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# Essential oils from condiment and medicinal plants in the control of contaminants from the micropropagation of *Myrciaria dubia*

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**Abstract –** The main limitation of the micropropagation of camu-camu (*Myrciaria dubia*) is related to *in vitro* contamination. In order to overcome contamination, the effect of essential oils was studied as an alternative to conventional chemical treatments. This study aimed to analyze the action of essential oils from four condiment and medicinal plants (Oregano, *Origanum vulgare L.*; Garlic, *Allium sativum L.*; Citronella, *Cymbopogon nardus L.*; and Ginger, *Zingiber officinale Rosc.*) in reducing microbial contamination growth and on the survival rate of explants in the micropropagation of camu-camu. The antimicrobial activity of essential oils was analyzed on the *in vitro* contamination of tissue culture from camu-camu at concentrations of 0.5, 1, 2, 3 and 4 µL $mL^{-1}$ in woody plant medium (WPM), emulsified with 1% Polysorbate 80 (Tween 80). The antibacterial activity of essential oils on strains isolated from camu-camu tissue culture was also analyzed using agar diffusion and broth microdilution methods. The use of essential oils allowed a reduction in the rate of *in vitro* contamination in tissue culture, it being observed that, from the concentration of 2 µL $mL^{-1}$, there was no manifestation of fungal contaminants, with a significant reduction in the rate of bacterial contamination, with the exception of ginger essential oil that showed significant contamination in all analyzed concentrations. Inversely in relation to the reduction in microbial growth *in vitro*, there is an increase in explant oxidation as concentrations increase, with citronella and oregano oils showing phytotoxic potential from the lowest concentrations. Garlic essential oil showed better balance, with lower oxidation rates and greater control of microorganisms in tissue culture at concentrations of 0.5 and 1 µL $mL^{-1}$. Oregano and citronella essential oils showed better antibacterial activity in the agar diffusion test.

**Index terms:** Camu-camu. Tissue Culture. Microbial Control. Microorganisms.

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# Óleos essenciais de plantas condimentares e medicinais no controle de contaminantes da micropropagação de *Myrciaria dubia*

**Resumo** – A micropropagação de camu-camu (*Myrciaria dúbia*) tem como principal limitação a contaminação *in vitro*. A fim de superar a contaminação, o efeito dos óleos essenciais foi estudado como alternativa aos tratamentos químicos convencionais. Este estudo teve como objetivo determinar a ação dos óleos essenciais de quatro plantas condimentares e medicinais (Orégano, *Origanum vulgare L.*; Alho, *Allium sativum L.*; Citronela, *Cymbopogon nardus L.*; Gengibre, *Zingiber officinale Rosc.*) na redução de contaminação microbiana e sobre a taxa de sobrevivência dos explantes na micropropagação de camu-camu. A atividade antimicrobiana dos óleos essenciais foi analisada sobre a contaminação *in vitro*, na cultura de tecidos de camu-camu, nas concentrações de 0,5, 1, 2, 3 e 4 µL $mL^{-1}$ em meio WPM, emulsificados com Polysorbate 80 (Tween 80) a 1%. Também foi analisada atividade antibacteriana dos óleos essenciais sobre cepas isoladas da cultura de tecidos de camu-camu, nos métodos de difusão em ágar e microdiluição em caldo. A utilização de óleos essenciais permitiu redução na taxa de contaminação *in vitro*, na cultura de tecidos, sendo observado que, a partir da concentração de 2 µL $mL^{-1}$, não houve manifestação de contaminantes fúngicos e redução significativa da taxa de contaminação bacteriana, à exceção de óleo essencial de gengibre, que apresentou contaminação expressiva em todas as concentrações analisadas. Em ordem inversa à redução no crescimento microbiano *in vitro*, percebe-se o aumento da oxidação dos explantes à medida que aumentam as concentrações, tendo os óleos de citronela e orégano apresentado potencial fitotóxico desde as mais baixas concentrações. O óleo essencial de alho apresentou melhor balanceamento, com menor taxa de oxidação e maior controle de microrganismos na cultura de tecidos, nas concentrações de 0,5 e 1 µL $mL^{-1}$. Os óleos essenciais de orégano e citronela apresentaram melhor atividade antibacteriana no teste de difusão em ágar.

