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Diaporthe Endophytes in the Control of Standard Bacterial Strains and Clinical Isolates

Endófitos de *Diaporthe* no Controle de Cepas Padrão e Isolados Clínicos Bacterianos

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Abstract

Endophytic microorganisms are widely exploited for their ability to produce compounds with biological activity, this allows to identify metabolites with potential for drug production, including antimicrobials. Considering the need to optimize the prospection of these microorganisms aiming the control of pathogenic bacteria resistant to antimicrobial drugs the present study selected the most promisor *Diaporthe* fungi in producing secondary metabolites against resistant microorganisms from an endophytic collection. With this intend, ten isolates of this genus were confronted against standard strains and clinical pathogenic bacteria. Endophytic fungi of the genus *Diaporthe* isolated from *Schinus terebinthifolius* leaves were directly confronted in Petri dishes containing potato dextrose agar by antagonism against eight bacterial strains (*Enterococcus faecalis*, *Staphylococcus aureus, Staphylococcus saprophyticus*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella typhimurium*) and seven resistant clinical isolates identified as *Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis* with different resistance profiles, including quinolones and cephalosporin, and ampicillin-resistant *Staphylococcus epidermidis*. Four endophytic fungi controlled the growth of Gram-positive and Gram-negative bacteria. One of the isolates, *Diaporthe endophytic* LGMF653, inhibited the growth of five bacteria isolated from clinical samples with characteristics of resistance to antibacterial drugs, including one Gram-positive and four Gram-negative microorganisms, being therefore considered the most promising among the endophytes evaluated. In addition to the prospection obtained, the data also show the importance of including clinical isolates in the screening of microorganisms with technological interest, allowing the selection of the prospected microorganism with the most adequate potential.

Keywords: Antimicrobial Resistance. Biological Activity. Endophytic Fungi.

Resumo

Microrganismos endofíticos são amplamente explorados por sua capacidade de produzir compostos com atividade biológica, o que permite identificar metabólitos com potencial para produção de fármacos, incluindo antimicrobianos. Considerando a necessidade de otimizar a prospecção desses microrganismos visando o controle de bactérias patogênicas resistentes a antimicrobianos, o presente estudo selecionou, a partir de uma coleção de endófitos, o fungo do gênero Diaporthe mais promissor na produção de metabolitos secundários contra microrganismos resistentes. Para isso, dez isolados deste gênero foram confrontados contra cepas padrão e bactérias patogênicas clínicas. Os fungos endofíticos do gênero Diaporthe isolados de folhas de Schinus terebinthifolius foram confrontados diretamente em placas de Petri contendo ágar batata dextrose por antagonismo contra oito cepas bacterianas (Enterococcus faecalis, Staphylococcus aureus, Staphylococcus saprophyticus, Enterobacter aerogenes, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli e Salmonella typhimurium) e sete isolados clínicos resistentes identificados como Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis com diferentes perfis de resistência, incluindo quinolonas e cefalosporinas, e Staphylococcus epidermidis resistente à ampicilina. Quatro fungos endofíticos controlaram o crescimento de bactérias Gram-positivas e Gram-negativas. Um dos isolados, Diaporthe endophytica LGMF653, inibiu o crescimento de cinco bactérias isoladas de amostras clínicas com característica de resistência a drogas antibacterianas, incluindo um microrganismo Gram-positivo e quatro Gram-negativos, sendo, portanto, considerado o mais promissor dentre os endófitos avaliados. Além da prospecção obtida, os dados também evidenciam a importância da inclusão de isolados clínicos na triagem de microrganismos com interesse tecnológico, permitindo selecionar o microrganismo prospectado com o potencial mais adequado.

