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Isolation, molecular identification, and *in vitro* control of *Campomanesia rufa* (O. Berg) Nied. endogenous bacteria

Isolamento, identificação molecular e controle *in vitro* de bacterias endógenas de *Campomanesia rufa* (O. Berg) Nied.

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ABSTRACT

Campomanesia rufa (O. Berg) Nied. is a Myrtaceae species native to the Brazilian Cerrado. As *C. rufa* is considered endangered, *in vitro* propagation is an alternative for its conservation. However, the lack of effective disinfection protocols for endophytic microorganisms naturally present in plant tissues hinders the success of micropropagation. In this context, the objective of this study was to isolate, molecular identify, and control endogenous occurring bacteria of *C. rufa* propagated *in vitro*. Purified PCR products of bacterial isolates were sequenced by the Sanger method and aligned with homologous sequences using the Basic Local Alignment Search Tool (BLAST) available in National Centre for Biotechnology Information (NCBI) database. MEGA7 software was used to align all sequences and to draw phylogenetic trees. Survival, shoot height, and efficiency of antibiotics (streptomycin, ampicillin, and chloramphenicol) at different concentrations in the culture medium were evaluated. Different *Bacillus* sp. strains were observed in the plant tissues. When testing the control of *Bacillus* sp. with antibiotics, 32 mg L^{-1} ampicillin caused a significant reduction in bacterial contamination with no effect on explant survival. On the other hand, 256 mg L^{-1} streptomycin caused the greatest reduction in contamination but was lethal to over 90% of the explants. This study is the first report on the occurrence of endogenous bacteria and their control in the context of *in vitro* native species conservation.

Index terms: '*Casaqueira*'; phylogenetic tree; *Bacillus* sp.; antibiotics.

RESUMO

Campomanesia rufa (O. Berg) Nied. é uma espécie mirtácea nativa do Cerrado brasileiro. Considerada uma espécie ameaçada de extinção, a propagação *in vitro* de *C. rufa* apresenta-se como uma alternativa para sua conservação. No entanto, a falta de protocolos eficazes para a desinfecção de microrganismos endofíticos naturalmente presentes nos tecidos vegetais dificulta o sucesso da micropropagação. Neste contexto, o objetivo deste estudo foi o isolamento, a identificação molecular e o controle de bactérias endógenas de *C. rufa* propagadas *in vitro*. Produtos de PCR purificados dos isolados bacterianos foram sequenciados pelo método de Sanger. As sequências obtidas foram alinhadas com sequências homólogas usando Basic Local Alignment Search Tool (BLAST) disponível na National Centre for Biotechnology Information (NCBI). As sequências obtidas neste estudo e as sequências homólogas listadas no GenBank foram alinhadas usando o software MEGA7 para elaborar árvores filogenéticas. Avaliaram-se a sobrevivência, a altura da parte aérea e a eficiência de antibióticos (estreptomicina, ampicilina e cloranfenicol) em diferentes concentrações no meio de cultura. A ocorrência de diferentes cepas de *Bacillus* sp. foi observada nos tecidos vegetais. Ao testar o controle de *Bacillus* sp. com antibióticos, o uso de 32 mg L^{-1} de ampicilina causou uma redução significativa na contaminação bacteriana sem afetar a sobrevivência do explante. Por outro lado, o uso de 256 mg L^{-1} de estreptomicina causou a maior redução na contaminação, embora esta concentração tenha sido letal para mais de 90% dos explantes. Este estudo é o primeiro relato sobre a ocorrência de bactérias endógenas e seu controle no contexto da conservação *in vitro* de espécies nativas.

Termos para indexação: '*Casaqueira*'; árvore filogenética; *Bacillus* sp.; antibióticos.

INTRODUCTION

The Brazilian savanna (Cerrado) biome is recognized as a global biodiversity hotspot (Sloan et al., 2014). Different native plant species of the Cerrado produce fruits with nutritional and pharmaceutical potential containing secondary compounds such as phenols, antioxidants, and antiproliferative agents (De Giffoni de Carvalho et al., 2019). The scarce information on the use and properties of these plants highlight the lack of studies aiming at understanding these species, their interactions and mechanisms with other microorganisms (Da Silva et al., 2019). In addition, almost 50% of the biome was devasted by the expansion of agriculture leading to significant losses of genetic diversity (Cohn et al., 2019), mainly from species of the Myrtaceae family (Da Silva et al., 2019; Abreu et al., 2020). Important nutritional traits of native species (e.g., phenolic content and antioxidant activity) make it possible for breeding programs to take advantage of these qualities more quickly (Belo et al., 2019; Barbosa et al., 2022). Studies have shown beneficial interactions between (endophytic) microorganisms and plants, which may also be valuable for agriculture (Tahir et al., 2017).

One species endemic to the Cerrado of Minas Gerais state (Villarroel et al., 2016) categorized as endangered by the (International Union for the Conservation of Nature - IUCN, 2023) is *Campomanesia rufa* (O. Berg) Nied., popularly known as '*casaqueira'* or '*gabiroba'* (Sant'Ana et al., 2018). The fruit of this woody Myrtaceae species has high vitamin C content and high *in vitro* antioxidant activity (Abreu et al., 2020).

One way to propagate endangered native plants is tissue culture. This technique allows a high multiplication rate (Oseni; Pande; Nailwal, 2018) and, if necessary, the maintenance of the species' genetic characteristics through micropropagation (Aaqib et al., 2022; Rodrigues et al., 2020). However, *in vitro* multiplication is still difficult for many native Cerrado species such as *C. rufa*. Some of the reasons include low seed germination rate (Sant'Ana et al., 2018) and the lack of effective disinfection protocols to eliminate epiphytic and endophytic microorganisms naturally present in plants. The use of silver nanoparticles (AgNPs), known for their antibacterial properties, did not affect the *in vitro* multiplication of *C. rufa* nodal segments (Timoteo et al., 2019). However, few asepsis protocols are available in cases of endogenous contamination (Ali et al., 2018), especially for woody species (Salles et al., 2017).