**Termos para indexação:** Camu-camu. Controle microbiano. Cultura de tecidos. Microrganismos.

## Introduction

Camu-camu (*Myrciaria dubia* (HBK) McVaugh) is a wild fruit that grows on the floodplains of rivers and lakes throughout the Amazon basin, its pink to dark purple fruit being of great commercial interest thanks to their nutritional, agro-industrial and pharmacological potential, which includes properties such as high concentrations of ascorbic acid; mineral compounds such as potassium, calcium, magnesium and sodium; and phenolic compounds (CHAGAS et al., 2015; SOUSA et al., 2015, GRIGIO et al., 2019; GRIGIO et al., 2021a, 2021b, 2021c). Due to the species having excellent levels of antioxidant activity, it is capable of minimizing the risk of incidence of a number of chronic diseases, which allows it to be included in the list of functional foods (PETERS; VÁSQUEZ, 1986; VILLACHICA, 1996; YUYAMA et al., 2011; SOUSA et al., 2015; CHAGAS et al., 2015).

Although the species is traditionally propagated by seeds, this technique is not interesting for the establishment of commercial agriculture due to the lack of uniformity generated by sexual reproduction, which impacts on the variability regarding fruiting, production cycle, and even alterations in the vitamin C content among the fruits of different plants (PASQUAL et al., 2012; CHAGAS et al., 2012). Cutting has been the most widely used method for camu-camu propagation, as it allows the maintenance of the genetic characteristics of the mother-plants, uniformity, reduced size, and precocity of production, the main difficulty,

however, comprising large-scale multiplication (MOREIRA FILHO, 2009; CHAGAS et al., 2012; PASCOAL et al., 2012).

Araújo et al. (2015) point to micropropagation as an alternative to the production process of camu-camu seedlings, which, through *in vitro* cultivation techniques such as organogenesis and somatic embryogenesis, allows the large-scale multiplication of identical plants throughout the year.

Camu-camu micropropagation, according to Araújo et al. (2016), offers difficulties in obtaining aseptic cultures due to the high rate of microbial contamination that has accelerated growth in the culture medium, favoring competition for nutrients. The study indicated the need to supplement the environment with antibiotics, considering that, even after disinfestation, a 70% contamination rate was observed. The study also suggests the presence of endophytic bacteria, which were detected in the medium only weeks after *in vitro* establishment. In the same sense, Palú et al. (2011) describe contamination of explants by endophytic bacteria as one of the main problems of fig tree micropropagation.

The use of essential oils in tissue culture is pointed out by Hamdeni et al. (2021) and Mokbel, Khalil and El Shazly (2017) as an important strategy in the control of contaminants, having demonstrated efficiency in the control of fungi, bacteria and viruses. Nevertheless, in order to observe this bioactivity of essential oils on microorganisms, a prior analysis of plant species with this inhibitory potential is necessary, thereby providing alternatives for inhibition on endophytic microorganisms (JASIM, SALIH, ATI, 2021; MEZIANI et al., 2019; ENNOURI et al., 2020).

Thus, this study aimed to analyze the effect of essential oils from four condiment and medicinal plants (Oregano, *Origanum vulgare L.*; Garlic, *Allium sativum L.*; Citronella, *Cymbopogon nardus L.*; Ginger, *Zingiber officinale Rosc.*) on reducing microbial contamination and on the survival rate of explants in camu-camu micropropagation.

## Materials and Methods

The experiment was carried out in two stages: in the first, the extraction of essential oils was performed; and in the second, the antimicrobial activity in camu-camu tissue culture was analyzed.

