Acta Scientiarum

http://periodicos.uem.br/ojs
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v43i1.49786

BIOTECHNOLOGY

Antimicrobial activity in cultures of endophytic fungi isolated from *Talinum triangulare*

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ABSTRACT. Endophytic fungi colonize the interior of plants without causing damage and act in symbiosis with their host. They are also potential sources of compounds with potential applications in industry, agriculture, and medicine, Thus, this study aimed to isolate and identify the endophytic fungi medicinal plant *Talinum triangulare* and evaluate its potential for the production of antimicrobial substances using the disk diffusion technique and testing in liquid medium front of *Staphylococcus aureus* ATCC SA 6538, *Pseudomonas aeruginosa* ATCC PA 0030, and *Corynebacterium diphtheria* ATCC 27012. *Corynebacterium diphtheria* was isolated from 3 fungi of the genus *Trichoderma* and *Penicillium*, with only the genus *Trichoderma* fungi showing antimicrobial activity.

Keywords: microorganism; pathogenic; fungi; secondary metabolites.

Received on August 31, 2019.
Accepted on October 16, 2020.

Introduction

Herbal medicine and the use of medicinal plants belong to a set of practices used in traditional folk medicine, forming a collection of internalized and disseminated knowledge, highlighted by oral tradition. It is considered an effective primary health care and can be performed in parallel to other treatments, although it is most commonly used by individuals with low incomes. Data released by the World Health Organization indicate that about 80% of the world population uses some kind of herb to relieve painful or unpleasant symptoms (Silva & Oliveira, 2018).

Cariru, also known as pigweed or John-Gomes, is part of the Portulacaceae family and has been reported to be of nutritional value scientifically. Unconventional vegetables have a limited distribution due to being grown in specific locations and regions, and have a great influence on food and medicine in traditional populations. The leaves and stems of cariru are typically consumed in stews. It is cultivated by sowing seeds or plant cuttings in sites spaced from 20×20 to 30×30 cm (Cardoso, Antonio, Berni, & Kano, 2017).

Endophytic fungi coexist with their hosts without causing damage and can assist in the adaptation of plants to harsh environments (*e.g.* excess salt, high temperatures, nutrient deficiencies). However, the complex relationship between endophytic fungi and their hosts remains poorly understood (Pádua, 2019). One of the first compounds to be isolated from endophytic fungi was *Taxus brevifolia*, which showed high anticancer activity, paving the way for further studies and applications of secondary metabolites produced by endophytic fungi (Bezerra et al., 2015).

The lack of antimicrobials in the market for treatment against increasingly strong microorganisms has become a critical issue, driven by the indiscriminate and irrational use of antibiotics, in addition to the evolution of the virulence mechanisms of microorganisms (Felix, Medeiros, & Medeiros, 2018). This has created the need for the identification of alternative antimicrobials.

In the context of increasing rates of microbial resistance and advances in the biotechnological potential of the endophytic fungi of medicinal plants, the present study explored biomolecules produced by fungi with antimicrobial activity against clinical pathogens. Ultimately, the objective of this study was to identify novel alternatives for the treatment of antibiotic-resistant infections and with fewer side effects than conventional drugs.

Material and methods

Collection and isolation of endophytic fungi *Talinum triangulare*

The seedlings of the medicinal plant *Talinum triangulare* were collected at a site in the peripheral region of São Luís, the state capital of Maranhão, Brazil, at a georeferenced position (latitude: –2.569896°, longitude: –44.205524°) during the dry season in August 2018. The collected seedlings were sent to the Environmental Microbiology Laboratory of the *Universidade Ceuma*.

The leaves were surface-disinfected with alcohol 70 (1 min.) and 2% sodium hypochlorite (over 3 min.), followed by immersion for 30 s in alcohol 70%, and washing in sterile distilled water. As a control, 1 mL of wash water was inoculated plate according to

After disinfecting, the leaves were fragmented and inoculated in Petri dishes (5 fragments per plate) containing potato dextrose agar (PDA) and streptomycin (50 mg mL^{-1}). The plates were incubated in an oven at 28°C for 5 days. Then, to isolate and purify the fungal colonies, the colonies were inoculated using the streaking depletion technique, cultured in Sabouraud plates, and incubated in an oven under the conditions described above (Petrini & Fisher, 1990).

