

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

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Abstract

Campomanesia rufa (O. Berg) Nied. (Myrtaceae) is a species native to the Cerrado. *In vitro* conservation is an option for endangered species such as *C. rufa*. The lack of effective protocols for the disinfection of endophytic microorganisms naturally present in the plant hinders the success of micropropagation. Within this context, the objective of this study was the molecular identification and *in vitro* control of contamination using antibiotics. Purified PCR products of the bacterial isolates were sequenced by the Sanger method. The sequences obtained were aligned with homologous sequences using the Basic Local Alignment Search Tool. The sequences obtained in this study and the homologous sequences listed in GenBank were aligned using MEGA7 software to draw phylogenetic trees. The survival, shoot height, and efficiency of antibiotics (streptomycin, ampicillin, chloramphenicol) at different concentrations (0, 32, 64, 128, 256 mg L⁻¹) in the culture medium were evaluated at 25 days. The occurrence of *Bacillus* sp. and of different strains was observed in the plant. When testing the control of *Bacillus* sp. with antibiotics, ampicillin at 32 mg L⁻¹ caused a significant reduction in bacterial contamination without affecting the survival of explants. On the other hand, streptomycin caused the greatest reduction in contamination at 256 mg L⁻¹ but was lethal to more than 90% of explants at this concentration. This study is the first report on the occurrence of endogenous bacteria and their control in the context of the conservation of native species.

1. Introduction

The Brazilian Cerrado (savanna) 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, so they can be used to obtain secondary compounds, such as phenols, antioxidants, and antiproliferative agents of human cancer cells (Carvalho et al., 2019). The scarce information on the use and properties of the plant species native to the Cerrado is a reflection of the lack of scientific studies aimed at understanding these species and their interactions (Silva et al., 2019). In addition, the devastation of almost 50% of the biome has led to the loss of genetic diversity of an invaluable number of native species (Cohn et al., 2019), mainly from the family Myrtaceae (Silva et al., 2019). The important economic characteristics of native species warrant breeding programs to take advantage of these qualities more quickly (Belo et al., 2019). Studies have shown beneficial interactions between (endophytic) microorganisms and plants, which may also be beneficial for agriculture (Tahir et al., 2017).

One of the species that deserves attention because it is categorized as endangered by the International Union for the Conservation of Nature (IUCN, 2020) is *Campomanesia rufa* (O. Berg) Nied., known in Brazil as 'casaqueira' or 'gabioba' (Sant'Ana et al., 2018). The fruit of this woody Myrtaceae plant endemic to the Cerrado of Minas Gerais (Villarroel et al., 2016) has high vitamin C content and high antioxidant activity *in vitro* (Ferreira et al., 2020).

One way to reproduce and repopulate endangered native plants is tissue culture, a technique that allows a high multiplication rate (Oseni; Veena; Kumar, 2018) and, if necessary, the maintenance of the genetic

characteristics of the species through micropropagation. *In vitro* multiplication, which would be a solution for rapid plant reproduction, is still difficult for many native Cerrado species, such as *C. rufa*. Some of the reasons for the technical difficulties include the low seed germination rate (Sant'Ana et al., 2018) and the lack of effective protocols for the disinfection of epiphytic and endophytic microorganisms naturally present in plants. When contamination is endogenous, few asepsis protocols are available (Ali et al., 2018), especially for woody species (Salles et al., 2017).

Plant–endophyte relationships can produce secondary metabolites and enzymes with potential biotechnological uses in the food, pharmaceutical, medicine, and textile industries (Gopinath et al., 2017; Orlikowska; Nowak; Reed, 2017; Savi; Aluizio; Glienke, 2019). In addition, plant–endophyte interactions can generate new metabolites that benefit of both species (Orlikowska; Nowak; Reed, 2017). Knowledge of the plant–endophyte interaction improves our understanding of plant ecology and provides support for studies on disease improvement and management in native plants with economic potential.