### Extraction of essential oils

The extraction of essential oils (OEs) was carried out by the hydrodistillation method, in a Clevenger apparatus. An amount of 500 g of the sample was used. After 2 h of extraction, the oil obtained was collected and placed in a glass bottle lined with previously sterilized aluminum foil. The extraction was performed in triplicate, and the yield of each species was evaluated. For the extraction of essential oils, fresh and dry leaves, rhizomes and bulbs were used, as described below (**Table 1**).

**Table 1.** Plants used for extracting essential oils

| Common Name | Parts Used | Scientific Name |
|---|---|---|
| Oregano | Dried leaves | *Origanum vulgare L.* |
| Garlic | Fresh bulbs | *Allium sativum L.* |
| Citronella | Fresh leaves | *Cymbopogon nardus L.* |
| Ginger | Fresh rhizomes | *Zingiber officinale Rosc.* |

### Isolation of bacteria from tissue culture of superior camu-camu clones

Bacterial strains of camu-camu were isolated at the Laboratory of Soil Microbiology (*Laboratório de Microbiologia do Solo* – LMS), at Embrapa Roraima, using tissue culture-dependent detection. In the method, the isolation was performed after bacterial manifestations in micropropagation of camu-camu in the Woody Plant Medium (WPM) culture (LLOYD, MCCOWN, 1980). The strains were isolated in nutrient agar culture medium in Petri dishes using the streak depletion method, incubated at 28ºC for 10 days for further morphological characterization (size, edge, surface, elevation, color, shape, brightness, and Gram color stain, among other characteristics) according to Hungary and Silva (2011).

## Essential oils in the control of contaminants isolated from camu-camu micropropagation

For this step, the diffusion method by perforation in agar was used, consisting of perforating the solid culture medium with the aid of cylinders of 6-8 mm in diameter to form wells where the essential oils to be analyzed. After the medium solidified, seedings were performed with dilution of each strain diluted in DYGS liquid medium without addition of agar, at 104 CFU $mL^{-1}$. After about 30 min, the 6 mm wells were drilled and filled with 35 µL of stock solution of each essential oil (Table 1), diluted to a concentration of 100 µL $mL^{-1}$ in distilled water with Tween 80® at 10 %. As a control, 10% polysorbate 80 (Tween 80®) was used, without essential oil (OSTROSKY et al., 2008).

This is considered a qualitative technique, making it possible to analyze whether microorganisms are susceptible or resistant to the substance to be analyzed. After incubation at 28 °C for 24 h, the reading is performed by measuring the diameter of the zone of inhibition with a digital caliper, which is able to classify the inoculum as susceptible when the zone is ≥ 8 mm, or resistant (r) when there is no zone formation or if said zone is less than 8 mm, according to the scheme shown in Figure 1 (MICHELIN et al., 2005).

