

Antagonistic activity of bacteria isolated from micropropagation of camu-camu

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Abstract: The micropropagation of camu-camu (*Myrciaria dubia*) faces significant challenges due to fungal contamination under *in vitro* conditions. This study investigated the potential of endophytic bacteria isolated from camu-camu as an alternative to chemical treatments for controlling contaminant fungi. The antagonistic activities of *Bacillus subtilis*, *Enterobacter* sp., *Brevundimonas* sp., *Bacillus* sp., and *Methylobacterium* sp. against the fungi *Colletotrichum* spp. and *Curvularia* spp. were evaluated using diffusion and antibiosis methods. In the diffusion method, the pathogen was cultured over the antagonist culture, while in the antibiosis method, fungal growth inhibition was assessed through the production of thermostable metabolites. The experimental design was completely randomized (CRD), with five bacteria, two fungi, five replications, and analysis performed using R software. Results demonstrated that the diffusion method is effective in inhibiting the mycelial growth of *Colletotrichum* spp. and *Curvularia* spp., isolated from camu-camu micropropagation. *Bacillus* sp. exhibited the highest inhibition rates for both fungi, while *Brevundimonas* sp. and *Methylobacterium* sp. were more effective against *Curvularia* spp., and *Enterobacter* sp. against *Colletotrichum* spp. It is concluded that endophytic bacteria are a promising alternative for controlling contaminant fungi in camu-camu micropropagation, highlighting the potential of the diffusion method as an antagonistic action mechanism.

Indexing Terms: microbial control, microorganisms, *Myrciaria dubia*, tissue culture.

Atividade antagonista de bactérias isoladas da micropropagação de camu-camuzeiro

Resumo: A micropropagação do camu-camu (*Myrciaria dubia*) enfrenta desafios significativos devido à contaminação fúngica em condições *in vitro*. Este estudo investigou o potencial de bactérias endofíticas isoladas do camu-camu como alternativa aos tratamentos químicos para o controle de fungos contaminantes. Foram avaliadas as atividades antagônicas das bactérias *Bacillus subtilis*, *Enterobacter* sp., *Brevundimonas* sp., *Bacillus* sp. e *Methylobacterium* sp. contra os fungos *Colletotrichum* spp. e *Curvularia* spp., utilizando métodos de difusão e

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antibiose. No método de difusão, o patógeno foi cultivado sobre a cultura do antagonista, enquanto no método de antibiose foi avaliada a inibição do crescimento fúngico pela produção de metabólitos termoestáveis. O delineamento experimental foi inteiramente casualizado (DIC), com cinco bactérias, dois fungos, cinco repetições, e análise realizada no Software R. Os resultados demonstraram que o método de difusão é eficaz na inibição do crescimento micelial de *Colletotrichum* spp. e *Curvularia* spp., isolados da micropropagação do camu-camu. *Bacillus* sp. apresentou as maiores taxas de inibição para ambos os fungos, enquanto *Brevundimonas* sp. e *Methylobacterium* sp. foram mais eficazes contra *Curvularia* spp., e *Enterobacter* sp. contra *Colletotrichum* spp. Conclui-se que bactérias endofíticas são uma alternativa promissora para o controle de fungos contaminantes na micropropagação do camu-camu, destacando o potencial do método de difusão como mecanismo de ação antagonica.

Termos para indexação: controle microbiano, microrganismos, *Myrciaria dubia*, cultura de tecidos

Introduction

Camu-camuzeiro (*Myrciaria dubia* (Kunth) McVaugh) of the *Myrtaceae* family stands out among the native fruit trees of the Amazon due to its great economic potential, given its agro-industrial and pharmacological characteristics. It includes high concentrations of ascorbic acid, mineral compounds such as potassium, calcium, magnesium, and sodium, as well as phenolic compounds (GRIGIO; DURIGAN; CHAGAS, 2019). It is known for its high vitamin C content, reaching 7,355.20 mg per 100 g of pulp (CHAGAS et al., 2015). Another significant advantage is its antioxidant potential, which minimizes the risk of some chronic diseases, allowing its classification as a functional food (CHAGAS et al., 2012; FRACASSETTI et al., 2013).

