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Endophytic fungi of *Euterpe edulis* (*açaí-do-sul*) fruits: antimicrobial activity and its relation with the growing medium

Vivien Patrícia Garbin[1], Cristiane Vieira Helm[2], Patrícia do Rocio Dalzoto[1], Andressa Katiski da Costa Stuart[1], Bruno Accioly Alves Romagnoli[3], Thiago de Farias Pires[4], Ida Chapaval Pimentel[1,*]

[1]Department of Basic Pathology, Division of Biological Sciences, Universidade Federal do Paraná, Curitiba, PR, Brazil.
[2]Embrapa Florestas, Colombo, PR, Brazil.
[3]ICC Postgraduate, Fundação Oswaldo Cruz, Curitiba, PR, Brazil.
[4]Faculty of Medicine, Universidade de São Paulo, São Paulo, SP, Brazil.
*Corresponding author: idinhaufpr@gmail.com

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Abstract: *Euterpe edulis* (*açaí-do-sul*) have wide nutritional, pharmaceutical and industrial applications. Its extensive exploitation threatens this species with extinction. The endophytic fungi can produce similar compounds to their hosts. Thus, the present study is aimed to isolate endophytic fungi from the fruits of *E. edulis* and to evaluate their antimicrobial activities against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, MRSA, and *Candida albicans* and the interference of the culture medium. Fungi were isolated in potato dextrose agar (PDA) medium. The isolates were characterized by morphology. The main genera isolated were *Fusarium*, *Alternaria*, *Penicillium*, *Trichoderma*, *Acremonium* and *Aspergillus*. The isolate *Penicillium* EFP1 shows antimicrobial activity against all the pathogens tested in PDA plus extract (PDAA). When in Sabouraud plus extract (SDAA), *Penicillium* EFP1 was able to inhibit all pathogens, except for *P. aeroginosa*. *Trichoderma* EFT. On the other hand, it only inhibited *E. coli, S. aureus* and MRSA, and the addition of the extract decreased the inhibition efficiency. The isolate *Penicillium* EFP1 presented significant inhibitory activity against pathogens, evidencing the potential for research papers in view of industrial interest and metabolites exploitation.

Keywords: Endophytes; biocontrol; *Trichoderma* sp. *Penicillium* sp.

Fungos endofíticos isolados de frutos de *Euterpe edulis* (açaí do sul): atividade antimicrobiana e sua relação com o meio de cultura

Resumo: Os frutos de *Euterpe edulis* (açaí-do-sul) possuem ampla aplicação nutricional, farmacêutica e industrial. Sua extensa exploração ameaça essa espécie de extinção. Os fungos endofíticos têm o potencial de produzir compostos semelhantes aos de seus hospedeiros. Dessa forma, este trabalho teve como objetivos isolar fungos endofíticos dos frutos de *E. edulis* e avaliar sua atividade antimicrobiana contra *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, MRSA e *Candida albicans*. Além disso, o trabalho buscou avaliar a interferência do meio de cultura sobre a atividade antimicrobiana visualizada. Os fungos foram isolados em meio ágar batata dextrosado (BDA). Os isolados foram caracterizados de acordo com sua morfologia. Os principais gêneros isolados foram *Fusarium*, *Alternaria*, *Penicillium*, *Trichoderma*, *Acremonium* e *Aspergillus*. O isolado *Penicillium* EFP1 apresentou atividade antimicrobiana contra todos os patógenos testados em BDA acrescido do extrato do fruto (BDAA). Quando em Sabouraud acrescido do extrato do fruto (SDAA), *Penicillium* EFP1 foi capaz de inibir todos os patógenos, exceto *P. aeroginosa*. O isolato *Trichoderma* EFT, por outro lado, inibiu apenas *E. coli*, *S. aureus* e MRSA, e a adição do extrato reduziu a eficiência de inibição. O isolado *Penicillium* EFP1 apresentou atividade inibitória significativa contra patógenos, evidenciando o potencial para trabalhos de pesquisa em função do interesse industrial e da exploração de metabólitos.