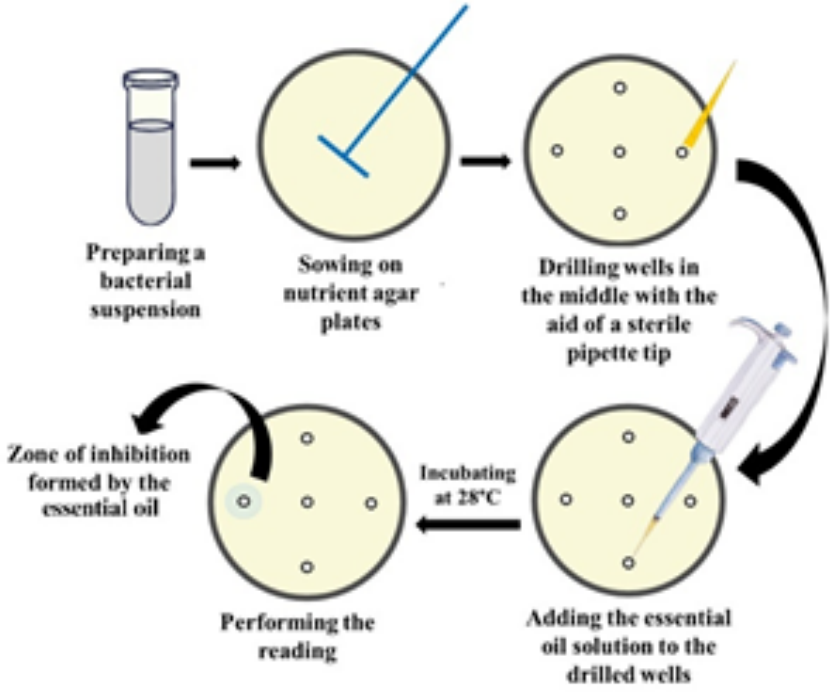


**Figure 1. Scheme of the Paper Disk Diffusion method (Source: Adapted from Carneiro, 2020)**

## Essential oils in the *in vitro* control of contaminants in camu-camu micropropagation

The camu-camu explants were collected at the Serra da Prata Experimental Camp (*Campo Experimental Serra da Prata* – CESP), belonging to Embrapa Roraima, located in the municipality of Mucajaí, Roraima. whose geographical location of the experimental area is located at 60°58'40'' W, 2°23'49'' N. Samples were collected at random points, prioritizing plants that did not show any signs of injury or damage caused by possible pathogens. After collection, the stem segments were taken to the laboratory and pre-cleaned as described below. Camu-camu explants, originating from new shoots with a pair of axillary buds and approximately 4 cm in length, were washed in running water, for partial leaching of phenolic compounds released as a result of tissue cutting.

Subsequently, the explants were immersed in a fungicide solution with 2 ml $L^{-1}$ Nativo® and 100 mg $L^{-1}$ of the antioxidant citric acid,

as recommended by Araújo et al. (2016), remaining under these conditions for 2 h.

After this step, in a laminar flow chamber, the stem segments underwent a disinfection process as described in the following sequence: the explants were immersed in 70% alcohol for 1 min; soon after, they were immersed in 1.5% sodium hypochlorite with 2 drops of neutral detergent for 10 min; at the end, the disinfected explants underwent triple washing with distilled, deionized and autoclave water (DDA) to completely remove the products from the surface of the tissues.

After asepsis, the treated explants were inoculated into test tubes containing 10 mL of WPM medium supplemented with 30 g $L^{-1}$ of sucrose, solidified by 7 g $L^{-1}$ of agar, with pH adjusted to 5.6 and autoclaved at 1.2 atm pressure and 120 °C for 15 min. After autoclaving, in a laminar flow chamber, the essential oils (garlic, ginger, citronella, and oregano) were added to the culture medium at different concentrations: 0.5; 1, 2, 3, and 4 µL $mL^{-1}$. For the emulsification of the oils in the culture medium, 10% Polysorbate 80 (Tween 80) was added. After complete homogenization of the oils in the culture medium, distribution in the test tubes was carried out. The essential oil was not added to the control.

The explants were evaluated every 7 days, to observe the appearance of microbial manifestations and conditions of the explant, and the final result was evaluated after 30 days of cultivation. All explants were maintained at 25 ± 2 °C with a 16 h photoperiod of 35-40 µmol $m^{-1}$ $s^{-1}$ provided by cool white fluorescent lamps. For each concentration of essential oil, a completely randomized design was carried out with 5 replications consisting of four explants each. The phytotoxic potential of the essential oil was evaluated according to the darkening and death of the explant, at the end of 30 days of *in vitro* cultivation.

Data were submitted to analysis of variance by F test ($p < 0.05$). Quantitative data were submitted to regression analysis, and qualitative data were submitted to the Tukey test ($p \leq 0.05$). The SISVAR software program was used to analyze the SISVAR data (FERREIRA, 2014).

## Results and Discussion

The use of essential oils was used in this study to discover the antimicrobial potential of camu-camu tissue culture. Initially, extraction of essential oils (EO) from garlic, ginger, citronella and oregano was performed, followed by antimicrobial evaluation on bacterial isolates from camu-camu tissue culture.