However, the production of high-quality seedlings still represents an obstacle to the development of the crop. As a woody plant in the process of domestication, it presents low propagation rate through conventional methods such as grafting and cutting. Additionally, the variability in vitamin C content through seed propagation hinders the uniformity and predictability of production (Lima et al., 2025). Thus, micropropagation emerges as strategic alternative for the cloning of superior genotypes with high productive potential (Araújo et al., 2021).

Micropropagation enables large-scale clonal multiplication with high genetic and phytosanitary uniformity, in addition to significantly contributing to genetic improvement programs (Zou et al., 2024). This technique allows for germplasm conservation and the propagation of promising lines, which is a fundamental aspect for species such as camu-camuzeiro (Xu et al., 2021). Accordingly, *in vitro* methods such as organogenesis and somatic embryogenesis have shown promising results in genotypes of this species, aiming at increased productivity, fruit quality, and adaptability to climatic conditions (Araújo et al., 2021; Lima et al., 2025). However, despite these advances, there is still no consolidated protocol for the species, which hinders its adoption on commercial scale. Therefore, it is recommended that existing protocols be refined according to each genotype, explant type, and concentrations of growth regulators (Araújo et al., 2021).

One of the main challenges faced during *in vitro* cultivation is microbial contamination, especially by phytopathogenic fungi present in tissues originating from tropical environments. In addition, phenolic oxidation of explants compromises the viability of the plant material (Dorighello et al., 2020). In this context, the application of beneficial

microorganisms, such as endophytic bacteria with antagonistic activity, emerges as an innovative alternative. The *Bacillus* genus, for instance, has been widely studied for its production of antimicrobial metabolites and its ability to induce defense responses in plants (Lopes et al., 2021; Zou et al., 2024). Thus, strengthening the production chain of camu-camuzeiro in the Amazon depends not only on the selection of promising genetic materials but also on the consolidation of *in vitro* propagation technologies that are safe, efficient, and economically viable. Therefore, the present study aims to evaluate the *in vitro* antagonistic potential of bacteria isolated during the micropropagation of camu-camuzeiro in controlling contaminating fungi, using different antagonism methods.

Materials and Methods

The experiment was conducted in the Soil Microbiology Laboratory of the Brazilian Agricultural Research Corporation - EMBRAPA-RR from January to March 2023.

The endophytic bacterial isolates were obtained from microorganisms present in tissue culture, selected based on observation

in test tubes, and chosen as the most frequent in camu-camuzeiro explants. These microorganisms were previously isolated and identified in experiments conducted by Barreto (2023). The identification of fungi was performed according to the classification key described by Barnett and Hunter (1972) (Figure 1).



Figure 1. Endophytic bacteria from *in vitro* multiplication of camu-camu trees

The antagonistic activities of *Bacillus subtilis* (17B), *Enterobacter* sp. (17C), *Brevundimonas* sp. (18J), *Bacillus* sp. (18K), and *Methylobacterium* sp. (18O) bacteria were tested against two fungal isolates, *Colletotrichum* spp. and *Curvularia* spp., using two different methods: diffusion and antibiosis (Figure 2A; Figure 2B; Figure 2C; Figure 2D; Figure 2E).