In most cases, contamination by microorganisms in the culture medium is a problem for the propagation or maintenance of *in vitro* cultures (Mahmoud; Al-ani, 2016; Metwaly; Salama; Ali, 2018). On the other hand, specific studies related to endophytes report some benefit to plants cultured *in vitro* (Santoyo et al., 2016). However, even these microorganisms must be carefully controlled because they compete for the nutrients available in the environment (Shehata et al., 2016). For these and other reasons, disinfection performed by immersing the explants in 70% ethanol and then in solution with another disinfectant substance, such as sodium hypochlorite, should ideally eliminate any contaminating microorganisms on the surface (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 known and used in tissue culture (Torres; Houllou; de Souza, 2016; Buckseth et al., 2017; Gerszberg; Grzegorzczak-Karolak, 2019). Ampicillin is a broad-spectrum aminopenicillin that inhibits the biosynthesis of cell wall mucopeptides (Teixeira 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 concentrations of the antibiotics ampicillin, streptomycin, and chloramphenicol for the control of endogenous bacteria in *C. rufa* shoots *in vitro*.

2. Materials And Methods

2.1 *Plant identification, germination and in vitro multiplication*

The *Campomanesia rufa* plant used in this study was located at 21°13'35.5" south latitude and 44°59'00.7" west longitude. Specimens of the plant were kept in the ESAL Herbarium of the Universidade

Federal de Lavras (UFLA) (Federal University of Lavras) under registration number ESAL21198.

Ripe fruits of *C. rufa* were collected and washed with running water and detergent before seed extraction. The seeds were established *in vitro* according to the protocol of Sant'Ana et al. (2018). The inoculated seeds were kept in the dark for 7 days and then transferred to the growth room, where they grew under a photoperiod of 16 hours, an irradiance of $36 \mu\text{mol m}^{-2} \text{s}^{-1}$, and a temperature of $25 \pm 2^\circ\text{C}$. The seeds were kept under these conditions for 4 to 6 months to obtain shoots.

The culture medium for shoots multiplication was composed of MS medium supplemented with 0.09 M sucrose, 2.5 g L^{-1} Phytigel®, and $5.6 \mu\text{M}$ benzylaminopurine. The culture medium pH was adjusted to 5.8 before the addition of Phytigel®. 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 $36 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance at $25 \pm 2^\circ\text{C}$ (Sant'Ana et al., 2018).

However, during the *in vitro* multiplication procedures, we observed shoots with symptoms of rot like those induced by bacteria. The procedures to detect and identify the microorganism causing seedling rot were performed at the Laboratory of Phytopathological Diagnosis of the Biological Institute in São Paulo.

2.2 Isolation of the bacteria present in in vitro culture of Campomanesia rufa

The bacterium was isolated from *C. rufa* shoots that were being multiplied *in vitro*. To isolate it, bacterial exudates and fragments of *C. rufa* seedlings were plated in Petri dishes containing 25 to 30 mL of nutrient agar medium (Kasvi, Madrid, Spain). The medium was previously 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. To maintain the colonies, subcultures were performed every 20–30 days in nutrient agar medium.

2.3 Extraction of genomic DNA from the bacterium

Because bacterial colonies formed, both in the exudate and in the seedling fragments, it was possible to extract the genomic DNA from these colonies. Bacterial DNA was extracted using two kits: the Wizard® Plus Miniprep-DNA purification kit (Promega, USA) and the DNAzol® Reagent - Genomic DNA isolation reagent kit (Thermo Fisher, United States). Both were used according to the manufacturer's instructions.

2.4 Amplification by PCR (polymerase chain reaction)

For the detection of bacteria and subsequent sequencing of the amplicon, the gene encoding the 16S ribosomal subunit (16S rRNA) was used as the target, which according to Kai et al. (2019) is strategic for the diagnosis of the presence of bacteria. The bacterial DNAs obtained by the two extraction procedures were subjected to PCR with the 16S rDNA forward primer Y1 described by Young et al. (1991) (5'-TGGCTCAGAACGAACGCTGCGGC-3') and the reverse primer Y3 described by Cruz et al. (2001) (5'-CTGACCCCACTTCAGCTTGTTCCAT-3'). PCR was performed with 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 up to 25 μL. The reaction cycle initial denaturation at 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 in an Axygen thermocycler.