Plant-endophyte relationships may produce secondary metabolites and enzymes that benefit both species (Orlikowska; Nowak; Reed, 2017) besides having potential biotechnological uses in the food, pharmaceutical, medicine, and textile industries (Gopinath et al., 2017; Orlikowska; Nowak; Reed, 2017; Savi; Aluizio; Glienke, 2019). A better understanding of the interaction between plants and endophytes enhances our knowledge of plant ecology and facilitates the management of disease resistance in native plants with economic potential.

Contamination by microorganisms in the culture medium is a common problem for the propagation or maintenance of *in vitro* cultures (Mahmoud; Al-Ani, 2016; Metwaly; Salama; Ali, 2018). In contrast, endophytes can promote the growth of plants cultured *in vitro* (Santoyo et al., 2016). However, even these beneficial microorganisms must be carefully controlled because they compete for the nutrients available in the environment (Shehata et al., 2016). Disinfection by immersing the explants in 70% ethanol and then in another disinfectant solution (e.g., sodium hypochlorite) should ideally eliminate surface contaminating microorganisms (Huang; Yuan; Chen, 2020).

When this is insufficient, the media can be supplemented with antibiotics (Khan et al., 2018). Among the known antibiotics, ampicillin, streptomycin, and chloramphenicol are widely used in tissue culture (Torres; Houllou, 2016; Buckseth et al., 2017; Gerszberg; Grzegorczyk-Karolak, 2019). Ampicillin is a broad-spectrum aminopenicillin that inhibits the biosynthesis of cell wall mucopeptides (Da Silva et al., 2003). Streptomycin and chloramphenicol are protein synthesis inhibitors (Pankhurst, 1977). This study aimed to isolate and molecularly identify endogenous bacteria from *in vitro* propagated *C. rufa* seedlings, as well as to evaluate the ideal concentration of the antibiotics ampicillin, streptomycin, and chloramphenicol for controlling endogenous bacteria on *C. rufa* shoots.

MATERIAL AND METHODS

Plant identification, germination, and *in vitro* multiplication

The *Campomanesia rufa* plants used in this study were located at 21°13′35.5″ south latitude and 44°59′00.7″ west longitude. Specimens are deposited in the School of Agricultural Sciences of Lavras (ESAL) at the Herbarium of the Federal University of Lavras (voucher ESAL21198).

Ripe fruits of *C. rufa* were collected and washed with running water and detergent before seed extraction. The seeds were germinated *in vitro* according to the protocol of Sant'Ana et al. (2018) and maintained in the dark for 7 days before being transferred to the growth room under a photoperiod of 16 hours, 36 μmol m^{-2} s^{-1}

irradiance, and temperature of 25 ± 2 °C. The seedlings were maintained under these conditions for 4 to 6 months.

Shoots were multiplied in MS medium (Murashige; Skoog, 1962) supplemented with 0.09 M sucrose, 2.5 g L^{-1} Phytagel®, and 5.6 µM benzylaminopurine. The pH was adjusted to 5.8 before the addition of Phytagel®. The medium was autoclaved at 121 °C, 1.5 atm for 20 minutes. The explants were transferred to a growth room with a 16-hour photoperiod under 36 µmol m^{-2} s^{-1} irradiance at 25 ± 2 °C (Sant'Ana et al., 2018).

As shoots showed rot symptoms, diagnostic tests to identify the causal agent were carried out at the Phytopathological Diagnostic Laboratory of the Biological Institute, São Paulo, Brazil.

Bacteria isolation

Bacteria were isolated from *C. rufa* shoots multiplied *in vitro* according to Verma and Sao (2018). Exudates and fragments of *C. rufa* seedlings were plated in Petri dishes containing 25 to 30 mL of nutrient agar medium (Kasvi, Madrid, Spain) prepared according to the manufacturer's instructions. The Petri dishes were sealed and incubated at 25 °C for 24 to 48 hours to form colonies. Subcultures were performed at intervals from 20-30 days using the same medium.

Extraction of genomic DNA from the bacterium

Bacterial DNA was extracted from colonies grown on plates containing exudates and seedling fragments using the Wizard® Plus Miniprep-DNA purification kit (Promega, USA) and the DNAzol® Reagent - Genomic DNA isolation reagent kit (Thermo Fisher, United States), according to the manufacturer's instructions.

Amplification by PCR

The gene encoding the 16S ribosomal subunit (16S rRNA) was used as target for detecting bacteria and sequencing the amplicon (Kai et al., 2019). The bacterial DNAs obtained by the two extraction procedures were subjected to PCR with the 16S rDNA forward primer Y1 described by Young, Downer and Eardly (1991) (5'-TGGCTCAGAACGAACGCTGCGGC-3') and the reverse primer Y3 described by Cruz et al. (2001) (5'-CTGACCCCACTTCAGCTTGTTCCAT-3'). PCR reactions contained 3 µL of DNA, 1 µL of each primer at 10 µM, 0.5 µL of dNTPs (10 mM), 2.5 µL of 10× buffer (GeneDireX, Taoyuan, Taiwan), 0.25 µL of *Taq* DNA Polymerase (GeneDireX, Taoyuan, Taiwan), and ultrapure water up to 25 µL. The reactions were carried out in an Axygen thermocycler following the thermal profile: 94 °C for 4 minutes, followed by 35 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 74 °C for 2 minutes, followed by a final extension at 74 °C for 10 minutes.