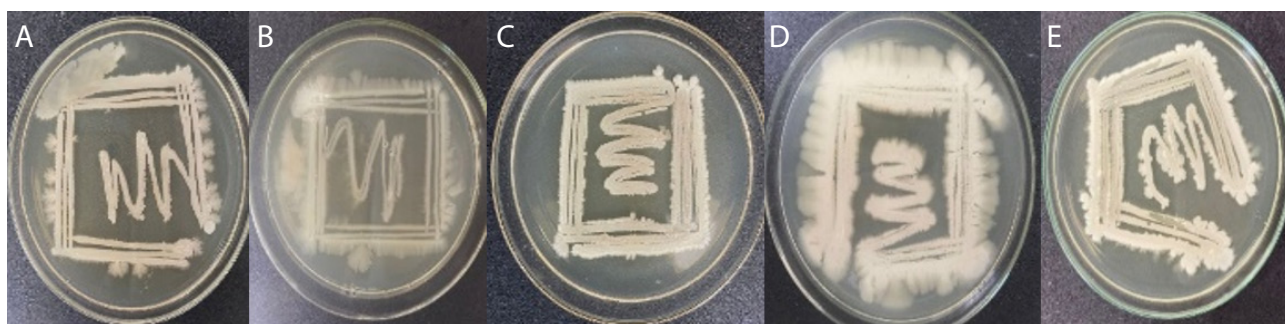


Figure 2. Antagonistic bacteria used in diffusion and antibiosis methods: *Bacillus subtilis* (17B), *Enterobacter* sp. (17C), *Brevundimonas* sp. (18J), *Bacillus* sp. (18K), and *Methylobacterium* sp. (18O)

In the first method, the diffusion technique was used, which consists of the cultivation of the pathogen over the antagonist culture. For this, a 7 mm disc of PDA (Potato Dextrose Agar) colonized by *Colletotrichum* spp. and *Curvularia* spp. was placed in the center of the Petri dish previously inoculat-

ed with the bacteria *Bacillus subtilis* (17B), *Enterobacter* sp. (17C), *Brevundimonas* sp. (18J), and *Bacillus* sp. (18K) (Figure 3).

In the second method, the inhibition of the growth of *Colletotrichum* spp. and *Curvularia* spp. was evaluated by the production of thermostable metabolites through antibi-

osis by *Bacillus subtilis* (17B), *Enterobacter* sp. (17C), *Brevundimonas* sp. (18J), *Bacillus* sp. (18K), and *Methylobacterium* sp. (18O) bacteria.

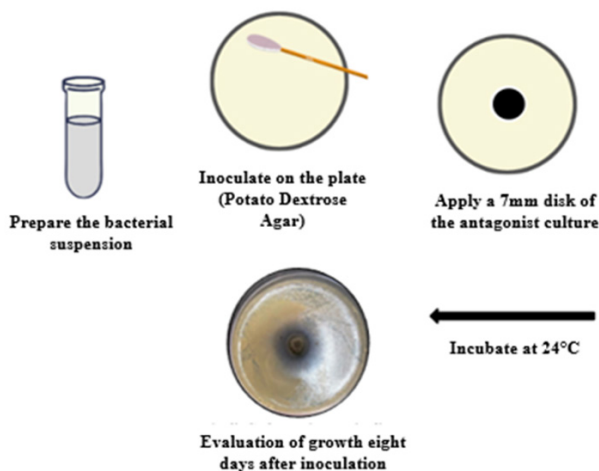


Figure 3. Cultivation of the pathogen over the antagonist culture in the diffusion method

For this, Erlenmeyer flasks containing 50 ml of PDA were used, inoculated with the an-

tagonistic strains, and incubated in a germination chamber for 48 hours. After this period, the Erlenmeyers were autoclaved at 121°C for 15 minutes and then maintained in a laminar flow chamber for solidification of the medium in an aseptic environment (Figure 4).

After the solidification of the medium, PDA discs colonized by *Colletotrichum* spp. and *Curvularia* spp. were placed in the center of the plates for subsequent growth evaluation.

As control treatment, PDA medium discs colonized by the *Colletotrichum* spp. and *Curvularia* spp. isolates were added to the center of Petri dishes containing PDA medium. All treatments were conditioned in a growth chamber with constant temperature of 25°C and photoperiod of 12 hours (Figure 5A; Figure 5B and Figure 5C).