The PCR product was analyzed on a 1.5% agarose gel in 1× TAE buffer (Tris, acetic acid, EDTA) in the presence of ethidium bromide. The electrophoretic run was performed at 80 V for 40 minutes. The images were recorded in a gel documentation system with a camera coupled to a UDV-312 Series Major Science transilluminator.

2.5 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 of the Biological Institute (São Paulo) using the Sanger method.

2.6 Sequence analysis and phylogeny

The sequences obtained were compared with homologous sequences using the Basic Local Alignment Search Tool at the National Center for Biotechnology Information (NCBI) website - www.ncbi.nlm.nih.gov. The sequences obtained in this study and the homologous sequences listed in GenBank were aligned using MEGA7 software (Kumar; Stecher; Tamura, 2016) and manually adjusted. The pairwise sequence alignments were used for the reconstruction of phylogenetic trees, both within MEGA7 software.

2.7 Use of antibiotics for *in vitro* control of endogenous bacteria of *Campomanesia rufa*

Shoots with two axillary buds, obtained from material under *in vitro* multiplication as described in items 2.1 and 2.2, were grown in fresh multiplication medium. The antibiotics ampicillin, chloramphenicol, and streptomycin were added to the multiplication medium. The concentrations used for each antibiotic were 32, 64, 128, and 256 mg L⁻¹. The control consisted of culture medium without antibiotics. The antibiotics were pre-sterilized by cold filtration (Millipore, 0.22 μm) and added to the culture medium when the medium was being cooled (40 to 50°C).

Each treatment consisted of 30 replicates. At 25 days, the percentage of explants with symptoms of bacterial rot, the percentage of survival of the explants, and the height of the shoots were evaluated. The *in vitro* material was kept in a growth room with a photoperiod of 16 hours and temperature of 25 ± 2°C.

2.8 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 when the regression was not significant. 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, and Nogueira, 2013).

3. Results And Discussion

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

3.1 Bacterial colonies in propagation medium

In the *in vitro* explants of *C. rufa*, the presence of endogenous bacteria was visible in the subcultures originating both from the propagation of shoots from adult plants and from seeds. In the wild, there were no symptoms of rot in the plant. Despite the disinfection protocol, the bacteria remained in the seeds and subsequently in the germinated seedlings, multiplying in the MS culture medium at pH 5.8. Initially, the presence of the bacterium was not harmful to seedling growth (Fig. 1).

Figure 1. Symptoms of rot of bacterial origin in *in vitro* seedlings of *Campomanesia rufa* (Bar = 1 cm).

The same has occurred with the species *Anthurium andraeanum* Lind., in which the bacterium *Xanthomonas campestris* pv. *dieffenbachiae* remained latent for 4 months to 1 year without causing symptoms or turbidity in the MS culture medium (McCulloch and Pirone, 1939).

3.2 Characteristics of bacterial colonies

The bacterial strains that multiplied in the MS culture medium at pH 5.8 during the micropropagation of the plant were isolated from exudates and fragments of *C. rufa* seedlings. The bacterial colonies obtained showed a milky, shiny appearance and had two types of edges: regular and smooth or irregular. In addition to this condition, in one of the Petri dishes, the colony obtained from plant exudates showed only regular and smooth edges. Subcultures of the former colony, i.e., colonies with both smooth edges and colonies with irregular edges, gave rise to what were termed bacterial strains 5SF and 5CF, respectively. The bacterial isolate from the colony with only the regular and smooth edges was named strain 5 (Fig. 2). The Gram stains performed with the bacterial strains showed them to be gram-positive bacteria.

Figure 2. Edges of bacterial colonies isolated from *in vitro* shoots of *Campomanesia rufa*. a. Smooth edges b. Rough edges (Bar = 1 cm).