Palavras-chave: endofitos; controle biológico; *Trichoderma* sp. *Penicillium* sp.

1. INTRODUCTION

Açaí-do-sul (*Euterpe edulis* Mart.) occurs in the Atlantic Forest region. In Brazil, it is distributed over the states of Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Rio de Janeiro, Minas Gerais and Bahia (BICUDO et al., 2014) and is widely used as feedstuffs in these regions (CUNHA JUNIOR et al., 2015). *Açaí-do-sul*, also known as *juçara*, produces round fruits which grow in bunches and contain a large variety of phenolic compounds, protecting against degenerative diseases due to their high antioxidant activity (BICUDO et al., 2014; PAREDES-LOPEZ et al., 2010). *Euterpe edulis* is therefore also one of the ten "super foods" (FREITAS et al., 2015) and has drawn the attention of nutraceutical and cosmetic industries; the species is used to fight inflammatory and oxidative mediators involved in aging (POULOSE et al., 2012).

In addition, *Euterpe edulis* provides high-quality palm heart (BORGES et al., 2011). However, its exploitation and export are difficult, because after cutting for palm heart extraction, the tree does not grow back, which represents significant issues for the preservation of the species (LIMA et al., 2012). Therefore, due to indiscriminate extraction, *E. edulis* is threatened with extinction in Brazil. One way to minimize this threat is the commercial use of highly nutritious fruits, which does not involve cutting down trees and represents a conservation strategy and an alternative income for rural communities (POLTRONIERI et al., 2014), besides adding more value to product with processing.

Among the various methods to preserve biodiversity, the study of endophytic microorganisms in tropical trees represents an alternative of great commercial and scientific importance (ARNOLD et al., 2001; BACKMAN & SIKORA, 2008). Endophytic microorganisms reside in living plant tissues, without causing immediate damage, and providing their host with various benefits, such as protection from pathogens, resistance to climatic variations, and the production of several secondary metabolites, which are also of interest to medicine, agriculture, and industry (HIGGINBOTHAM et al., 2013; STROBEL & DAISY, 2003). Previous studies show that endophytic fungi can produce a significant number of secondary metabolites (ALY et al., 2011; SURYANARAYANAN et al., 2009). The relation between the endophytes and the origin plant is being extensively researched, and several studies show there are interactions (NIELSEN & NIELSEN, 2017).

2. MATERIALS AND METHOS

2.1. Biological material

The samples were collected in the municipalities of Antonina (25°25'43"S and 48°42'43"W) and Morretes (25°28'37"S and 48°50'04"W), state of Paraná. Healthy fruits with no disease symptoms were separated from the branches, and those that were at their maximum maturation stage (purplish to black color) were selected, washed in distilled water, and kept under refrigeration at 4°C.

2.2. Isolation and identification of endophytic fungi

For the isolation of endophytic fungi, we used the methodology described by Araújo et al. (2002). Three fragments of approximately 0.5 × 0.5 cm were removed from the pulp and placed in Petri dishes (9 cm diameter) containing potato dextrose agar (PDA Himedia®) tetracycline (100 $\mu L mL^{-1}$) and cultured in a chamber incubator (BOD) at 28 ± 0.5°C with a photoperiod of 12 hours for seven days. Purified colonies were cultivated in Sabouraud dextrose agar medium (Himedia®) plus tetracycline (100 $\mu L mL^{-1}$), incubated under the conditions described above, for further identification. Identification of fungi was carried out by determining the morphological species (morphospecies) of the colonies, based on fungal growth rate, colony shape, coloration and effects on the culture medium. Observation of reproductive structures (asexual and sexual) was made with light microscope examination (KERN & BLEVINS, 1999; DE HOOG et al., 2000).