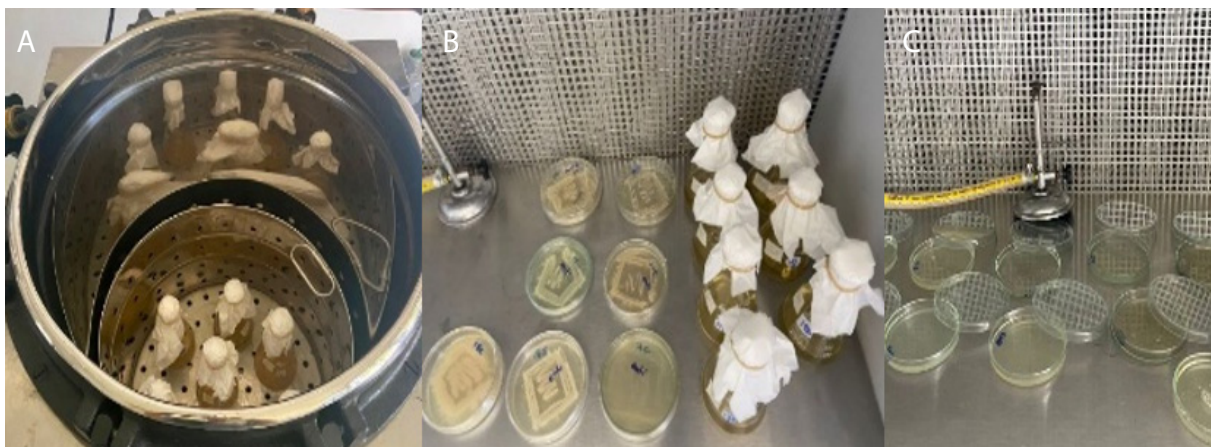


Figure 4. Autoclaved bacteria placed in a laminar flow chamber

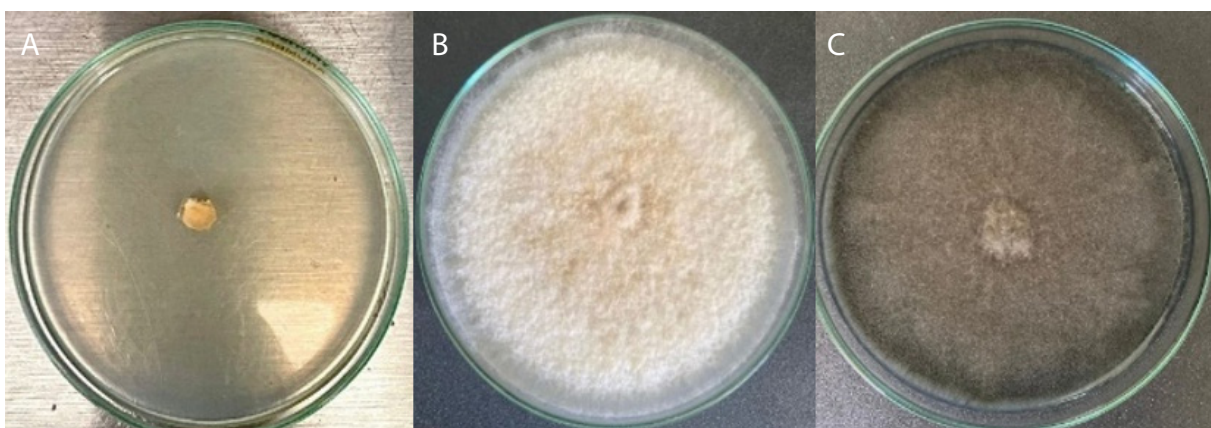


Figure 5. PDA medium discs (A) colonized by *Colletotrichum* spp. isolates (B) and *Curvularia* spp. (C) in the control treatment.

The evaluation consisted of measuring the average diameter of *Colletotrichum* spp. and *Curvularia* spp. colonies using a caliper for eight days after inoculation, during which one of the isolates in the control treatment reached the edges of the plates. Subsequently, the percentage of inhibition (%) of the treatments relative to the control was calculated using the following formula (RIUNGU et al., 2008):

$$\%I = \frac{(C - T)}{C} \times 100$$

Where %I: percentage of inhibition, C: average diameter of the control's mycelial growth, and T: average diameter of the control's mycelial growth.

The experimental design used was completely randomized (DIC), with two methods to evaluate antagonism and a control treatment; five bacteria (*Bacillus subtilis*, *Enterobacter* sp., *Brevundimonas* sp., *Bacillus* sp., *Methylobacterium* sp.); two fungi (*Colletotrichum* spp. and *Curvularia* spp.); and five repetitions. Data were subjected to the non-parametric Kruskal-Wallis test for the different antagonism tests and variance analysis for the diffusion method, F test ($p < 0.05$) of probability, and data were subjected to Tukey's test ($p \leq 0.05$). The analysis was performed using R software version 4.2.2 (R Development Core Team, 2024). Statistics and graphical representation were analyzed and generated by the AgroR package.