The PCR product was analyzed on a 1.5% agarose gel in 1× TAE buffer (40 mM Tris-acetate, pH 8.0, 20 mM sodium acetate, 1 mM EDTA) in the presence of ethidium bromide. The electrophoretic run was performed at 80 V for 40 minutes. The gel was photographed using a UDV-312 transilluminator imaging system (Major Science, CA, United States).

Purification of the PCR product and sequencing

The products obtained from the PCR of three bacterial isolates were cut from the agarose gel and purified with the kit Wizard PCR Preps DNA purification system (Promega, United States) according to the manufacturer's instructions. The purified products were sent for sequencing at the Laboratory of Bovid Viruses, Biological Institute (São Paulo, Brazil) using the Sanger method.

Sequence analysis and phylogeny

The sequences obtained in this study were compared with homologous sequences listed in GenBank using the Basic Local Alignment Search Tool (BLAST) available in National Center for Biotechnology Information (NCBI) database. Pairwise and multiple sequence alignments were performed using MEGA7 software (Kumar; Stecher; Tamura, 2016) and were manually adjusted. The pairwise sequence alignments were used for the reconstruction of phylogenetic trees.

Antibiotics for *in vitro* control of endogenous bacteria

Shoots with two axillary buds obtained from *in vitro* multiplication were grown in a fresh multiplication medium. Different concentrations (32, 64, 128, and 256 mg L^{-1}) of antibiotics ampicillin, chloramphenicol, and streptomycin were pre-sterilized by cold filtration (Millipore, 0.22 µm) and added to the multiplication medium when being cooled (40 to 50 °C). The control consisted of a culture medium without antibiotics.

Each treatment consisted of 30 replicates. At 25 days, the percentage of explant survival, rot symptoms, and shoot height were evaluated.

Statistical analysis

The experiments were conducted in a completely randomized design. The data were subjected to analysis of variance and the means were analyzed at 5% probability by regression or by the Scott-Knott test. The statistical

analyses were performed using the statistical software R version 3.5.2 (R Core Team, 2019) and the ExpDes.pt package (Ferreira; Cavalcanti; Nogueira, 2013).

RESULTS AND DISCUSSION

Different microorganisms can live endogenously in plants and have a neutral, negative, or positive impact on their host (Ribeiro; Pamphile, 2017). The diversity of endophytic microorganisms in native plants such as *C. rufa* is underexplored and may lead to the discovery of species of interest to the medical and agricultural fields (Contesini; Melo; Sato, 2018).

Endogenous bacteria were visible in the *in vitro* subcultures originated from the propagation of shoots from both adult plants and seedlings. In the wild, no symptoms of rot were observed in the plants. Despite the disinfection protocol, the bacteria remained in the seeds and subsequently in the seedlings. Initially, the presence of the bacterium was not harmful to seedling growth (Figure 1).

Endophytic bacteria also limited the already efficient *in vitro* establishment of nodal segments in *Guadua angustifolia* (Tejada et al., 2022). Axenic (germ-free) plant cultures are inexistent since all plants have endophytes but some do not cause any apparent disease (Espósito, 2020).

Bacterial strains that multiplied during plant micropropagation were isolated from exudates and fragments of *C. rufa* seedlings. The bacterial colonies were shining and creamy white in color and had two types of edges: regular/smooth or irregular/rough whose subcultures gave rise to strains named 5SF and 5CF, respectively. Moreover, in one of the Petri dishes derived from seedling exudates, the colonies showed only regular/smooth edges and was named strain 5. The different strain morphology is shown in (Figure 2). The Gram stain test showed that gram-positive bacteria were present in each strain.

Figure 1: Symptoms of bacterial rot in seedlings of *C. rufa* grown *in vitro* (Scale bar = 1 cm).

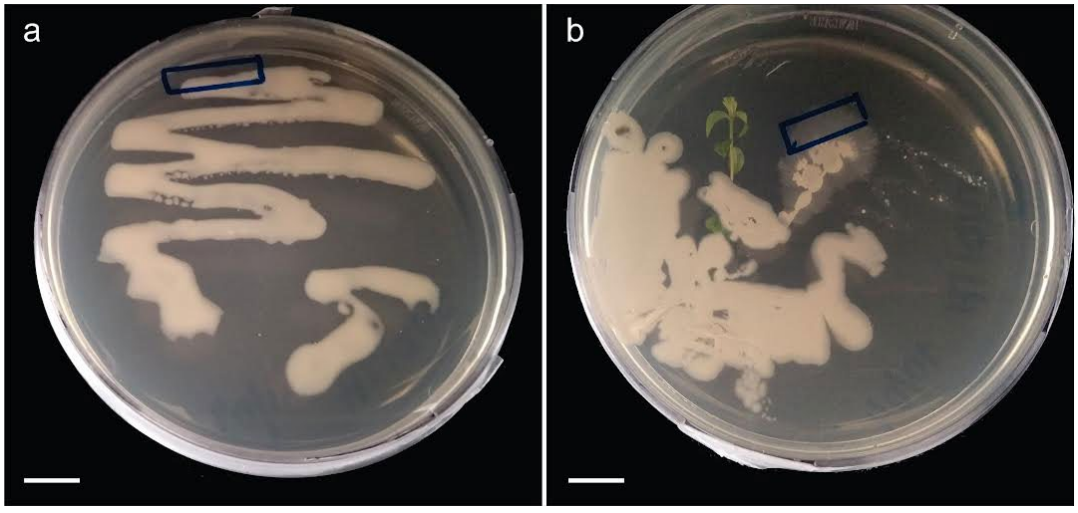

Figure 2: Edge morphology of bacterial colonies isolated from shoots of *C. rufa* cultured *in vitro*. (a) Regular/smooth edge; (b) Irregular/rough edge (Scale bar = 1 cm).