2.3. Evaluation of antimicrobial activity

The obtained isolates were cultivated in potato dextrose agar (PDA) and Sabouraud medium (SDA) to verify if the media influences antimicrobial activity. The method of choice was agar drilling (adaptation of the M2-A8 manual standardized by the Clinical and Laboratory Standards Institute — CLSI, 2003). The isolates were tested against five pathogenic microorganisms: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, MRSA (*methicillin-resistant Staphylococcus aureus*), and inoculants of *Candida albicans* ATCC 10231, purchased from Curitiba's Clinical Hospital, state of Paraná, Brazil. The strains were previously inoculated onto plates containing Mueller Hinton medium (Himedia®) and incubated for 24 hours at 35°C (DEUSCHLE et al., 2007). After the incubation period, four inoculum colonies were selected and transferred to a tube containing 5 mL of 0.8%

saline solution until the concentration of 1×10^7 CFUmL^{-1} was obtained, except for *Candida albicans*, in which the concentration used was 1×10^8 CFUmL^{-1}. Suspensions of the strains were then inoculated using swabs or spread across the entire surface of the culture medium. The PDA or SDA media were used as negative control; as positive control for antimicrobial potential, we established media with vancomycin 0.2 μg/mL^{-1} for MRSA, nystatin 100,000 IUmL^{-1} for *Candida albicans*, and gentamicin 0.2 μgmL^{-1} for other bacteria. After 24 hours, growth inhibition halos were formed (OSTROSKY et al., 2008).

2.4. Modified medium with hydroalcoholic extract of fruits

The isolates that presented antimicrobial activity were tested in PDA or SDA media, modified by the addition of hydroalcoholic extract of the fruits of *E. edulis*, to test the influence of the fruit compounds on antimicrobial activity. The extract tested was prepared with pure ethanol and fresh fruits, considering the humidity indexes of the fruit, protected from light, and concentrated in a rotaevaporator at 75ºC. The product obtained was an intense odorless purple paste, with optimum water solubility, which was maintained under UV light (375 nm) for 1 hour for decontamination. The PDA and SDA media were sterilized and modified at a ratio of 1:1 to fruit extract and homogenates, yielding a dark purple culture medium. To evaluate antimicrobial activity, we used agar drilling (CLSI, 2003).

2.5. Statistical analysis

Maximum and minimum inhibition interval data from each group were used; all experimental combinations of inhibitors with inhibitory targets were performed in replicate. In the study using statistical inference, non-parametric statistical techniques were applied, because they dismiss the assumption of probabilistic distribution of the sample, besides providing parsimonious results in small samples (HOLLANDER et al., 2015). The analysis was performed using the Statistical Environment R, in which a value of $p < 0.05$ was considered significant, and the calculations were performed with the statistical software R 3.0.2 (R CORE TEAM, 2014). A final analysis was conducted to test for significant differences in the inhibition with the presence of the extract in the media for all pathogens tested, considering the inhibitory fungus applied as sample extracts of the experiment. We used the van Elteren test in R 3.02.2 in the Sanon library (KAWAGUCHI & KOCH, 2015).

3. RESULTS AND DISCUSSION

3.1. Fungal isolation

Fusarium spp., *Alternaria* spp., *Penicillium* spp., *Trichoderma* spp., *Acremonium* spp., and *Aspergillus* spp. were the main genera identified in endophytic isolatesinthis study. To the best of our knowledge, this is the first report of endophytic fungi isolation of this species. In a previous study on endophytic fungi of this genus, Rodrigues (1994) described fungi found in leaves of *E. oleraceae,* collected in the city of Belém, state of Pará, Brazil, and identified the genera *Xylaria* and *Letendraeopsis* as the two most abundant. Benchimol et al. (2017) demonstrate the importance of the family Areaceae in the botanical community of the Atlantic Forest, in which its absence affects pollination and dispersion, among other ecological processes.