Results and Discussion

Statistical results showed a significant difference between the different antagonism methods by the Kruskal-Wallis test ($p < 0.0001^{***}$).

When using the diffusion method, bacteria *Bacillus subtilis* (17B), *Enterobacter* sp. (17C), *Brevundimonas* sp. (18J), *Bacillus* sp. (18K), and *Methylobacterium* sp. (18O) presented significantly higher inhibition averages of the fungi, with 74.82%, compared to the

antibiosis method, which showed lower results with only 9.84% inhibition (Figure 6).

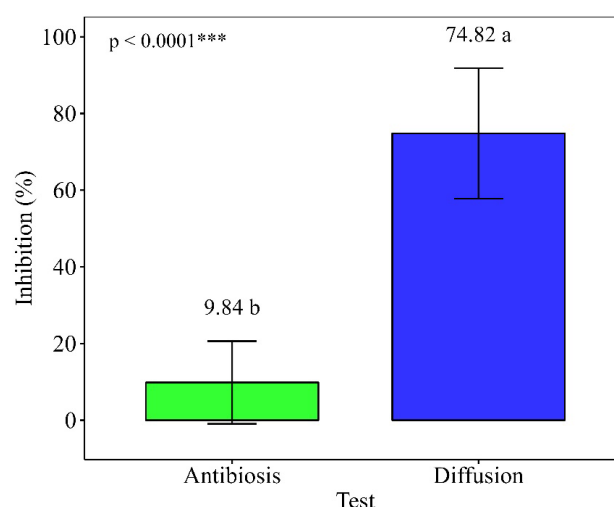


Figure 6. Action of endophytic bacteria *Bacillus subtilis* (17B), *Enterobacter* sp. (17C), *Brevundimonas* sp. (18J), *Bacillus* sp. (18K), and *Methylobacterium* sp. (18O) on contaminating fungi (*Colletotrichum* spp., *Curvularia* spp.) by the methods of antibiosis and diffusion isolated from the micropropagation of camu-camuzeiro. Lowercase letters differ significantly from each other by the Kruskal-Wallis test ($p < 0.05$).

Previous studies by Arora et al. (2020) and Lopes et al. (2021) demonstrated that endophytic bacteria of the genera *Bacillus* sp., *Brevundimonas* sp., *Enterobacter* sp., and *Methylobacterium* sp. can act as bactericides, fungicides, insecticides, herbicides, and nematocides in direct contact (diffusion method) due to the production of hydrolytic enzymes that degrade cell wall components of other microorganisms. These bacteria have antifungal metabolic characteristics, including lipopeptides from the surfactin, iturin, and fengycin families (LOPES et al., 2021). These results highlight the importance of these genera of endophytic as producers of antifungal compounds by the diffusion method. Santoyo et al. (2019) and Santos-Torres et al. (2021) also highlighted the use of these microorganisms for biocontrol and plant growth promotion, as well as their capacity in phosphate solubilization.

Compared to the antibiosis test, these results evidence the high intraspecific vari-

ability of the isolates studied, highlighting the influence of methodology and the antagonistic potential of bacteria on the inhibition of phytopathogen structures. The metabolites of these isolates had their antifungal activity altered after exposure to a temperature of 121°C for 15 minutes. This differs from studies conducted by Zengerer et al. (2018), which discuss the genus *Pseudomonas orientalis* as a strain with metabolic versatility and genetic plasticity, characteristics that contribute to its high

potential as antagonist in different biological control methods

The results presented in the graph indicate significant inhibition of fungi *Colletotrichum* spp. and *Curvularia* spp. by different bacterial isolates *Bacillus subtilis* (17B), *Enterobacter* sp. (17C), *Brevundimonas* sp. (18J), *Bacillus* sp. (18K), and *Methylobacterium* sp. (18O). The statistical analysis revealed p -value < 0.0001 , indicating statistically significant differences between treatments (Figure 7).