To establish the bacterial DNA extraction protocol, colonies with regular/smooth and irregular/rough edges were mixed and processed by two methods. PCRs resulted in a product of approximately 1500 bp, as expected for the Y1/Y3 primer pair. Based on this result, the DNA of each strain (5, 5SF, and 5CF) was then individually extracted with DNAzol. DNA extraction methods usually vary according to the sample or matrix (Nascimento et al., 2017). However, the solutions must contain a buffer substance to stabilize the pH, a salt to dissociate the proteins, a detergent to solubilize the membranes, and an agent to inactivate the DNases and protect the genomic DNA (Nascimento et al., 2017).

The highly conserved 16S rRNA gene is often used in the reconstruction of phylogenetic relationships of microorganisms (Cruz et al., 2001; Ali et al., 2018). The pairwise analysis of the three 16S rRNA gene sequences obtained from bacteria isolated from *C. rufa* and homologous sequences in GenBank is shown in Figure 3.

Colonies with different edge types (i.e., strains 5CF and 5SF) showed 90.5% identity between their sequences, while those with regular/smooth edges from different Petri dishes (i.e., strains 5SF and 5) had 96.8% identity. The highest percentage identity between the three bacterial strains obtained from *C. rufa* and those deposited in GenBank was 97.3%, 98.9%, and 98.7% for 5SF, 5, and 5CF strains, respectively.

The 5SF strain showed a high percentage identity not only with *Bacillus thuringiensis* strains but also with *B. cereus* and *Bacillus* sp. A high percentage identity of strain 5 with *B. thuringiensis*, *Bacillus* sp., and *Bacterium* strains was also observed. In turn, the 5CF strain showed a more significant identity with the *B. altitudinis*, *B. pumilus*, *Bacillus* sp., and *Bacterium* strains.

The three bacterial isolates identified in this study were not in the same subtree. Strain 5CF, with irregular edges, shared a common ancestor with *B. altitudinis*, while colonies with smooth and regular edges, 5 and 5SF, shared the same ancestor. The results obtained in the pairwise and phylogenetic analyses indicate that the three bacterial strains belong to the genus *Bacillus*, but it was not possible to identify the species.

Species of the genus *Bacillus* can be anaerobic, aerobic, or facultative (Villarreal et al., 2018). The bacteria are gram-positive, have a rod morphology, and form spores under adverse conditions, allowing long survival in different habitats with a wide pH range (2 to 10). (Shafi; Tian; Ji, 2017). These bacteria also produce heat-resistant spores because they have inducible heat shock genes (Calvo; Zuñiga, 2010). Although they can live in soil, *Bacillus* can be isolated from various hosts, from plants to humans (Villarreal et al., 2018), and can be both phytopathogens and opportunists and produce toxins (Elshaghabee et al., 2017). In addition, they have a great diversity of mechanisms that promote plant health (Lanna Filho; Ferro; Pinho, 2010). For example, species such as *B. thuringiensis*, *B. megaterium*, *B. cereus*, and *B. subtilis* have been used to improve the development of *Chrysophyllum cainito* L. and *Litchi chinensis* Sonn

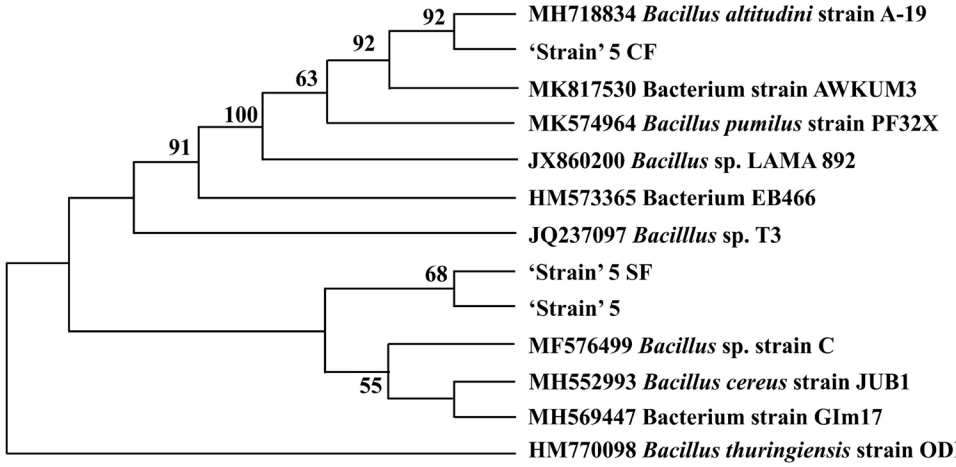

Figure 3: Phylogenetic tree for bacterial strains based on the nucleotide sequences of the 16S rRNA gene. A total of 1135 positions were analyzed and all positions with gaps were excluded. The numbers shown next to the nodes indicate percentage bootstrap values (1000 replicates). Bootstrap values below 50% are not shown in the tree. Distances were inferred by the maximum likelihood method using Kimura two-parameter model. Phylogenetic analysis was performed with MEGA7 software.

seedlings (Santos et al., 2018). *Bacillus* Rh219 was shown to increase the biometric parameters and defense enzyme activity in rice plants (Yasmin et al., 2016). Under field conditions, some endogenous bacteria can promote plant hormone production, plant growth, phosphate solubilization, nitrogen fixation, pathogen resistance, and heavy metal degradation (Liu et al., 2017). However, in tissue culture, endogenous bacteria can compete with the plant for nutrients (Cheong; Na; Jeong, 2020).

Interestingly, the phylogenetic analysis revealed the presence of strains with different ancestors coexisting within the same *C. rufa* plant. Two isolates with the same colony type, 5SF and 5, shared the same ancestor, which was different from that of 5CF. Thus, studies aimed at understanding the relationship not only between endogenous bacteria and host plants but also between bacterial strains coexisting in the same plant will help us to properly use these bacteria for biological control.