3.3 Analysis of bacterial DNA and PCR

To establish the bacterial DNA extraction protocol, colonies with smooth edges and rough edges were mixed and processed by two methods. The methods used for bacterial DNA extraction were efficient because the PCRs of the DNAs obtained from the two procedures resulted in a product of approximately

1500 bp, as expected for the Y1/Y3 primer pair. Based on this result, the DNA of the 5, 5SF, and 5CF strains was extracted with DNAzol. The 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 to protect the genomic DNA (Nascimento et al., 2017).

3.4 Sequence analysis and phylogeny

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 between the three 16S rRNA gene sequences obtained from bacteria isolated from *C. rufa* and those obtained in GenBank is shown in Fig. 3. Colonies with mixed edge types, strains 5CF and 5SF, had 90.5% identity between their sequences, while the colonies with smooth and regular edges from different Petri dishes, 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% for the 5SF strain, 98.9% for the 5 strain, and 98.7% for the 5CF strain (Fig. 3).

The 5SF strain had high percentage identity not only with *Bacillus thuringiensis* strains but also with *B. cereus* and *Bacillus* sp. High percentage identity of strain 5 with *B. thuringiensis*, *Bacillus* sp., and *Bacterium* strains was also obtained. In turn, the 5CF strain showed more significant identity with the *B. altitudinis*, *B. pumilus*, *Bacillus* sp., and *Bacterium* strains (Fig. 3).

In the phylogenetic tree built from the alignment of the 16S rRNA gene sequences, the three bacterial isolates were not put in the same subtree. Strain 5CF, with irregular edges, shared a common ancestor with *B. altitudinis*, while the strains obtained from colony colonies with smooth and regular edges, 5 and 5SF, share the same ancestor. According to the results obtained in the pairwise and phylogenetic analyses, the three bacterial strains were considered to belong to the genus *Bacillus*, but it was not possible to identify the species (Fig. 3).

Species of the genus *Bacillus* can be anaerobic, aerobic, or facultative (Villarreal-Delgado 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 and Zuñiga, 2010). Although their habitat may be soil, *Bacillus* can be isolated from various hosts, from plants to humans (Villarreal-Delgado et al., 2018), and can be both phytopathogens and opportunists and can produce toxins (Elshaghabe 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 seedlings (Santos et al., 2018), and *Bacillus* Rh219 has been used 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 do harm by competing for nutrients with the plant (Cheong, Na, and Jeong, 2019).

An interesting fact observed in the phylogenetic analysis was the presence of strains with different ancestors coexisting within the same plant of *C. rufa*, as two isolates with the same type of colony, 5SF and 5, shared the same ancestor, which was different from that of 5SF. 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 properly use these bacteria for biological control purposes.

Despite the high economic potential of Cerrado species such as *C. rufa*, there are no records on the microbiota associated with this species. 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).

Figure 3. Phylogenetic tree inferred by the maximum likelihood method, based on the Kimura two-parameter model, with the nucleotide sequences of the 16S rRNA gene. A total of 1135 positions were analyzed, and positions with gaps were excluded. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bootstrap values below 50% are not shown in the tree. Phylogenetic analysis was performed with MEGA7 software.

3.5 Antibiotics for the in vitro control of endogenous bacteria in Campomanesia rufa shoots

The antibiotic experiment showed that ampicillin, streptomycin, and chloramphenicol reduced the occurrence of bacterial contamination in *C. rufa* without compromising the survival of the plant material up to a concentration of 64 mg L⁻¹ (Fig. 4–5). These results are important because they indicated the elimination of the microbial infestation and contributed to the asepsis and maintenance of the culture established *in vitro*. In fact, even the lowest antibiotic concentration reduced the bacteria to levels below 30% in the case of ampicillin and streptomycin and to levels below 40% when using chloramphenicol. Complete bacterial elimination was not observed at any concentration tested (Fig. 4a; Fig. 5).

