{bB}	36.00
T5	36.68^{cA}	18.91^{cB}	48.45	16.55^{aA}	9.19^{bB}	44.47
T6	38.27^{bA}	25.17^{bB}	34.23	12.02^{bA}	9.68^{bB}	19.47
T7	33.40^{cA}	25.88^{bB}	22.51	11.24^{bA}	9.53^{bA}	15.21
T8	24.63^{dA}	19.66^{cA}	20.18	14.00^{aA}	7.73^{bB}	44.79
T9	19.78^{dA}	15.40^{dA}	22.14	8.26^{cA}	9.01^{bA}	-

Means followed by the same letter do not differ statistically from each other, lowercase in the column (comparison between treatments) and uppercase in the line (comparison between collection days) using the Scott-Knott test (p>0.05); *Bio[1]: organic product containing strains of *Bacillus subtilis*, *B. licheniformis* and *Lactobacillus* spp.; Bio[2]: 100% organic commercial fluid rich in organic matter containing humic and fulvic acids. **T1 - Control: negative control; T2 - Cadusafos (15 L/ha: Positive control); T3 - Fluensulfone (1.0 L/ha); T4 - Bio[1] (10 L/ha); T5 – Bio[2] (20 L/ha); T6 - Bio[1] 10 L/ha + Fluensulfone (1.0 L/ha); T7 – Bio[2] 20 L/ha + Fluensulfone 1.0 L/ha; T8 - Bio[1]10 L/ha + Bio2 20 L/ha and T9 - Bio[1] 10 L/ha + Bio[2] 120 L/ha + Fluensulfone 1.0 L/ha. ***Original values transformed into √x – 1

Bio[1] (T4) and Bio[2] (T5) showed similar results to Cadusafos (T2), a chemical nematicide recommended for controlling phytonematodes in coffee crops. The use of biofertilizers for the management of phytonematodes has been studied in recent years, due to concern about the high risks to human health and the environment caused by commercial nematicides and the search for products that have a lower environmental impact (Jones et al. 2017; Gupta et al. 2024).

The Relative Efficiency (RE) (%) of the treatments evaluated on coffee roots is shown in Figure 4A. The highest ER values were observed in plants treated with Bio[1]+Bio[2]+Fluensulfone (T9), Bio[2] (T5), Bio[1]+Bio[2] (T8), and Bio1 (T4), respectively, with approximately 75% to 80% efficiency, demonstrating the effectiveness of biofertilizers used alone or in combination in reducing the *M. exigua* population. While in the soil, it is observed that the Relative Efficiency (RE) (%) of the treatments underwent greater variation, with higher values for treatments with Bio[1]+Bio[2] (T8), Cadusafos (T2), Bio[1] (T4) and Bio[2] (T5), in this order, with a variation between 60% and 40%. Lower efficiencies were observed in other treatments (Figure 4B).

4. Discussion

The morphological characterization of bacterial colonies is important, as it indicates the diversity of species present in a sample, as an initial form of screening, for subsequent biochemical, physiological and genetic tests (Rocha et al. 2023). This screening is the first way to evaluate the diversity of microbial populations, especially in large-scale studies, as in this work, because it allows grouping of organisms with similar characteristics, optimizing the identification and classification process (Martins et al. 2020).

The absence of Pseudomanas spp. in the fluorescent group does not rule out the hypothesis that bacteria of the genus Pseudomanas spp. exist, since this genus is heterogeneous, presenting two distinct groups, classified based on the ability to produce pigments (pyoverdine) or to accumulate poly-α-hydroxybutyrate (not fluorescent) (Ghssein and Ezzeddine 2022).

Through UPGMA cluster analysis, for the 61 groups of isolates in the present study, it was possible to generate a dendrogram of similarity with the cutoff point of 70%. Based on the analysis of the dendrogram generated (Figure 3), it was observed that isolates V11, V12, V13, V15, V16, V18, V19 and V20 were considered similar, and they were grouped together in the three pairs of primes. The same is observed for isolates V26 and V27. It is worth highlighting that isolates V11, V12, V13, V15, V16, V18, V19 and V20 showed similarities in morphological characteristics, in terms of size (average), shape of the colony (circular), with variations in relation to the surface, ranging between smooth and rough and in color varying from white to

cream. Isolates V26 and V27 are similar in terms of size (average), elevation (high) and surface (rough) of the isolated colonies.

Another piece of information obtained from the dendrogram analysis was the organization of the number of isolates present in the two biofertilizers. Isolates M7 and M9, which were found only in Bio2 biofertilizer, were grouped with isolates present in Bio1 or with isolates found in both biofertilizers. With the aid of REP-PCR it was noted that isolates V4, V5, V7, V8, V9, V33, V34, V37, V39, V40, V41, V45 and V46 are only present in Bio1, since they were grouped with isolates found only in this biofertilizer. Thus, the use of Rep-PCR made it possible to expand knowledge about the biodiversity of the microbiota present in biofertilizers, in addition to enabling genetic screening of isolates.