Palavras-chave: *Resistência Antimicrobiana. Atividade Biológica. Fungos Endofíticos*

1 Introduction

The emergence and spread of multidrug-resistant organisms (MDROs) is a serious global health threat. Experts in infectious diseases, clinical microbiology, epidemiology, public health and pharmaceutical research and development and World Health Organization (WHO) representatives selected a group of antibiotic resistant bacteria of international concern, the list of microorganisms includes: *Acinetobacter baumanni* carbapenem-resistant, *Campylobacter* spp. fluoroquinolone-resistant, *Enterococcus faecium* vancomycin-resistant, Enterobacteriaceae carbapenem-resistant, Enterobacteriaceae third-generation cephalosporin-resistant, *Haemophilus influenzae* ampicillin-resistant, *Helicobacter pylori* clarithromycin-resistant, *Neisseria gonorrhoeae* fluoroquinolone-resistant third-generation cephalosporin-

resistant, non-typhoid *Salmonella* fluoroquinolone-resistant, *Pseudomonas aeruginosa* carbapenem-resistant, *Salmonella typhi* fluoroquinolone-resistant, *Shigella* spp. fluoroquinolone-resistant, *Staphylococcus aureus* methicillin-resistant vancomycin-resistant and *Streptococcus pneumoniae* penicillin non-susceptible (WHO, 2017). The combined proportions of resistance to Gram-negative pathogens were higher in lower-middle-income countries, carbapenem and third-generation cephalosporin resistance. The expansive ability to acquire resistance to various drugs makes them a threat to global health (AYOBAMI *et al*., 2022; TACCONELLI *et al*., 2018).

The antimicrobial resistance among microorganisms that commonly cause infections, combined with the lack of pharmacological alternatives for treatment, dramatically impacts on control efforts and increases health care costs (ALVIN *et al*., 2014; MOREL *et al*., 2020; SANCHEZ; DEMAIN, 2014). These challenges highlight the need to surveillance and monitoring of changes in epidemiology (WANG *et al*., 2022) and the critical demand for alternative active compounds, safe for human use, with low environmental impact (ANG *et al*., 2004) and active against multidrug-resistant bacteria (TACCONELLI *et al*., 2018), that could be used as an effective antimicrobial control strategy.

A promising pathway for discovering new pharmacologically significant compounds is by isolating bioactive compounds from biological sources, including endophytic fungi from medicinal plants (ALY *et al.,* 2010). The interaction between endophytic fungi and medicinal plants promotes production of a wide diversity of secondary metabolites (LUDWIG-MÜLLER, 2015) with antimicrobial, antioxidant and antitumor activities (TANVIR *et al*., 2017). Therefore, bioprospecting of endophytic fungi from medicinal plants for new bioactive compounds is a promising pathway to find new pharmacologically important alternatives for treating infections caused by MDROs (MANE; ANAND; VEDAMURTHY, 2020; STROBEL; DAISY, 2003).

Schinus terebinthifolius is a medicinal plant with antimicrobial properties (DEGÁSPARI *et al.,* 2005). Also, the endophytic fungi community of this plant, consisted by fungi of the genus *Alternaria*, *Bjerkandera*, *Colletotrichum*, *Diaporthe*, *Penicillium* and *Xylaria*, controls the development of *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas aeruginosa* (TONIAL *et al.,* 2015).

Diaporthe sp. is a fungus genus of endophyte that produces bioactive metabolites with antimicrobial activity (FERREIRA *et al*., 2017; MEDEIROS *et al*., 2018; NORILER *et al*., 2018; SAVI *et al*., 2019; TONIAL *et al*., 2017), including the carboxamide, vochysiamides B, that presented antibacterial activity against the Gram-negative bacterium *Klebsiella pneumoniae* producer of carbapenemases (NORILER *et al*., 2019). This genus has gained increasing attention on bioprospecting researches due to their ability to produce a wide diversity of secondary metabolites, which make them interesting candidates for drug discovery (UDAYANGA *et al*., 2011). In addition to activity against microorganisms, studies using extracts of *Diaporthe* sp. have had a significant impact on other fields, like the diaporthols A and B, that act as inhibitors of tumor metastasis (NAKASHIMA *et al*., 2018) and the CYP3A4 inhibitor, diaporthichalasinand (PORNPAKAKUL *et al.,* 2007). Therefore, in order to select the most promisor *Diaporthe* fungi in producing secondary metabolites against resistant microorganisms from an endohytic collection, ten isolates of this genus were confronted against standard strains and clinical pathogenic bacteria.