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

From the toxicological point of view, it is desirable that the disinfecting agent act only on pathogenic organisms, preserving the vitality of the plant (Quambusch and Winkelmann, 2018). This was observed up to a concentration of 64 mg L^{-1} of all three antibiotics evaluated. However, at a concentration of 128 mg L^{-1} , the survival of the explants was reduced (Fig. 4b; 5). Streptomycin and chloramphenicol caused the highest percentages of explant loss (Fig. 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 noncultivated plants (MINDEN et al., 2017). High concentrations of antibiotics can be toxic to plants, inhibit growth and photosynthesis, and cause oxidative stress (Bao, Gu, and Zhang, 2016; Minden et al., 2017; Riaz et al., 2017; Liu et al., 2018). Along these lines, streptomycin and chloramphenicol negatively affected the growth of the shoots regenerated in 128 mg L^{-1} antibiotic. 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 (Fig. 4c; Fig. 5).

Antibiotics have a biphasic effect on growth characterized by hormesis, where a low dose has a stimulatory or beneficial effect and a high dose has an inhibitory or toxic effect (Pan; Chu, 2017). 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.

Therefore, considering that the contamination was not completely eliminated and considering the variety of microorganisms that may proliferate in the culture medium, future studies should test a lower concentration of more than one antibiotic individually in subcultures to see if this could yield a contamination-free culture.

Figure 4. Effect of antibiotic concentration on the percentage of bacterial occurrence. (a) Bacteria occurrence (b) Survival of explants (c) Shoot height. Bars: standard error. The same uppercase letters denote nonsignificant differences between the different concentrations, and the same lowercase letters denote nonsignificant differences under the same concentration, according to the Scott-Knott test at the 5% level.

Figure 5. Shoots of *Campomanesia rufa* inoculated *in vitro* subjected to different concentrations of antibiotics. Circumference shows the presence of the bacterium.

4. Conclusions

This is the first report of the occurrence of endogenous bacteria (*Bacillus* sp.) in shoots of *C. rufa* and of the presence of different strains coexisting in this plant. The soft rot in *C. rufa* plants cannot be definitively associated with the presence of one, two, or all three strains of *Bacillus* sp.

Regarding the control of *Bacillus* sp. with antibiotics, it was evident that ampicillin (32 mg L^{-1}) caused a significant reduction in bacterial contamination without affecting the survival of explants, in addition to promoting plant growth. On the other hand, streptomycin (256 mg L^{-1}) caused the greatest reduction in contamination but was lethal to more than 90% of explants at this concentration.

Declarations

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Contributions

JGAM: Conceptualization, Validation, Investigation, Writing – Original Draft, Visualization and Writing – Review & Editing. RP: Conceptualization, Methodology, Resources, Supervision, Project administration and Funding acquisition. E.B.R.: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – Original Draft and Writing – Review & Editing, Supervision. RH: Formal analysis, Investigation, Resources, Methodology and Data Curation. MVR: Conceptualization, Validation, Supervision and Review. LBS: Formal analysis, Investigation, Writing – Original Draft and Writing – Review & Editing. MAGSD: Conceptualization, Validation, Methodology, Resources. All the authors read and approved the manuscript.

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Ethics declarations

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures



Figure 1

Symptoms of rot of bacterial origin in *in vitro* seedlings of *Campomanesia rufa* (Bar = 1 cm).