Characterization macromorphological and micromorphological endophytic fungi

After growing on plates, the fungal colonies were characterized and cataloged in terms of their macromorphology, observing characteristics such as surface mycelium color and the colony reverse, texture, pigmentation, and edge structure (Pitt, 1991).

The cultured fungi were isolated and their genus was identified based on the microscopic characteristics of the colonies after the preparation of the microcultivation blades. The cultures were then stained for observation by optical microscopy (Whitaker & Long, 1971)

Inoculum of clinical pathogens

The bacteria *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 0030, *Pseudomonas aeruginosa* ATCC 0026, *Corynebacterium diphtheria* ATCC 27012, and *Corynebacterium diphtheria* ATCC 27010 were grown on Mueller Hinton agar plates for 24 hours at 37°C, and then diluted with 0.9% saline according to standard turbidity of 0.5 McFarland tube (10^8 CFU mL^{-1}).

Assay medium solid

The antimicrobial activity test was performed according to the methodology described by Ichikawa, Ishikura, and Ozaki (1971), which calls for the diffusion of bioactive compounds in agar. This methodology is also known as the ‘agar block method’. Briefly, the fungal isolates were initially grown in a Sabouraud culture petri dish. After incubating for 10 days in 28 circular agar blocks with a diameter of 6 mm, the isolates were transferred to plates containing Mueller Hinton and Sabouraud inoculated with the test microorganisms: one standardized cell suspension of test microorganisms in the concentration of approximately 1.2×108 CFU mL^{-1} was used for the bacteria. The test was performed in triplicate for all strains isolated. The plates were incubated with respect to the physiological characteristics of each test microorganism, as described above. After the incubation period, the diameters of inhibition zones of each block were measured (in mm), and the arithmetic mean, and standard deviation were used as the results. The control of the culture medium was performed by inoculating only Sabouraud agar blocks (without the isolated fungi) on the test microorganisms.

Assay half net

The diffusion test in agar described by Bauer, Kirby, Sherris, and Turck (1966) was used to determine the fungus selected, established according to the Clinical and Laboratory Standards Institute (CLSI). Briefly, 10 L of fermented filter paper disks (6 mm in diameter) were soaked in the test solution and transferred to Petri plates seeded with the microorganisms of clinical interest (Gram-positive and Gram-negative) for incubation at 37°C (bacteria clinical) for up to 72 hours. Then, the inhibition halos were measured using calipers. For the negative control, a disk impregnated with DMSO (dimethyl sulfoxide) was used.

Results and discussion

Fungal endophytes isolation

Three cultures were isolated from the endophytic fungi fragments of the leaves of *Talinum triangulare*.

Macromorphological and micromorphological characterization of endophytic fungi

A macromorphological predominance of white aerial mycelium stained gray with a velvety texture was observed, followed by isolated gray aerial mycelium with a cotton-like texture (Figure 1).

Micromorphological analysis confirmed that the *Trichoderma* isolates could be recognized based on their macroscopic characteristics, including rapid growth in culture, hyaline aerial mycelium network, septate, highly branched appearance, sparsity, and the production of Conidiogenous differential blisters (white or green). Spores were abundantly produced and appeared either loose or compressed into tufts. The genus *Penicillium* presented typical characteristic of the presence of specific conidiophores, as shown in Figure 2.