Despite the high economic potential of Cerrado species such as *C. rufa*, no information is available on the microbiota associated with them. Therefore, knowledge of other microorganisms and their relationships with native species would support research focused on genetic resources or derived products for agronomic, industrial, pharmaceutical, cosmetic, and food purposes (Costessi et al., 2018).

The use of ampicillin, streptomycin, and chloramphenicol reduced the occurrence of bacterial contamination in *C. rufa* without compromising the survival of the explant up to a concentration of 64 mg L^{-1} (Figure 4).

These results indicate the elimination of the microbial infestation and contributed to the asepsis and maintenance of the culture established *in vitro*. Even the lowest antibiotic concentration reduced the bacteria to levels below 30% in the case of ampicillin and streptomycin, and to 40% when using chloramphenicol. Total bacterial elimination was not observed at any concentration tested (Figure 4a and Figure 5).

Thus, the combination of more than one antibiotic could be a viable option for the complete asepsis of the plant material (Orlikowska; Nowak; Reed, 2017). Two basic strategies can be used: combined (mixture) or sequential (successive cycles) addition of antibiotics (Quambusch; Winkelmann, 2018). Ampicillin has activity against gram-positive and gram-negative bacteria and is, therefore, a broad-spectrum antibiotic (Eslami; Sarlak, 2018). Streptomycin is indicated against infections caused by gram-negative or gram-positive bacteria if combined with other antibiotics (Ramirez; Tolmasky, 2017; Tertigas; Barber, 2019). Chloramphenicol is also a broad-spectrum antibiotic (Ansiliero; Candiago; Gelinski, 2018).

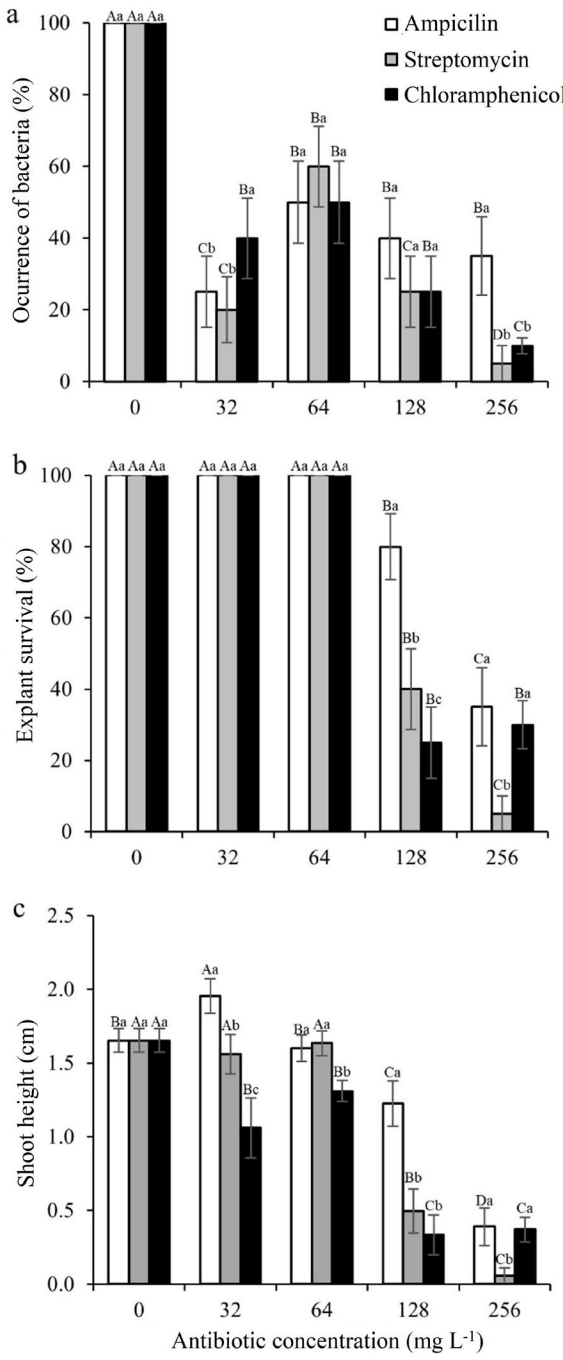

Figure 4: Effect of antibiotic concentration on (a) Bacteria occurrence, (b) Explant survival, and (c) Shoot height. Values shown are means ± standard error (n = 30). Significant differences (Scott-Knott test for comparison of the means, P < 0.05%) are indicated by letters. Capital letters compare different concentrations of each antibiotic, and small letters compare the same concentration among antibiotics.