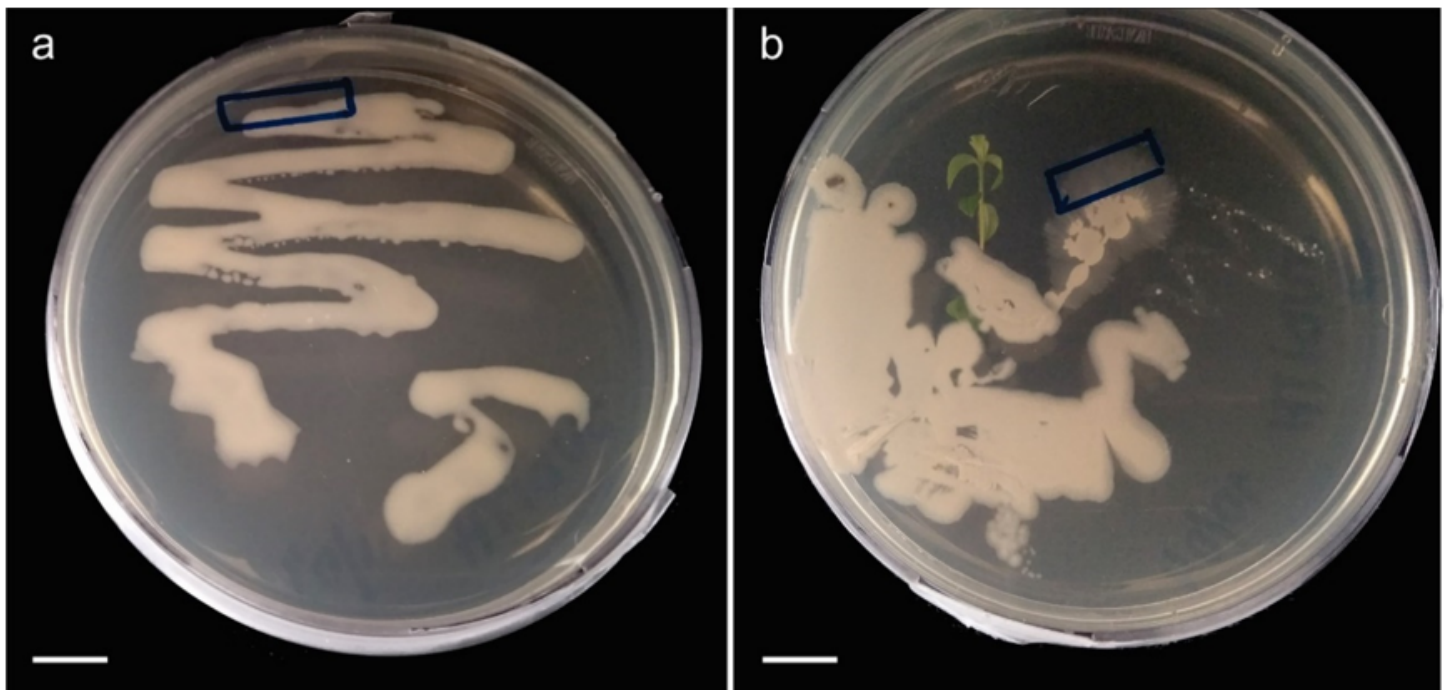


Figure 2

Edges of bacterial colonies isolated from *in vitro* shoots of *Campomanesia rufa*. a. Smooth edges b. Rough edges (Bar = 1 cm).