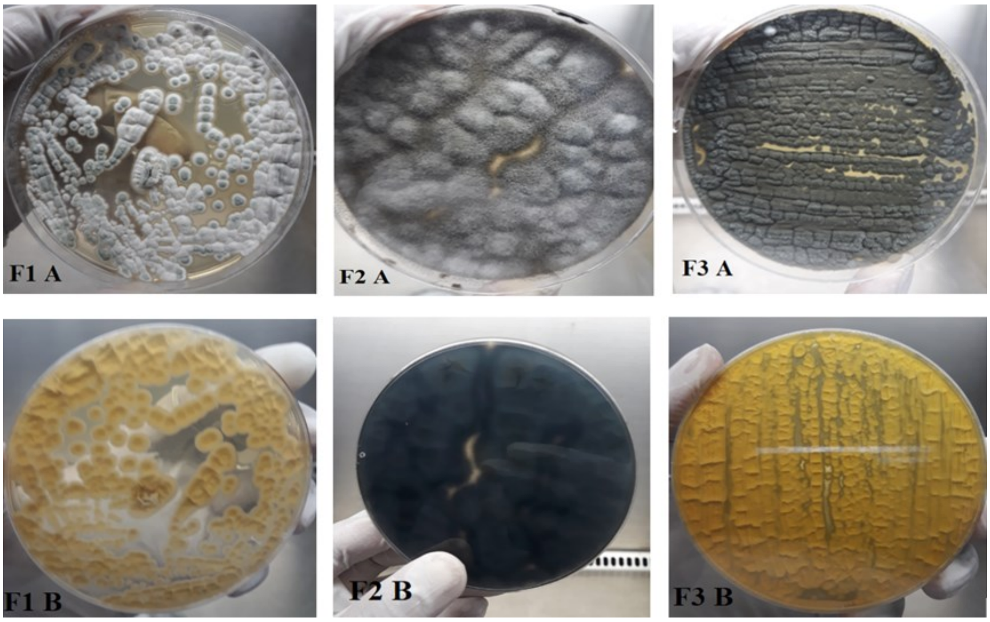

Figure 1. Macromorphological characteristics of endophytic fungi isolated from *Talinum triangulare* plant: F1 (*Trichoderma* sp.), F2 (*Penicillium* sp.), and F3 (*Trichoderma* sp.).

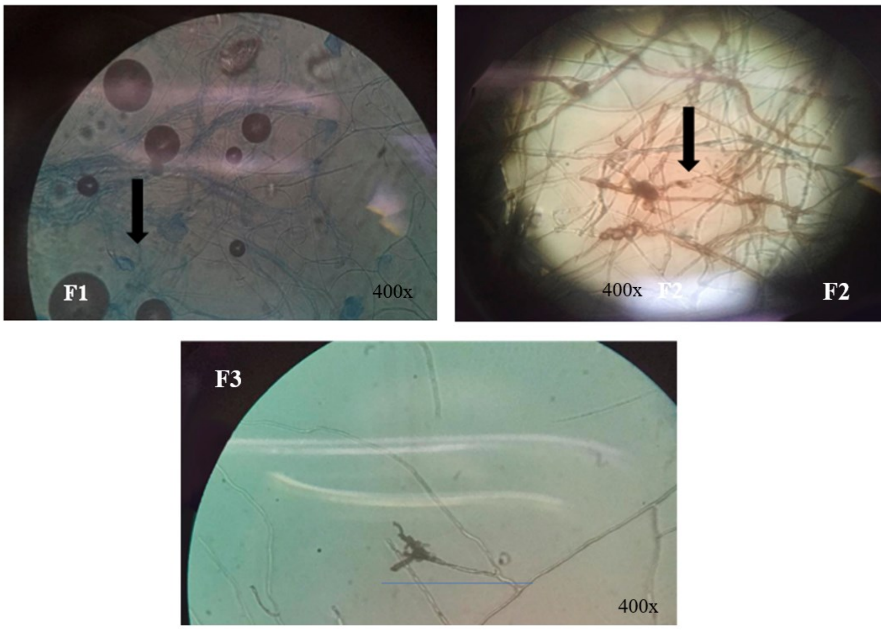

Figure 2. Micromorphological characteristics of endophytic fungi isolated from *Talinum triangulare* plant: F1 (*Trichoderma* sp.), F2 (*Trichoderma* sp.), and F3 (*Penicillium* sp).

F1 and F3 were classified cultures of the genus *Trichoderma*, while F2 was classified as *Penicillium*.

Antimicrobial activity of endophytic fungi against pathogens by clinical testing on solid medium

Among the endophytic fungi, only the F1 and F3 cultures showed inhibitory activity against the tested pathogens, as shown in Figure 3.

Antimicrobial activity of endophytic fungi against pathogens by clinical testing in liquid medium

None of the fungal isolates showed inhibitory activity against clinical pathogens. Although the extracellular secondary metabolites of these fungi showed no antimicrobial activity, the results of the inhibition test on solid medium indicated that the isolates exerted inhibitory activity via primary metabolites, competition fungus by synergistic nutrient medium, or both.