### Essential oils in the control of contaminants isolated from camu-camu micropropagation

The evaluation of the antibacterial action of citronella, oregano, ginger and garlic essential oils was conducted primarily by the agar well diffusion method. **Table 2** shows the isolates that were considered susceptible and those that were resistant to the tested oils. In zones smaller than 8 mm, or considered as NR (no

**Table 2** – Bactericidal action of essential oils on bacteria isolated from camu-camu tissue culture, by the agar well diffusion method*.

| Isolated | Gram Color | Citronella (mm) | Oregano (mm) | Ginger (mm) | Garlic (mm) |
|---|---|---|---|---|---|
| 07A | + | 12.69 ± 1.97(s) | 13.27 ± 1.01(s) | NR (r) | NR (r) |
| 07B | + | 9.45 ± 1.25(s) | 10.81 ± 1.88(s) | NR (r) | NR (r) |
| 07C | + | NR (r) | 13.09 ± 1.00(s) | NR (r) | NR (r) |
| 10A | + | 21.55 ± 4.05(s) | 10.41 ± 1.52(s) | 11.01 ± 0.79(s) | NR (r) |
| 10B | + | 24.99 ± 4.99(s) | 17.61 ± 2.16(s) | 14.07 ± 2.91(s) | 10.23 ± 1,63(s) |
| 15A | + | 9.28 ± 3.39(s) | 11.76 ± 1.31(s) | NR (r) | NR (r) |
| 15B | + | 39.03 ± 3.57(s) | 9.98 ± 0.12(s) | NR (r) | NR (r) |
| 17A | + | 20.95 ± 1.32(s) | 12.25 ± 3.78(s) | 11.59 ± 0.39(s) | NR (r) |
| 18A | + | 10.33 ± 0.41(s) | 12.87 ± 1.69(s) | NR (r) | NR (r) |
| 18B | + | 12.44 ± 1.25(s) | 12.72 ± 0.95(s) | 13.34 ± 1.44(s) | NR (r) |
| 18O | + | 9.30 ± 0.93(s) | 10.95 ± 0.383(s) | NR (r) | NR (r) |

* Each value represents the mean of three replicates and standard deviations from the mean; (r): resistant; (s): susceptible; NR: there was no formation of zones of inhibition.

resistance), bacterial isolates were considered resistant to the essential oil used.

Similar to the results found by Santos et al. (2011) in their study, oregano essential oil offered control over all analyzed microorganisms, while garlic EO showed selectivity, providing the largest zones of inhibition on some species of bacteria and no inhibition on others.

Citronella essential oil showed selective control over the analyzed bacterial isolates – a result similar to that found by Silveira et al. (2012) and Martins et al. (2009). Ginger and garlic essential oils showed low efficiency in controlling these microorganisms by the diffusion method at the concentrations used. Even so, it is observed that the 07C isolate was considered susceptible only to oregano.

Ginger EO showed selective antimicrobial activity, with effectiveness on only four of the 11 bacterial isolates analyzed. In their study, Cutrim et al. (2019) state that it is essential to identify the Gram stain of the isolates, considering that many studies indicate greater resistance of Gram-negative bacteria to essential oils than Gram-positive bacteria. This resistance is commonly related to an external membrane to the cell wall of Gram-negative bacteria, composed of polysaccharides, which offers less permeability to hydrophobic compounds, which is the case of essential oils (CUTRIM et al., 2019; PROBST, 2012; BARBOSA et al., 2015). In this study, it was possible to observe, in Table 2, that all isolates were identified as Gram-positive staining. This characteristic, however, does not explain the low effectiveness of ginger essential oil, being possibly related to the bacterial species.

## Essential oils in the control of contaminants in camu-camu micropropagation

In the following step, the essential oils were analyzed for their *in vitro* antimicrobial potential in camu-camu tissue culture. The study made it possible to observe that the contamination rate reduction in the micropropagation of the species was proportional to the increase in the concentration of the essential oil used, with the exception of essential oil of ginger, which presented a high significant microbial contamination in all analyzed concentrations (Figures **Figure 2**A and **Figure 2**B).