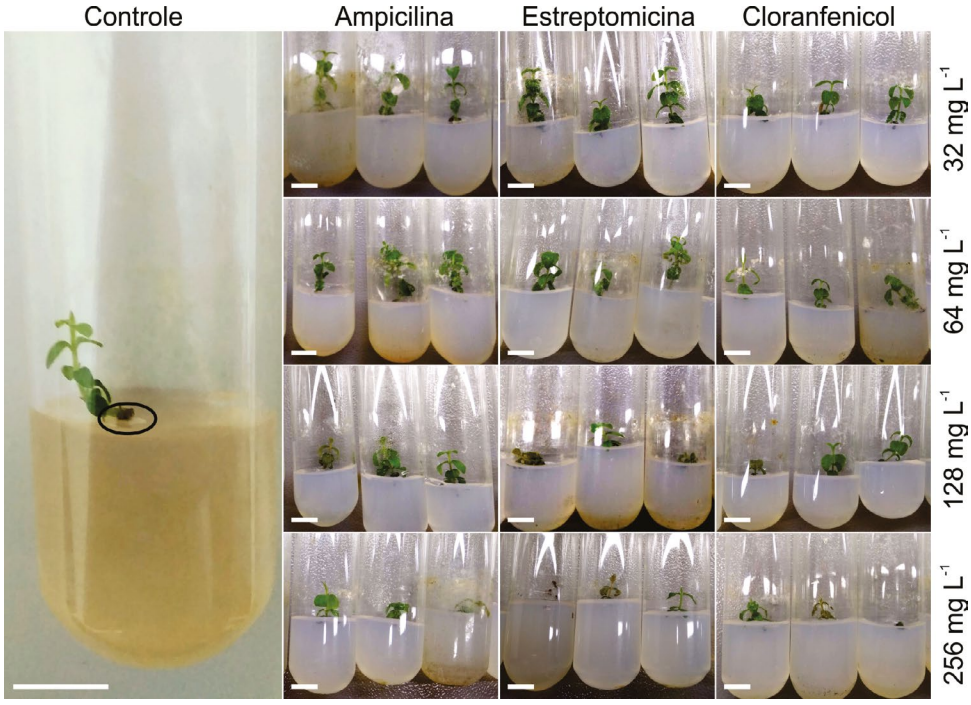

Figure 5: Shoots of *C. rufa* subjected to different concentrations of antibiotics. The circle shows the presence of the bacterium.

From the toxicological point of view, the disinfecting agent should act only on pathogenic organisms, preserving the vitality of the plant (Quambusch; Winkelmann, 2018). This was observed up to a concentration of 64 mg L^{-1} of all three antibiotics evaluated. However, the survival of the explants was reduced at 128 mg L^{-1} (Figure 4b; 5). Streptomycin and chloramphenicol caused the highest percentages of explant loss (Figure 5). Although the effects of residual antibiotics in plants on human health are well studied, little attention has been given to their effects on the plants themselves, especially the uncultivated (Minden et al., 2017). High concentrations of antibiotics can be toxic to plants, inhibit growth and photosynthesis, and cause oxidative stress (Bao; Gu; Zhang, 2016; Minden et al., 2017; Riaz et al., 2017; Liu et al., 2018). Streptomycin and chloramphenicol negatively affected the growth of the shoots regenerated at 128 mg L^{-1}. In turn, ampicillin at 32 mg L^{-1} caused a slight increase in growth, resulting in higher shoots than in the control, whereas higher concentrations had an inhibitory effect (Figure 4c; Figure 5).

Antibiotics have a biphasic effect on growth characterized by hormesis, a low-dose response that has an opposite effect than high doses. Their phytotoxicity varies according to the antibiotic and plant species (Pan; Chu, 2017). The threshold survival levels under exposure to different antibiotics in tobacco (*Nicotiana tabacum* Samsun SS) and two chrysanthemum cultivars (*Dendranthema* × *Grandiflora*) are distinctly different (Silva et al., 2003). However, in that study, the ranking of the toxicity of the antibiotics was the same as found here: chloramphenicol > streptomycin > ampicillin.

Considering that the contamination was not eliminated and the variety of microorganisms that may proliferate in the culture medium, future studies should test a lower concentration of combined antibiotics to check the possibility of a contamination-free culture.

CONCLUSIONS

This is the first report of the occurrence of endogenous bacteria (*Bacillus* spp.) in shoots of *C. rufa* grown *in vitro* and the presence of different coexisting strains. The soft rot in *C. rufa* plants cannot be definitively associated with the presence of one, two, or all three *Bacillus* sp. strains. Ampicillin was able to reduce *Bacillus* sp. contamination with no effect on explant survival and promoted plant growth. The highest reduction in contamination was achieved using streptomycin although it was lethal to over 90% of the explants.

AUTHOR CONTRIBUTION

Conceptual idea: Mosqueira, J.G.A.; Paiva, R.; Rivas, E.B.; Reis, M.V.; Methodology Design: Mosqueira, J.G.A.; Paiva, R.; Rivas, E.B.; Harakava, R.; Souza-Dias, M.A.G.; Reis, M.V.; Data collection: Mosqueira, J.G.A.; Paiva, R.; Rivas, E.B.; Harakava, R.; Souza-Dias, M.A.G.; Data Analysis and Interpretation: Mosqueira, J.G.A.; Paiva, R.; Rivas, E.B.; Harakava, R.; Reis, M.V., and Writing and Editing: Mosqueira, J.G.A.; Paiva, R.; Rivas, E.B.; Reis, M.V.

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REFERENCES

AAQIB, S. et al. Synergistic effect of benzylaminopurine and meta-topolin combination for micropropagation of gerbera 'Pink Melody. Ciência e Agrotecnologia, 46:e017521, 2022.

ABREU, L. A. F. et al. Antioxidant activity and physico-chemical analysis of *Campomanesia rufa* (O. Berg) Nied. fruits. Ciência e Agrotecnologia, 44:e016720, 2020.

ALI, M. et al. Endogenous bacterial contamination of plant tissue culture materials: Identification and control strategy. Plant Tissue Culture & Biotechnology, 28(1):99-108, 2018.

ANSILIERO, R.; CANDIAGO, N. T.; GELINSKI, J. M. L. N. Caracterização bioquímica e potencial de biocontrole de rizobactérias de feijão preto (*Phaseolus derasus*) contra patógenos bacterianos um estudo preliminar. Anuário Pesquisa e Extensão Unoesc Videira, 3:e19684, 2018.

BAO, C. Y.; GU, G. P.; ZHANG, M. K. Effects veterinary antibiotics stress on growth and antibiotics accumulation of *Oenanthe javanica* DC. Chinese Journal of Soil Science, 47(1):164-172, 2016.

BARBOSA, F. J. V. et al. Potencial anti-inflamatório e antioxidante dos frutos do Tocantins. Multidebates, 6(3):17-27, 2022.