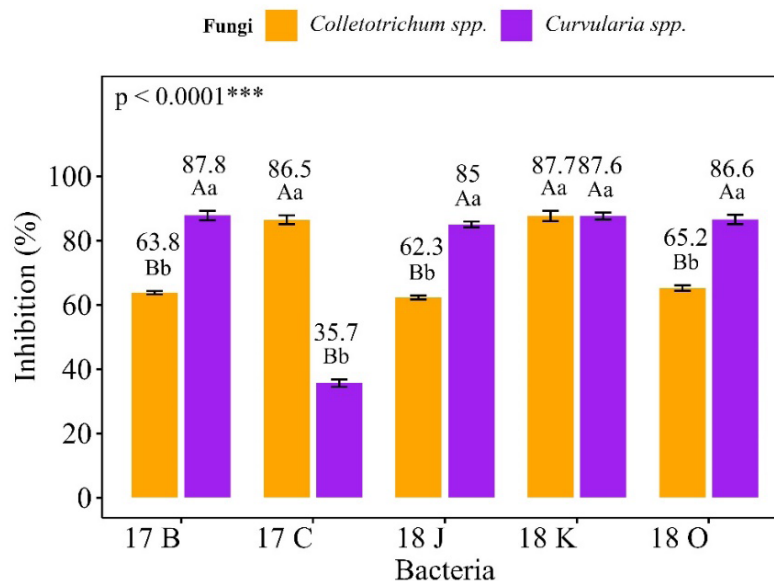


Figure 7. Antagonism tests of diffusion of endophytic bacteria (*Bacillus subtilis* (17B), *Enterobacter* sp. (17C), *Brevundimonas* sp. (18J), *Bacillus* sp. (18K), *Methylobacterium* sp. (18O)) on contaminating fungi (*Colletotrichum* spp., *Curvularia* spp.). Uppercase letters do not differ significantly from each other for different bacteria, lowercase letters do not differ significantly from each other for different fungi ($p < 0.05$) in the Tukey test.

Inhibition of *Colletotrichum* spp. ranged from 62.3% for *Brevundimonas* sp. (18J) to 87.7% for *Bacillus* sp. (18K). It was observed that the isolates *Enterobacter* sp. (17C) and *Bacillus* sp. (18K) were more effective in inhibiting *Colletotrichum* spp. with percentages above 85%. Isolates *Bacillus subtilis* (17B) 63.8%, *Methylobacterium* sp. (18O) 65.2%, and *Brevundimonas* sp. (18J) 62.3%, on the other hand, showed lower inhibition rates, significantly differing from the other strains for fungus *Colletotrichum* spp. (Figure 7).

Kazerooni et al. (2020), suggest that *Enterobacter cloacae* has potential to be used as biocontrol agent to suppress cu-

cumber rot disease caused by *Pythium aphanidermatum*, where it significantly reduced disease incidence by 63% through direct contact. Consequently, the genus *Enterobacter*, in addition to presenting antifungal compounds, can be found in plant species endophytically.

Sánchez et al. (2014) noted that in diffusion tests with direct confrontation of fungal culture over antagonist culture, bacteria of the genus *Bacillus* sp. expressed the ability to inhibit the growth of *Colletotrichum gloeosporioides* with variations of 62% and 80%. Moreira et al. (2014) demonstrated that *Brevundimonas* sp. was able to inhibit

approximately 60% of the mycelial growth of *Colletotrichum* spp., corroborating with the present study.

For fungus *Curvularia* spp., the results showed inhibition ranging from 35.7% to 87.8%. The isolate *Enterobacter* sp. (17C) presented the lowest inhibition rate with 35.7%, while the other isolates obtained the best inhibition results with 87.8%, 85%, 87.6%, and 86.6% for *Bacillus subtilis* (17B), *Brevundimonas* sp. (18J), *Bacillus* sp. (18K), and *Methylobacterium* sp. (18O) strains, respectively (Figure 7).

For the action of bacteria on fungi, the strain *Bacillus subtilis* (17B) showed significant difference in inhibition between *Colletotrichum* spp. (63.8%) and *Curvularia* spp. (87.8%), indicating that the strain *Bacillus subtilis* (17B) is more effective against *Curvularia* spp. than against *Colletotrichum* spp. Species of the genus *Bacillus* sp., such as *Bacillus velezensis*, were able to inhibit the growth of *Botrytis cinerea* when cultivated *in vitro* with inhibition rate of 72.23% (XU et al., 2021).