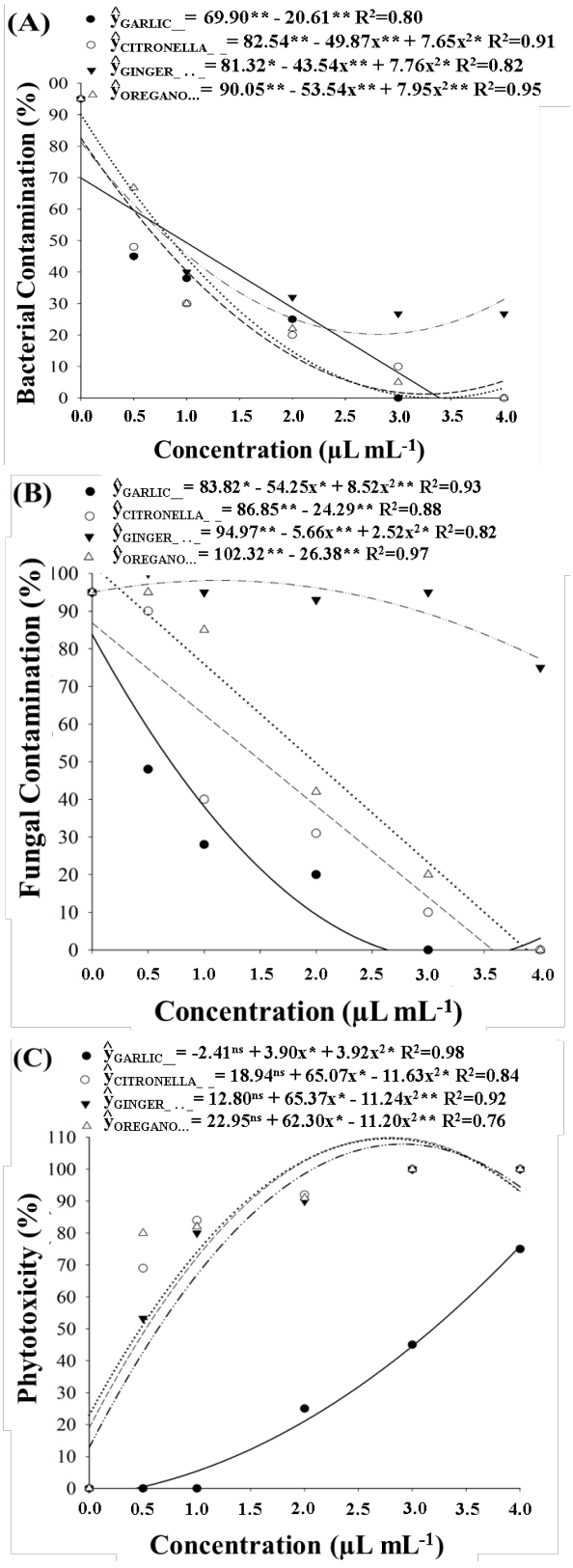


**Figure 2** – Antimicrobial activity and oxidation of essential oils in camu-camu tissue culture.

Garlic essential oil was effective in fungal control from a concentration of 1 µL $mL^{-1}$ (**Figure 2**B). A reduction in contamination by bacteria was also observed, although there was no total control, as observed for fungal contamination, with the exception of citronella essential oil at concentrations of 3 and 4 µL $mL^{-1}$ in which bacterial control was effective (**Figure 2**A).

Inversely in relation to the reduction of contamination following the increase in the concentration of essential oils, an increase in the oxidation of the explants was noticed as the concentrations increased, the darkening of the explant being observed, with citronella and oregano oils presenting phytotoxic potential starting at the lowest concentrations (**Figure 2**C).