Bonfim et al. (2016) describe endophytic septate fungi, isolated from roots of *E. edulis*, found in a see-saw pattern in the state of São Paulo, Brazil, observing the genera *Alternaria*, *Ascochyta*, *Cladosporium*, *Coniothyrium*, *Nigrospora*, *Microdiplodias*, and *Phoma*. In decomposing parts from *E. oleraecea* (sheaths, curls, spathes, stipes, leaflets, petioles, and roots), 45 taxa of conidial fungi were isolated, including *Coleodictyospora*, *Helminthosporium*, *Spadicoides*, among other genera (CASTRO et al., 2012). Arbuscular mycorrhizal fungi spores were described in *E. edulis* (by MEDINA et al., 2012), and leaf hyphomycetes, by Grandi (1999). *Colletotrichum gloeosporioides*, isolated from diseased fruits of *E. edulis*, was studied as to the mycelial development, production, and germination of conidia (POLTRONIERI et al., 2014). From the soil of Savana and Atlantic Forest sites in the state of São Paulo, Brazil, Barbosa et al. (2017) isolated *Trichoderma* spp., *Fusarium* spp., *Acremonium* spp., *Penicillium* spp., and *Paecilomyces* spp. with bactericidal and bacteriolytic activity.

3.2. Antimicrobial tests

Of the isolated fungi, seven isolates, namely *Penicillium* EFP1, *Penicillium* EFP2, *Penicillium* EFP3, *Trichoderma* EFT, *Mycelia sterilia* EFT1, *Mycelia sterilia* EFT2, and *Alternaria* EFA were selected for testing against human pathogens. In PDA medium, we observed activity against *S. aureus* (*Penicillium* EFP1 with an inhibition halo of 6 mm, *Penicillium* EFP2 with halo of 4 mm, *Trichoderma* EFT with a 9-mm halo, and *Mycelia sterilia* EFT1 with a 5-mm halo,

respectively). Against MRSA, *Penicillium* EFP1 showed an inhibition halo of 4-mm diameter. When in SDA medium, *Penicillium* EFP1 presented a 4-mm inhibition halo to MRSA. *Trichoderma* EFT demonstrated activity against *E. coli*, *S. aureus*, and MRSA, with inhibition halos of 8 cm for the first two species and of 14 mm for the latter (Figure 1). The metabolites produced by fungi had a diffusion capacity in PDA and SDA medium, as halos up to 14 mm in diameter were observed. The isolate with the highest activity was *Trichoderma* EFT, against *E. coli*, *S. aureus*, and MRSA.

Penicillium EFP1, *Penicillium* EFP2, *Trichoderma* EFT, and *Mycelia sterilia* EFMS2 were grown in medium supplemented with *E. edulis* fruit hydroalcoholic extract in order to evaluate their morphology and antimicrobial activity. In PDA medium with added extract (PDAA), *Penicillium* EFP1 demonstrated a 10-mm inhibition halo against *E. coli*, 8-mm halo against *S.* aureus, 1-mm halo against *P. aeruginosa*, 10-mm halo against MRSA, and 3-mm halo against *C. albicans*. In SDA with added extract (SDAA), *Penicillium* EFP1 showed a 10-mm inhibition halo against *E. coli* and *S. aureus*, 11 mm against MRSA, and 5 mm against *C. albicans* (Figure 1).

As shown in Figure 1, inhibition by the fungus *Penicillium* EFP1, for the studied pathogens, was higher in relation to the others, especially for the medium with added hydroalcoholic extract. *Trichoderma* EFT also promoted an inhibitory effect for *E. coli*, *S. aureus*, and MRSA. However, in this case, the addition of the extract resulted in lower inhibition. *Mycelia sterilia* EFMS2 and *Penicillium* EFP2 showed chance inhibition. The Wilcoxon test showed a statistical difference for the addition of the extract proposed for *Penicillium* EFP1 and *Trichoderma* EFT. However, for *Penicillium* EFP1, the difference increased with the addition of fruit extract, whereas in *Trichoderma* EFT, this activity was negative (Table 1).

Several metabolites of the genus *Penicillium* demonstrated important antimicrobial and antioxidant activity (RADHAKRISHNAN & LEE, 2015). Two important metabolites were isolated from fungi of this genus (penicilenol B1 and B2), which demonstrated an important anticancer activity and activity against *Staphylococcus aureus* (KEMPF et al., 2015). In different species of *Trichoderma*, their antimicrobial activity has been recently described (MUTAWILA et al., 2016). Therefore, biofungicides are being developed from fungi of this genus.