BELO, A. P. M. et al. Fenologia, biometria e precocidade de plantas de caju arbóreo do Cerrado (*Anacardium othonianum* Rizz.). Ciência Florestal, 29(4):1672-1284, 2019.

BUCKSETH, T. et al. Effect of streptomycin and gentamycin on *in vitro* growth and cultural contaminants of potato cultivars. International Journal of Current Microbiology and Applied Sciences, 6(12):4038-4043, 2017.

CALVO, P.; ZUÑIGA, D. Caracterización fisiológica de cepas de *Bacillus* spp. aisladas de la rizósfera de papa (*Solanum tuberosum*). Ecología Aplicada, 9(1):31-39, 2010.

CHEONG, E. J.; NA, M.; JEONG, U. The effect of endophytic bacteria on *in vitro* shoot growth of *Prunus yedoensis* and its identification and elimination. In Vitro Cellular & Developmental Biology-Plant, 56:200-206, 2020.

COHN, A. S. et al. Forest loss in Brazil increases maximum temperatures within 50 Km. Environmental Research Letters, 14:084047, 2019.

CONTESINI, F. J.; MELO, R. R.; SATO, H. An overview of *Bacillus* proteases: From production to application. Critical Reviews in Biotechnology, 38(3):321-334, 2018.

COSTESSI, A. et al. Novel sequencing technologies to support industrial biotechnology. FEMS Microbiology Letters, 365(16):1-13, 2018.

CRUZ, L. M. et al. 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (*Musa* spp.) and pineapple (*Ananas comosus* (L.) Merril. Applied and Environmental Microbiology, 67(5):2375-2379, 2001.

DA SILVA, J. A. T. et al. The effect of antibiotics on the *in vitro* growth response of chrysanthemum and tobacco stem transverse thin cell layers (tTCLs). Scientia Horticulturae, 97(3-4):397-410, 2003.

DE GIFFONI DE CARVALHO, J. T. et al. Medicinal plants from brazilian Cerrado: Antioxidant and anticancer potential and protection against chemotherapy toxicity. Oxidative Medicine and Cellular Longevity, Article ID 3685264, 2019.

ELSHAGHABEE, F. M. F. et al. *Bacillus* as potential probiotics: Status, concerns, and future perspectives. Frontiers in Microbiology, 8:1490, 2017.

ESLAMI, S.; SARLAK, M. Forced degradation tests of ampicillin in medication forms. MedBioTech Journal, 2(3):113-117, 2018.

ESPÓSITO, N. P. P. Contaminação versus manifestação endofítica: Implicações no cultivo *in vitro* de plantas. Rodriguésia, 71:1-15, 2020.

FERREIRA, E.; CAVALCANTI, P.; NOGUEIRA, D. ExpDes.Pt: Experimental Designs Package. R Package (Português) Version 1.1.2, 2013. Available in: <http://CRAN.R-project.org/package=ExpDes.pt>. Access in: August 16, 2023.

GERSZBERG, A.; GRZEGORCZYK-KAROLAK, I. Influence of selected antibiotics on the tomato regeneration in *in vitro* cultures. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 47(3):558-564, 2019.

GOPINATH, S. C. B. et al. Biotechnological processes in microbial amylase production. BioMed Research International, Article ID 1272193, 2017.

HUANG, Y. L.; YUAN, S. C.; CHEN, F. C. Establishment of an efficient micropropagation system in *Anthurium* hybrids through *in vitro* callogenesis and suspension culture. The Horticulture Journal, 89(1):54-60, 2020.

INTERNATIONAL UNION FOR CONSERVATION OF NATURE - IUCN. The IUCN red list of threatened species. Version 2022-2. 2023. Available in: <www.iucnredlist.org>. Access in: February, 14, 2023.

KAI, S. et al. Rapid bacterial identification by direct PCR amplification of 16S rRNA genes using the MinION TM nanopore sequencer. FEBS Open Bio, 9(3):548-557, 2019.

KHAN, T. et al. Molecular identification and control of endophytic contamination during *in vitro* plantlet development of *Fagonia indica*. Acta Physiologiae Plantarum, 40:150, 2018.

KUMAR, S.; STECHER, G.; TAMURA, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution, 33(7):1870-1874, 2016.

LANNA FILHO, R.; FERRO, H. M.; PINHO, R. S. C. Controle biológico mediado por *Bacillus subtilis*. Revista Trópica: Ciências Agrárias e Biológicas, 4(2):12-19, 2010.

LIU, H. et al. Inner plant values: Diversity, colonization and benefits from endophytic bacteria. Frontiers in Microbiology, 8:2552, 2017.

LIU, X. et al. Response of ginger growth to a tetracycline-contaminated environment and residues of antibiotic and antibiotic resistance genes. Chemosphere, 201:137-143, 2018.

MAHMOUD, S. N.; AL-ANI, N. K. Effect of different sterilization methods on contamination and viability of nodal segments of *Cestrum Nocturnum* L. International Journal of Research Studies in Biosciences, 4:4-9, 2016.

METWALY, A.; SALAMA, G. M.; ALI, G. A. Using hydrogen peroxide for reducing bacterial contamination in date palm tissue culture. International Journal of Advances in Agricultural Science and Technology, 5(4):25-33, 2018.

MINDEN, V. et al. Antibiotics impact plant traits, even at small concentrations. AoB Plants, 9(2):1-19, 2017.

MURASHIGE, T.; SKOOG, F. A. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15(3):473-97, 1962.

NASCIMENTO, A. L. S. et al. Extração de DNA em mangabeira (*Hancornia Speciosa* Gomes). Nucleus, 14(2):97-106, 2017.

ORLIKOWSKA, T.; NOWAK, K.; REED, B. Bacteria in the plant tissue culture environment. Plant Cell, Tissue and Organ Culture, 128(3):487-508, 2017.