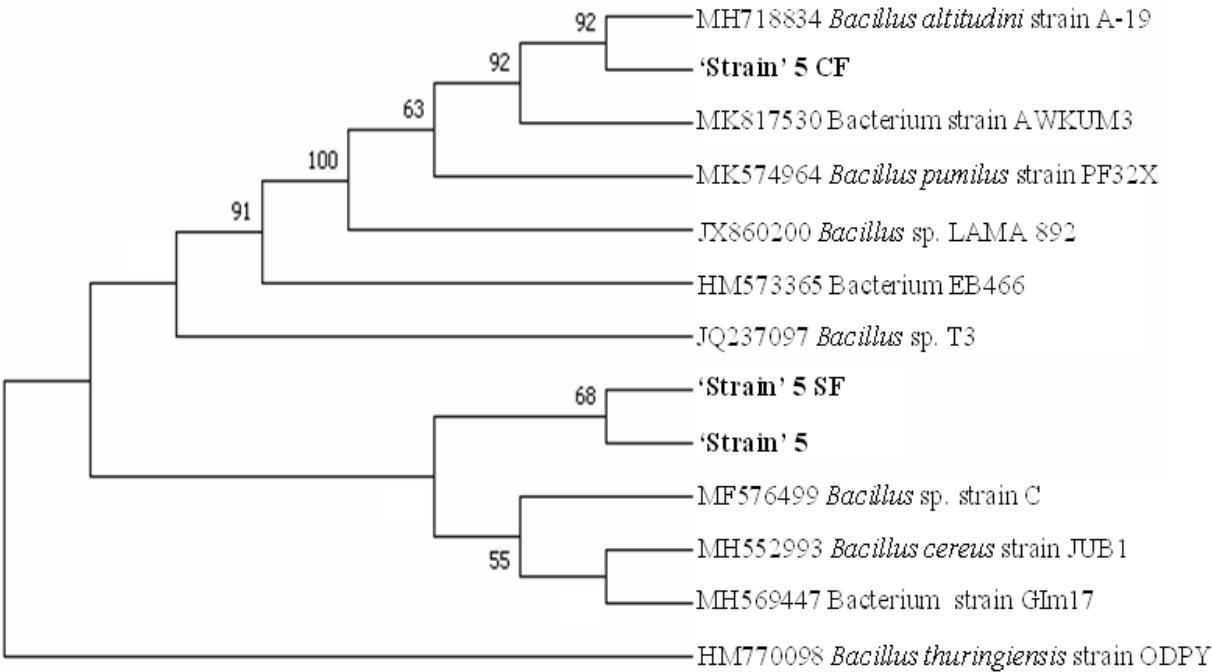


Figure 3

Phylogenetic tree inferred by the maximum likelihood method, based on the Kimura two-parameter model, with the nucleotide sequences of the 16S rRNA gene. A total of 1135 positions were analyzed, and positions with gaps were excluded. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bootstrap values below 50% are not shown in the tree. Phylogenetic analysis was performed with MEGA7 software.

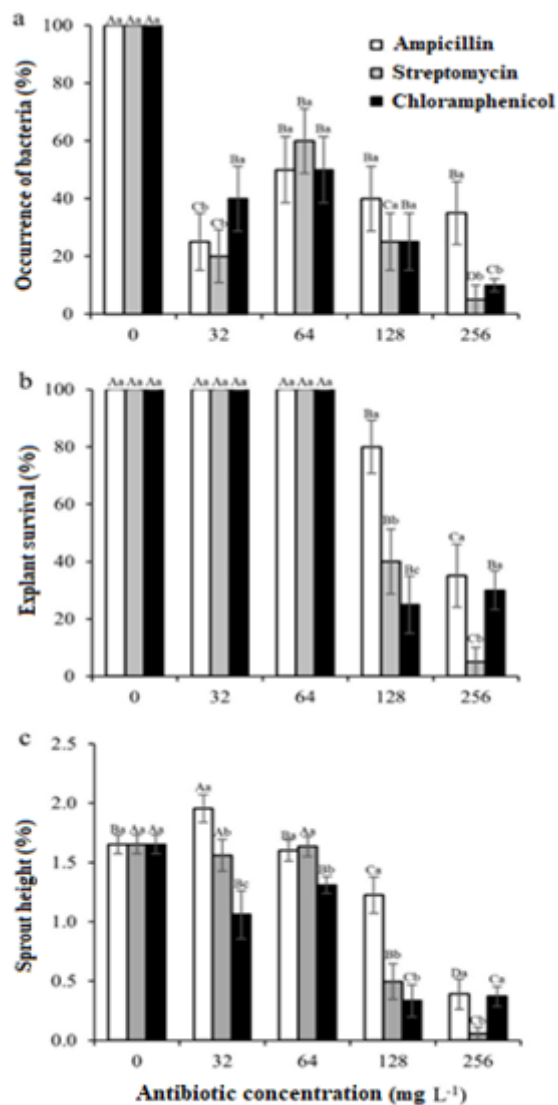


Figure 4

Effect of antibiotic concentration on the percentage of bacterial occurrence. (a) Bacteria occurrence (b) Survival of explants (c) Shoot height. Bars: standard error. The same uppercase letters denote nonsignificant differences between the different concentrations, and the same lowercase letters denote nonsignificant differences under the same concentration, according to the Scott-Knott test at the 5% level.