The use of the Rep-PCR technique has been described in the literature to characterize the diversity of symbiotic and pathogenic bacteria to plants. According to Louws et al. (1994), the use of Rep-PCR primers promoted the formation of distinct genomic patterns in strains of Pseudomonas syringae, Xanthomonas campestris (Pammel) Dowson and X. oryzae (ex Ishiyama). Bouzar et al. (1999) studied the genetic diversity of 400 strains of Xanthomonas spp. at 32 points in Central America and the Caribbean using Rep-PCR, making it possible to group them into two large groups.

REP-PCR has been promoting a better understanding of the genetic diversity of the microbiota present in the rhizosphere, as well as helping to identify potential antagonistic microorganisms that can be used in Brazilian agriculture (Shin et al. 2023).

The application of the Rep-PCR technique allows rhizobacteria to be characterized with greater safety, because this characterization is not subject to risks such as the action of the environment (culture medium, temperature, pH), since the genotypic characteristics are the same (Thomassen et al. 2021, 2023). With the genetic screening of the isolates present in the two biofertilizers, it was possible to select representatives to conduct bioassays in order to understand how the isolates act in multitrophic relationships between pathogen-host-antagonist. In this way, it will be possible in the future to optimize biofertilizer formulas by adding them alone or enriching the product with specific strains that will not have to compete with strains that do not optimize the product's ability to reduce the population of phytonematodes in the field.

Thus, Rep-PCR provides more concrete support in information about the microbial communities present in biofertilizers, making it possible to compile this information, with morphological and biochemical characteristics, and understand how rhizobacteria act in the multitrophic interaction between rhizobacteria-host-pathogens (Ngamau 2012).

Some authors state that the use of biofertilizers can reduce the number of nematodes in the soil and in the root system, also reducing the number of galls, egg masses and females. Indeed, this effect was observed in this work in all treatments, some with greater effectiveness than others (Hemmati, S. and Saeedizadeh 2019; Rani et al. 2022; Kisaakye et al. 2024).

The success in managing the phytonematodes observed in this work using biofertilizers is related to the metabolites produced by bacteria, such as volatile compounds, fatty acids, hydrogen sulfide, enzymes, hormones, alcohol and phenolic compounds. Such products may be toxic to nematodes or may indirectly suppress the nematode population through modification of the rhizosphere (Ayaz et al. 2024; Deeikshana et al. 2025).

The application of Bio1 + Bio2 (T8) did not present results different from those observed in the positive control (T2) for both the number of nematodes in the soil and in the roots. However, the association of these different biofertilizers (T8) resulted in reductions in the number of M. exigua in the roots and soil (Table 2 and Figure 3). According to Pantigoso et al. (2022) the association between different microorganisms promotes a change in the rhizosphere through co-toxicity, resulting in a reduction in the number of phytonematodes. There may have been a synergistic interaction between Bio1 + Bio2, which resulted in a reduction in the number of M. exigua in the soil, since both biofertilizers have in their composition groups of bacteria that can act in different ways in the multitrophic interactions between antagonist-pathogen-plant. Furthermore, the process of colonization of host plant roots by rhizobacteria occurs through multiple steps that involve bacterial genes and metabolites. Among these metabolites are antimicrobial agents that act in biological control, which may explain the reduction of M. exigua in Arabica coffee in our research (Pradana et al. 2023).

Chemical control with Fluensulfone (T3) and combinations Bio1 + Fluensulfone (T6) and Bio2 + Fluensulfone (T7) showed lower efficiency in reducing the population of M. exigua in the soil. Although the association between chemical and biological control is reported in the literature, it is believed that the interaction between the two components did not optimize the process; however, it still resulted in a reduction in the nematode population compared to the negative control. The efficiency of the combined use of biological and chemical products, in some cases, is due to the fact that some nematicides have a nematostatic effect, paralyzing phytonematodes and exposing them to antagonists, causing these nematode biocontrol agents to increase their efficiency (Siddiqui et al. 1999; Alves et al. 2021; Abd-Elgawad 2025).

5. Conclusions

Colony morphology and genetic characterization made it possible to group 218 bacteria isolated from two biofertilizers (Bio[1] and Bio[2]) into 61 groups, as well as identify the presence of some isolates in both products. This screening provided more concrete insights into the microbial diversity present in biofertilizers and their effectiviness in field applications, both alone and in combination with chemical control, demonstrating their ability in reducing the population of *M. exigua* in coffee trees cv Catuaí.

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