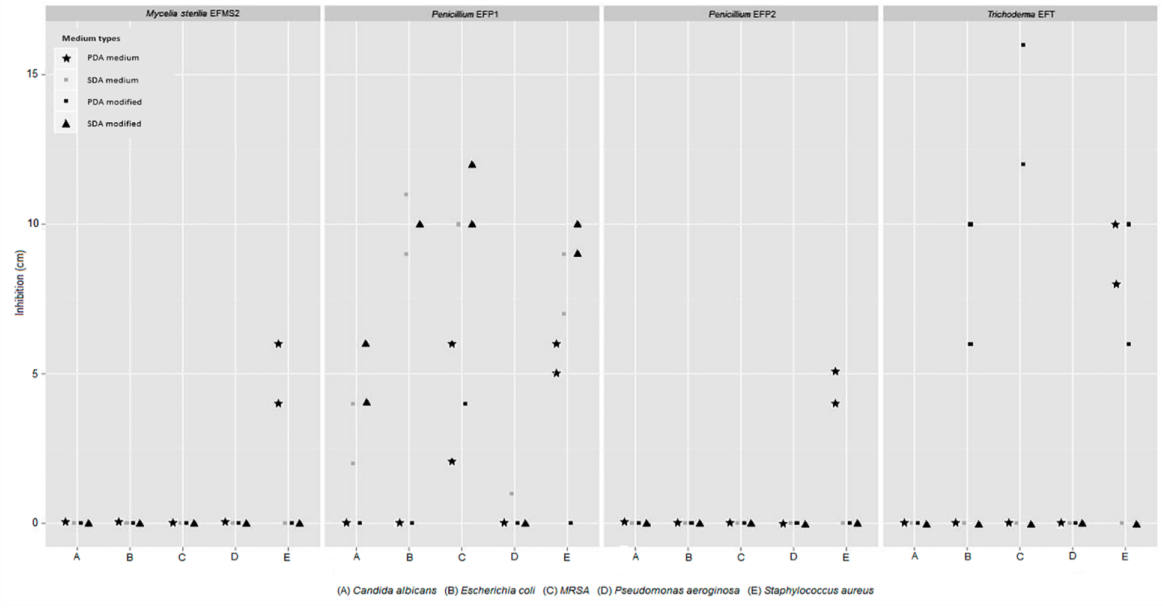

Figure 1. Antimicrobial activity presented by endophytic fungi against *Candida albicans, Escherichia coli,* MRSA, *Pseudomonas aeruginosa and Staphylococcus aureus* in different culture media.

Table 1. Values of p associated with Wilcoxon test for the inhibition of each isolated fungus.

Fungi	p
Penicillium EFP1	3.773e-05*
Penicillium EFP2	0.1626
Mycelia sterilia EFMS1	0.1626
Trichoderma EFT	0.002075*

*Significant statistical difference.

Based on our results, we suggest that the presence of fruit extracts in culture medium acts in three different ways. The first hypothesis suggests that the modified culture medium activates a previously inactive metabolic pathway of the fungus, causing it to produce bioactive compounds, thereby presenting inhibitory activity against strains that were not inhibited previously. Suryanarayanan et al. (2009) have described that the composition of the culture medium, humidity, pH, and the presence of certain enzymes can significantly alter the profile of secondary metabolites secreted by an endophytic fungus, as well as induce the production of several new metabolites by the activation of previously inactive genes. Another possibility is that the extract compounds participate in the primary metabolism of the fungus, therefore being used during its development. Its increase in the medium caused a greater availability of compounds, causing an increase in the production of active principles. The third hypothesis is that the absence of the fruit pulp compounds leads to the production of fungal metabolites for self-protection, because the environment in which it is found is different from its natural habitat. When the fungus is cultivated in medium containing the extract, such metabolites can be produced (YANTO et al., 2017).