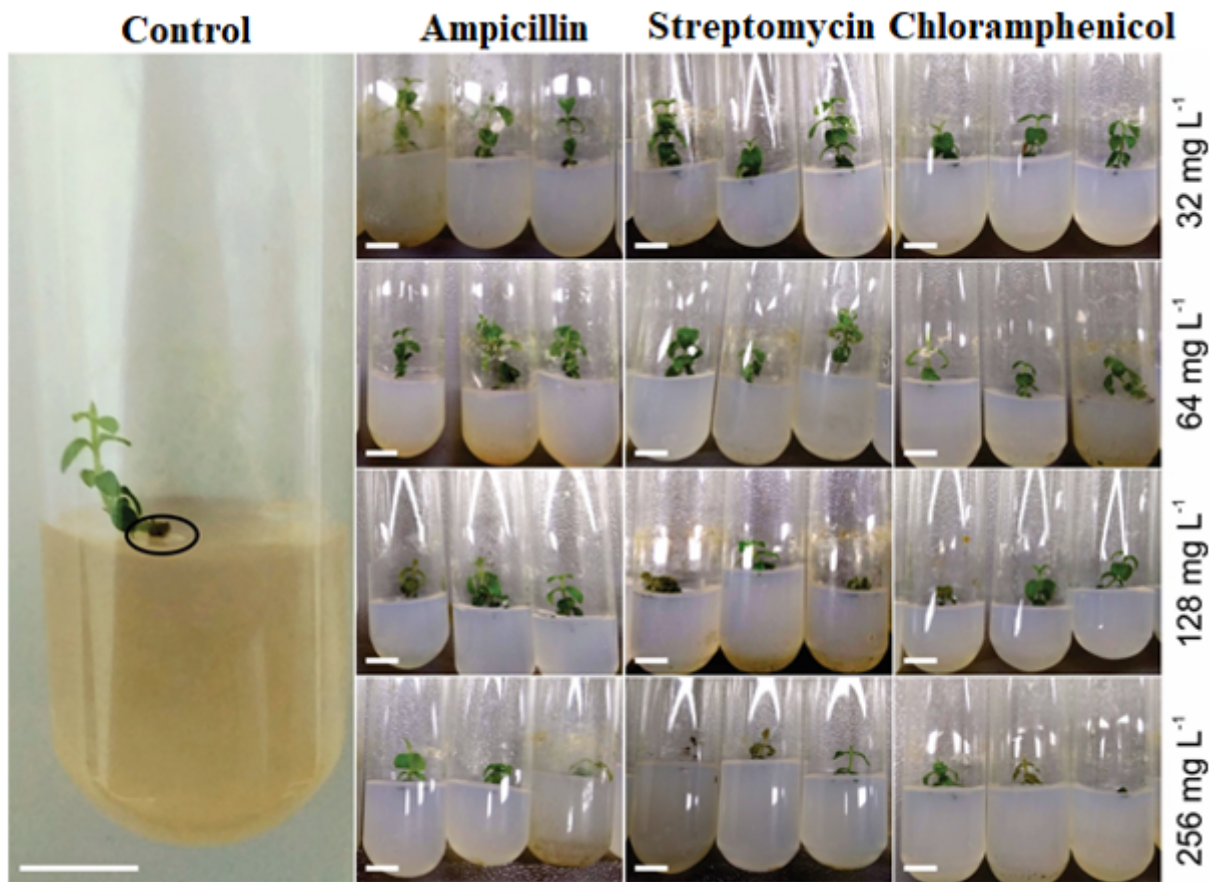


Figure 5

Shoots of *Campomanesia rufa* inoculated *in vitro* subjected to different concentrations of antibiotics. Circumference shows the presence of the bacterium.