2 Material and Methods

2.1 Microorganisms

Ten endophytes of the genus *Diaporthe* isolated from leaves of *Schinus terebinthifolius* (TONIAL *et al.,* 2015) had the biotechnological potential evaluated in this study. The probable species of *Diaporthe* were: *D. terebinthifolii* - LGMF625, LGMF655, LGMF657 and LGMF658; *D. endophytica* - LGMF653; *D. helianthi* - LGMF694; *D. infecunda* - LGMF627, LGMF700 and LGMF701; and *Diaporthe* sp. - LGMF695 and LGMF714. Methods describing the isolation of endophytic fungi and identification by standard morphological techniques (macro- and micromorphology) and by direct sequencing of fungal internal transcribed spacer (ITS) are described in Tonial *et al*. (2015). The endophytic fungi are stored at the mycoteca of Bioprospecting and Molecular Genetics of Microorganisms Laboratory (BIOGEMM) from Federal University of Paraná (UFPR) and are maintained viable by subculture on potato dextrose agar.

The Gram-positive bacterial standard strains used in the biological assay were: *Enterococcus faecalis* (NEWP 0012), *Staphylococcus aureus* (NEWP 0038) and *Staphylococcus saprophyticus* (ATCC 15305). The Gram-negatives used in the assay were: *Enterobacter aerogenes* (NEWP 0048), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (NEWP 0083), *Escherichia coli* (ATCC 25922) and *Salmonella typhimurium* (NEWP 0028).

Samples from diverse anatomical sites of patients under health care with infections were collected and identified by the hospital's clinical analysis laboratory. Six Gram-negative (S1, S2, S3, S4, S5 and S7) and one Gram-positive (S6) clinical pathogens were selected: S1 (*Klebsiella pneumoniae* from urine), S2 (*Escherichia coli* from urine), S3 (*E. coli* from urine), S4 (*Proteus mirabilis* from muscle tissue - left triceps), S5 (*E. coli* from urine), S6 (*Staphylococcus epidermidis* from bone) and S7 (*E. coli* - urine ESBL producer). Antibiotic resistance pattern was assessed. The assay to determine the resistance of bacterial isolates to antibacterial drugs followed the Clinical and Laboratory Standards Institute (*CLSI*) protocols.

2.2 Antimicrobial assay – Challenge method

Endophytic fungi were grown on Petri plates with potato dextrose agar (PDA; 39 g PDA Merck per litter of distilled water) at 28 °C for 5-7 days. Bacteria were grown on Mueller-Hinton (MH Himedia) agar plates at 36 °C for 24 hours. Bacterial suspensions were prepared in MH broth at 10^8 CFU mL^{-1}, visually adjusted with 0.5 standard of the McFarland scale.

An adaptation of the disk-diffusion technique proposed in 1966 by Bauer *et al.* was performed. The screening for activity of endophytic fungi against pathogenic microorganisms were done in triplicates. Mycelial discs, 5 mm in diameter, of each endophyte were cut from the edge of a colony and placed in the center of a new PDA plates. Pathogenic microorganism suspensions were inoculated streaking with a platinum loop from the edge of mycelial disc to the edge of the plate. Co-culture plates were incubated at 36 °C for 24 hours.

Endophytic fungi were considered biologically active against a pathogenic microorganism when the bacterial growth was not detected close to the mycelium. If any growth was observed, it was considered that the endophytic isolate did not express biological activity against the pathogen. All co-cultures indicating growth inhibition were repeated.