OSENI, O. M.; PANDE, V.; NAILWAL, T. K. A review on plant tissue culture, a technique for propagation and conservation of endangered plant species. International Journal of Current Microbiology and Applied Sciences, 7(7):3778-3786, 2018.

PAN, M.; CHU, L. M. Fate of antibiotics in soil and their uptake by edible crops. Science of the Total Environment, 599:500-512, 2017.

PANKHURST, C. E. Symbiotic effectiveness of antibiotic-resistant mutants of fast-and slow-growing strains of *Rhizobium* nodulating *Lotus* species. Canadian Journal of Microbiology, 23(8):1026-1033, 1977.

QUAMBUSCH, M.; WINKELMANN, T. Bacterial endophytes in plant tissue culture: Mode of action, detection, and control. In: LOYOLA-VARGAS, V. M.; OCHOA-ALEJO, N. Methods in Molecular Biology, New York, United States: Human Press, p. 69-88, 2018.

R CORE TEAM. R: A Language and environment for statistical computing. R foundation for statistical computing, Version 4.3.1. Vienna, Austria, 2019. Available in: <https://www.R-project.org/>. Access in: August 16, 2023.

RAMIREZ, M. S.; TOLMASKY, M. E. Amikacin: Uses, resistance, and prospects for inhibition. Molecules, 22(12):2267, 2017.

RIAZ, L. et al. Physiological and antioxidant response of wheat (*Triticum aestivum*) seedlings to fluoroquinolone antibiotics. Chemosphere, 177:250-257, 2017.

RIBEIRO, A. S.; PAMPHILE, J. A. Microorganismos endofíticos e seu potencial biotecnológico. Uningá Review, 29(3):88-93, 2017.

RODRIGUES, F. R. et al. *In vitro* callus induction and development of *Vernonia condensata* Baker with embryogenic potential. Ciência e Agrotecnologia, 44:e026719, 2020.

SALLES, E. A. P. B. et al. Desinfestação e introdução *in vitro* de segmentos nodais de *Acacia mearnsii*. Pesquisa Florestal Brasileira, 37(92):485-491, 2017.

SANT'ANA, C. R. O. et al. *In vitro* propagation of *Campomanesia rufa*: An endangered fruit species. Ciência e Agrotecnologia, 42(4):372-380, 2018.

SANTOS, C. H. B. et al. Promoting fruit seedling growth by encapsulated microorganisms. Revista Brasileira de Fruticultura, 40(3):e-179, 2018.

SANTOYO, G. et al. Plant growth-promoting bacterial endophytes. Microbiological Research, 183:92-99, 2016.

SAVI, D. C.; ALUIZIO, R.; GLIENKE, C. Brazilian plants: An unexplored source of endophytes as producers of active metabolites. Planta Medica, 85(8):619-636, 2019.

SHAFI, J.; TIAN, H.; MINGSHAN, J. *Bacillus* species as versatile weapons for plant pathogens: A review. Revista Mexicana de Fitopatología, 31(3):446-459, 2017.

SHEHATA, H. R. et al. Relevance of *in vitro* agar based screens to characterize the anti-fungal activities of bacterial endophyte communities. BMC Microbiology, 16:8, 2016.

SILVA, D. L. et al. Antioxidant capacity of cerrado fruits. DESAFIOS - Revista Interdisciplinar da Universidade Federal do Tocantins, 6:127-133, 2019.

SILVA, J. T. et al. The effect of antibiotics on the *in vitro* growth response of *Chrysanthemum* and *Tobacco* stem transverse thin cell layers (TTCLs). Scientia Horticulturae, 97(3-4):397-410, 2003.

SLOAN, S. et al. Remaining natural vegetation in the global biodiversity hotspots. Biological Conservation, 177:12-24, 2014.

TAHIR, H. A. S. et al. Plant growth promotion by volatile organic compounds produced by *Bacillus subtilis* SYST2. Frontiers in Microbiology, 8:171, 2017.

TEJADA, J. J. A. et al. Efecto de biocidas y consistencia del medio de cultivo para el establecimiento *in vitro* de *Guadua angustifolia*. Bosque (Valdivia), 43(2):117-123, 2022.

TERTIGAS, D.; BARBER, G. Determining the rate of development of antibiotic resistance to streptomycin and doxycycline in *Escherichia Coli*. Sciential-McMaster Undergraduate Science Journal, 1(3):2-7, 2019.

TIMOTEO, C. D. et al. Silver nanoparticles in the micropropagation of *Campomanesia rufa* (O. Berg) Nied. Plant Cell, Tissue Organ Culture, 137:359-368, 2019.

TORRES, G. R. C.; HOULLOU, L. M. Control of contaminants during introduction and establishment of *Bambusa vulgaris in vitro*. Research in Biotechnology, 7:58-67, 2016.

VERMA, K. K.; SAO, S. Isolation and identification of endophytic bacteria from rare medicinal plant genera of Bilsapur city of Chhattisgarh. International Journal of Science and Research, 8(8):2211-2213, 2018.

VILLARREAL, M. F. D. et al. El género *Bacillus* como agente de control biológico y sus implicaciones en la bioseguridad agrícola. Revista Mexicana de Fitopatología, 36(1):95-130, 2018.

VILLARROEL, D. et al. New botanical discoveries in *Eugenia* (Myrtaceae) from Bolivia and Brazil. Phytotaxa, 253(4):266-274, 2016.

YASMIN, S. et al. Plant growth promotion and suppression of bacterial leaf blight in rice by inoculated bacteria. PLoS ONE, 11(8):e0160688, 2016.

YOUNG, J. P.; DOWNER, H. L.; EARDLY, B. D. Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. Journal of Bacteriology, 173(7):2271-2277, 1991.