The results obtained in the present study can be explained by the interaction between the endophyte and the extract from the host plant. The type of interaction between the endophyte and a plant is controlled by the genes of both organisms and modulated by the environment (DEEPIKA et al., 2016). Therefore, a change in the environment of the endophyte also implies a change in its metabolic profile. A hostile environment or one closer to the natural environment may contribute to the increased capacity of synthesized endophytes, which may explain the variation observed when an endophyte species isolated from a host plant produces a bioactive compound, but fails to do so when isolated from another plant species or in a standard culture medium (SURYANARAYANAN et al., 2009). This means that the host plant influences the synthetic capacity of an endophyte. Thus, endophytes may manifest their antimicrobial activity—or produce remarkable secondary metabolites—in the presence of inducers or substrates from where they live (LUDWIG-MÜLLER, 2015). In this way, the activity of *Penicillium* EFP1 increased, which also increased its antimicrobial activity after supplementation with the fruit extract, considering that several essential compounds, such as carbohydrates, proteins, lipids, and various minerals, added via the fruit extracts, can be used as energy sources. In addition, the decreased activity of some fungi, *Trichoderma* EFT, for example, can be explained with the lower production of metabolites because the medium is more similar to the conditions in the natural habitat. The opposite also occurs, plants begin to produce different compounds in the absence or presence of endophytes (KOEGEL et al., 2015). All common secondary metabolites are derived from relatively few primary precursors, and conditions affecting the availability of these precursors may also affect the production of certain classes of metabolites (GUL et al., 2014). It is suggested that long-term adaptation and co-evolution of endophytes lead to the production of metabolites similar or equal to those of the host plant (XU et al., 2009). Studies show that endophytic microorganisms can develop genetic information transfer systems with their hosts (ABRAHÃO et al., 2013). However, this does not seem to apply to the present study, because extracts from the plant alone have no antimicrobial activity, which indicates that the activity performed by the fungus is derived from the metabolism of other compounds present in the environment.

The presence of the pulp extract in the culture media may be regulating gene expression, as well as some enzymatic pathways of fungal metabolism. The structural interaction, physiological or at the level of gene pools, of the endophytic fungi with their host plant is remarkable. An example of genetic linkage between antibiotic production and morphological development has been described (CALVO et al., 2002), which reported the snail-regulating elements and antibiotic production in endophytic bacteria and *Streptomyces* spp., stating that genes containing all or most of information needed for metabolite biosynthesis are regulated (expressed/suppressed) throughout evolution. Sequencing of the *Fusarium graminearum* genome has revealed that the number of genes supposedly dedicated to secondary metabolism is much greater than the number of compounds produced by a particular species (ALY et al., 2011). In addition, a number of endophytes demonstrated their ability to produce more active compounds than those observed in the laboratory (HIGGINBOTHAM et al., 2013). The use of molecular markers and techniques using cDNA would provide some clarification, leading to the elaboration of procedures to obtain metabolites of interest (SURYANARAYANAN et al., 2009; NIELSEN & NIELSEN, 2017).

Future tests with these organisms could demonstrate interesting biochemical components within the endophytic community of *E. edulis*. Several bioactive natural products isolated from endophytic fungi have been reported, including alkaloids, peptides, steroids, triterpenoids, quinones, flavonoids, aliphatic compounds, and phenols (YU et al., 2010). Given the antimicrobial activity of fungi, research aiming at the isolation of the active principle would be the initial step for the possible industrial application of these compounds. Using molecular techniques would enable us to see where, within the genome of the species, the genes that probably regulate the production of the compound are located. It would also allow verification of how the extract acts on the metabolism of these organisms and how the genes regulate their response. Further interdisciplinary studies with this plant may not only depict new industrial or pharmaceutical possibilities, but, above all, encourage sustainable exploitation of this species.

4. CONCLUSION

Our study demonstrates that the isolated fungi from *E. edulis* can present a difference in antimicrobial activity, according to the medium in which they are being cultivated, demonstrating a direct relation of the fungi metabolism with the growth medium. We also show that the endophytic fungus *Penicillium*, isolated from *E. edulis*, presented significant inhibitory activity against pathogens of medical interest, evidencing its potential for research on metabolites of industrial interest.

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