Various species of the genus *Bacillus* are important agents in disease control due to their metabolic versatility, which allows them to produce biologically active molecules capable of inhibiting plant pathogens or promoting plant development and systemic resistance (PANDIN et al., 2018). The efficacy of these agents can vary depending on the specificity of the target fungus, as seen in cases of pathogen inhibition such as *Colletotrichum* spp. and *Curvularia* spp.

Corroborating the studies of Zengerer et al. (2018), the genus *Brevundimonas* sp. presents metabolic versatility and genetic plasticity, encompassing endophytic bacteria with high antagonistic potential. Previous studies such as that by Poorniammal et al. (2009), which evaluated the antagonistic potential of bacteria such as *Methylobacterium* sp., showed that these bacteria can reduce the mycelial growth of

Fusarium udum in diffusion tests, achieving an inhibition of over 58%.

Evaluating the action of the strain *Enterobacter* sp. (17C), it was possible to observe significant inhibition of *Colletotrichum* spp., reaching 86.5%. This result significantly differs from the inhibition rate observed for *Curvularia* spp., which was 35.7%. (Figure 7). These results can be observed in the morphological development of fungi cultivated in Petri dish (Figure 8-1-18-17; Figure 8-2-18-17).

With this, it is possible to infer that there were variations in the sensitivity of the isolates of *Curvularia* spp. to the volatile metabolites produced by *Enterobacter* sp., given that there is high genetic variability among fungi, which may confer distinct characteristics. Similar results were conducted by Dariva et al. (2015), who confirmed that there was no inhibition of *Fusarium solani* mycelial growth by volatile metabolites produced by *Bacillus Subtilis* for the same reason.

Panigrahi et al. (2021) confirmed that *Enterobacter cloacae* inhibited the mycelial growth of contaminating fungi such as *Rhizoctonia solani* by 73.75%, where the results obtained, along with literature information, indicate that the antimicrobial constituents of the endophytic bacterium *E. cloacae* have potential to contribute to antimicrobial activity. Kazerooni et al. (2020) demonstrated that *Enterobacter cloacae* can be used as a biocontrol agent to suppress cucumber rot disease caused by *Pythium aphanidermatum*, reducing disease incidence by 63%. These endophytic strains, in addition to producing antifungal and plant growth-promoting characteristics, offer various benefits to plants. They can be considered environmentally safe, compatible, and effective in both the short and long term, helping plants to survive and resist various types of stresses (TIMMUSK et al., 2017).