Similar results were found by Taghizadeh, Solgi and Shahrjerdi (2016), in which the use of essential oils based on eugenol, carvacrol and thymol reduced *in vitro* contamination by bacteria and fungi in strawberry micropropagation. Nevertheless, the authors also report that this addition to the culture medium increased the oxidation of the explants, with a significant interaction between the concentration of essential oils and the symptoms of phytotoxicity, i.e., the higher the concentration of EO, the greater the percentage of oxidation. In this study, oregano and citronella oils showed phytotoxicity starting at the lowest concentrations, thus requiring the identification of minimum inhibitory concentration that does not cause damage to the explants and which is effective in controlling contaminants (**Figure 2**C).

The use of essential oils from medicinal and aromatic plants was also used by Meziani et al. (2019) in tissue culture of date palms. Oils based on rosemary, thyme, artemisia and other species with antimicrobial potential were used. The authors observed that all concentrations used were capable of inhibiting the growth of endophytic bacteria in the multiplication phase, although the highest concentrations were toxic for the explants. Ennouri et al. (2020) report that the essential oils of oregano and thyme were effective in fungal control without showing phytotoxicity to the explant at concentrations of 0.15 µL $mL^{-1}$, as they did not interfere with the process of regeneration and differentiation of plant tissues.

In **Figure 2**C, it was possible to observe that the use of garlic essential oil in camu-camu tissue culture resulted in a lower percentage of oxidation when compared to the other essential oils used in this study. This result may be due to the majority presence of organosulfur compounds, as highlighted by Mnayer et al. (2014) and Amagase (2006), as being constituents with significant antioxidant activity. The study by Botas (2017), evaluating the antioxidant and antibacterial potential of four varieties of garlic, identified that white garlic had the highest antioxidant properties, although their antimicrobial activity was not satisfactory, showing low bactericidal activity.

The ginger EO was not satisfactory in any of the concentrations, showing phytotoxic activity and low efficiency in antimicrobial activity (**Figure 2**). Unlike the study by Cutrim et al. (2019), who identified antioxidant and antimicrobial activity in essential oils and hydroalcoholic extracts of ginger and rosemary.

In the study reported by Jasim, Salih, and Ati (2021), the use of essential oils based on thyme (*Thymus vulgaris L.*), mint (*Mentha piperita L.*), camphor (*Cinnamomm camphora*), Colocynthis (*Citrullus colocynthis*), and arugula (*Eruca vesicaria*) was employed against fungal contamination in tissue culture. The study indicated effective inhibition of mycelial growth from 2 mL 100 $mL^{-1}$ for thyme and mint oils, while essential oils of camphor, colocynth and arugula showed less potential for *in vitro* inhibition. Nevertheless, similarly to this work, a high percentage of phytotoxicity was identified on the explants, resulting in significant mortality.

Mokbel, Khalil and El Shazly (2017) attribute the antimicrobial activity of the essential oil to its lipophilic character, which may facilitate absorption by plant tissues, improving the control of endophytic contaminants. Nevertheless, depending on the composition

and increase in concentration, this same characteristic may result in phytotoxicity to the explants. The authors describe the use of eugenol nanoemulsions as a high oxidation rate strategy, which were effective in controlling fungi and viruses contaminating tissue culture and did not show any negative effect on regeneration. The use of eugenol nanoemulsions favored the inhibition of fungal contamination, as well as allowing virus-free seedlings after five weeks of *in vitro* cultivation. As a final result, the study resulted in improved growth and rooting in the presence of indole-3-butyric acid, and a higher survival rate in the acclimatization phase.