Pires et al. (2015) evaluated the antimicrobial activity of the Cacti endophytic fungi Caatinga and found that *Trichoderma* strains showed antibiotic activity against the clinical pathogens *Bacillus subtilis*, *Staphylococcus aureus*, and *Salmonella enteritidis* by forming inhibition zones varying from 8-20 mm. In agreement with these results, the largest inhibition zone in the present study was associated with a *Trichoderma* strain inhibiting *Staphylococcus aureus* with halos between 24-29 mm in disk diffusion tests.

In another study conducted by Souza, Trocoli, and Monteiro (2016), 109 strains of endophytic *Trichoderma* were isolated from four Caatinga native plants. When evaluating their antifungal activity against the pathogen *Fusarium guttiforme* in pineapple, three isolates were found to decrease the symptoms of disease by 68-84% in the field. This is in agreement with Lopes, Junior, Neves, Chapla, and Batistella (2017), who demonstrated the potential of antagonistic *Trichoderma* strains isolated from grass citronella against phytopathogenic fungi. Thus, several studies have shown the great inhibitory potential of *Trichoderma* isolates against the pathogens *Fusarium oxysporum*, *Curvularia scope*, and *Bipolaris oryzae*.

Previous studies have also reported on the colonization of Brazilian medicinal plants by *Penicillium*. Souza et al. (2018) investigated the diversity of endophytic fungi associated with medicinal plant *Kalanchoe pinnata* and found that the fungi of the genus *Penicillium* represented 1.2% of isolates from plant leaves. Batista, Raposo, and Silva (2018) investigated the diversity and antimicrobial activity of separate açaizeiro endophytic fungi and obtained similar results. They isolated *Penicillium* fungi and found it did not exert any antibiotic activity against the clinical pathogen *Staphylococcus aureus* in disk diffusion testing.

Candeias et al. (2016) isolated endophytic fungi from the roots of *Agave sisalana*, popularly known as sisal, which is found in semi-arid regions in Brazil. Among the genera of fungi isolated, *Penicillium* was found to exert an antagonistic effect against *Aspergillus niger*, the main causative agent of red rot disease in sisal plantations.

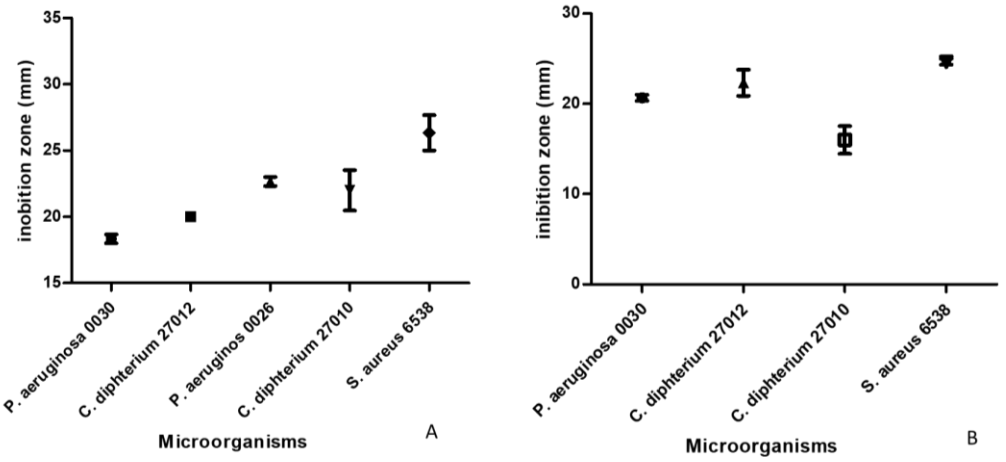

Figure 2. Antibacterial activity (mean and standard deviation of the inhibition zone) of endophytic fungi F1 (A) and F3 (B) compared to the clinical test pathogens on solid medium.

Conclusion

The medicinal plant *Talinum triangulare* has a high potential to host a diverse range of endophytic fungi with various biotechnological applications. Among the fungi isolated belonging to the genera *Trichoderma* and *Penicillium*, only *Trichoderma* fungi showed antibiotic activity against pathogens. Clinically, however, the fungi showed no extracellular production of secondary metabolites. Thus, further studies will be needed to evaluate the activity exerted by the isolated fungi against agricultural plant pathogens according to their potential reported in the literature.

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