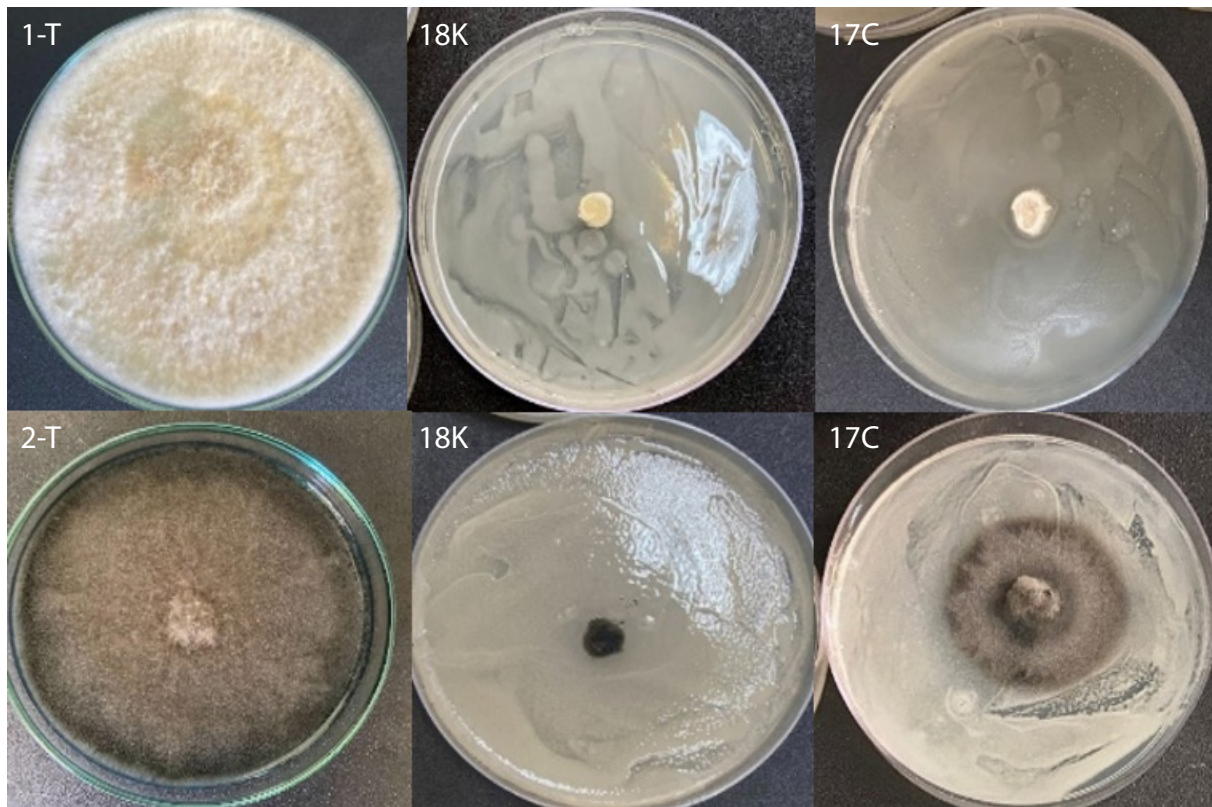


FIGURE 8. Antagonism of strains *Enterobacter* sp. (17C), *Bacillus* sp. (18K) between *Colletotrichum* spp. (1) and *Curvularia* spp. (2) after seven days of incubation in a growth chamber by the diffusion method (using the technique of fungal culture over antagonist culture) and control (T).

Strains *Brevundimonas* sp. (18J) and *Methylobacterium* sp. (18O) show high inhibition for fungus *Curvularia* spp., differing from *Colletotrichum* spp. On the other hand, *Bacillus* sp. (18K) is highly effective against fungi *Colletotrichum* spp. and *Curvularia* spp., with inhibition rates above 87%. Chen et al. (2008) identified 14 volatile antifungal compounds produced by *Bacillus subtilis* that inhibited the development of *Curvularia lunata* and *Bipolaris* sp. by up to 82.7%. These compounds, along with other secondary metabolites such as antibiotics and phytohormones, promote plant growth, induce systemic resistance, inhibit pathogens, and increase nutrient bioavailability (ZHENG et al., 2015; ZOU et al., 2024).

Thus, species of *Bacillus* act in the inhibition of phytopathogens through different mechanisms, including competition for resources, and production of soluble antimicrobial compounds with antimicrobial properties (GUEVARA-AVENDAÑO et al., 2018). This diversity of actions makes *Bacillus* sp. valu-

able allies in the management of agricultural pests and diseases in the laboratory. Accordingly, Salazar et al. (2017) showed that the fungicidal action of *Bacillus amylo-liquefaciens* exhibited significant antifungal activity *in vitro* against *Fusarium oxysporum*, *Fusarium avenaceum*, and *Mucor* sp., causing cellular damage in various structures of these fungi.

Conclusion

The diffusion method proved to be an effective mechanism of antagonistic action compared to antibiosis. All bacteria tested in the diffusion method exhibited significant antagonistic activity in inhibiting the mycelial growth of contaminating fungi *Colletotrichum* spp. and *Curvularia* spp. isolated from the micropropagation of camu-camuzeiro. Among the tested bacteria, *Bacillus* sp. showed the highest inhibition rates of mycelial growth for both fungi. *Brevundimonas* sp. and *Methylobacterium* sp. showed better inhibition rates for

Curvularia spp., while *Enterobacter* sp. had the best inhibition rate for *Colletotrichum* spp.

Therefore, the use of endophytic bacteria evaluated from camu-camuzeiro for biological control of contaminating fungi is recommended. Future work can explore the production of bioproducts derived from endophytic bacteria for plant disease control in micropropagation, focusing on isolates from the same fruit species.

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