Based on the developed study, it was possible to observe the effect of essential oils in the control of contaminants and in the phytotoxicity rate on explants in tissue culture of camu-camu. Below, it is possible to observe the phytotoxic effect of essential oils of garlic (Figure 3A) and citronella (Figure 3B) at a concentration of 1 µL $mL^{-1}$ in camu-camu explants:

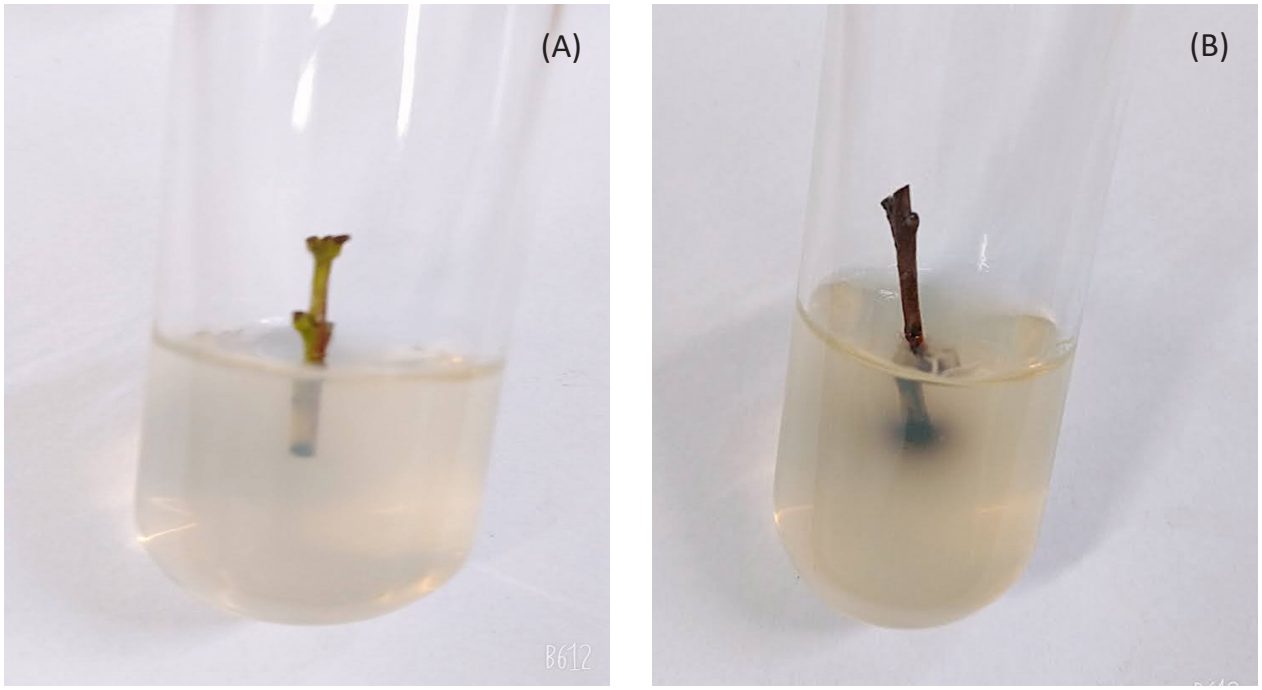


**Figure 3** – Evaluation of the phytotoxic effect of essential oils on the explant in camu-camu tissue culture.

Garlic essential oil proved to be the best *in vitro* treatment, with a low oxidation rate and better effectiveness against microorganisms in camu-camu micropropagation, although it did not correspond to the best antibacterial treatment in the agar diffusion analysis. This can be attributed to the fact that the garlic essential oil is resinous and denser than the others. The low phytotoxicity of garlic essential oil favors its use in tissue culture, enabling its use in supplementation, replacing or added to other antimicrobial agents, in the successful *in vitro* cultivation of camu-camu due to its antioxidant, antibacterial and antifungal properties.

## Conclusion

Essential oils of oregano and citronella showed better antibacterial activity in the agar well diffusion test. In tissue culture, the essential oils based on garlic, citronella and oregano showed better control in the inhibition of mycelial growth and a significant reduction in bacterial contamination. Despite the effectiveness in the microbial control of these oils, garlic essential oil was the one that presented the lowest phytotoxicity to the explants and the best balance between phytotoxicity and microbial control, being thus indicated as the best *in vitro* treatment at concentrations between 0.5 and 1 µL $mL^{-1}$.

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