3 Results and Discussion

Antibiotic resistance pattern of clinical pathogens tested in this study were described in Table 1. The pathogen S1 (*K. pneumoniae*) was resistant to quinolones, penicillin, cephalosporin and sulfonamides and susceptible to carbapenem and aminoglycosides. S2 and S5 (*E. coli*) were susceptible to all groups tested. The isolate S3 (*E. coli*) and S4 (*P. mirabilis*) were resistant to ampicillin (penicillin) and, respectively, to cephalexin and cefuroxime (cephalosporin), both β-lactams. The S6 (*S. epidermidis*) was resistant to ampicillin (penicillin), and susceptible to others β-lactams, included amoxicillin + clavulanic acid, and quinolones, aminoglycosides and sulfonamides. Isolate of *E. coli* – ESBL (S7) are resistant to sulfonamides, quinolones, cephalosporin and cefepime (penicillin) and were susceptible to nitrofurantoin, gentamicin, amoxicillin + clavulanic acid and ampicillin. Every pathogen tested were susceptible to gentamicin (aminoglycoside) and just S1 are resistant to amoxicillin + clavulanic acid (Table 1).

Table 1 - Antibiotic resistance pattern of clinical pathogens tested in this study

	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**
	Klebsiella pneumoniae	*Escherichia coli*	*Escherichia coli*	*Proteus mirabilis*	*Escherichia coli*	*Staphylococcus epidermidis*	*Escherichia coli* – ESBL
Nalidixic acid	R	S	S	NT	S	NT	R
Amikacin	S	NT	NT	S	NT	NT	NT
Ampicillin	R	S	R	R	S	R	S
Amoxicillin + Clavulanic acid	R	S	S	S	S	S	S
Cephalexin	R	S	R	S	S	S	R
Ceftriaxone	R	S	S	S	S	S	R
Cefuroxime	R	S	NT	R	S	S	R
Ciprofloxacin	R	S	S	S	S	S	R
Cefepime	R	S	S	S	S	NT	R
Gentamicin	S	S	S	S	S	S	S
Imipenem	S	NT	NT	S	NT	NT	NT
Nitrofurantoin	R	S	NT	NT	S	NT	S
Norfloxacin	R	S	S	NT	S	NT	R
Sulfazotrim	R	S	S	S	S	S	R
Piperacillin / Tazobactam	R	NT	NT	S	NT	NT	NT
Meropenem	S	NT	NT	S	NT	NT	NT

Legend: (R) resistant; (S) sensitive; (NT) not tested.
Source: Resource data.

Four *Diaporthe* endophytic of *Schinus terebinthifolius* leaves inhibited the development of pathogenic bacteria. Two of them showed antimicrobial activity only when confronted with standard strains and two were also active against pathogens isolated from clinical samples (Table 2). The LGMF653 (*Diaporthe endophytica*) inhibited the growth of standard strains *S. saprophyticus*, *E. coli* and *S. typhimurium*, and of clinical isolates *K. pneumoniae* (S1), *P. mirabilis* (S4), *E. coli* (S5), *S. epidermidis* (S6) and *E. coli* ESBL producer (S7). In the antibiotic resistance test S1, S4, S6 and S7 were resistant to 12 of the 16, 2/13, 1/8 and 8/12 antibiotics tested, respectively (Table 1). Strain LGMF694 of *Diaporthe helianthi* inhibited *S. saprophyticus*, *E. coli* and *S. epidermidis* (S6), ampicillin resistant. Also, the growth of *K. pneumoniae* and *S. saprophyticus* was inhibited by *D. infecunda* LGMF701 and *D. terebinthifolii* LGMF658 strains. The results show the potential of *Diaporthe* endophytes to control the growth of Gram-positive and Gram-negative bacteria and highlight LGMF653 *D. endophytica* isolate as a promising source for the isolation of new bioactive compounds against MDROs.

Table 2 - Microorganisms inhibited by *Diaporthe* endophytic of *Schinus terebinthifolius* by direct challenge method

Diaporthe	Standard Strain	Clinical Isolated
LGMF653 *D. endophytica*	*Staphylococcus saprophyticus* (ATCC 15305) *Escherichia coli* (ATCC 25922) *Salmonella typhimurium* (NEWP 0028)	*Klebsiella pneumoniae* (S1) *Proteus mirabilis* (S4) *Escherichia coli* (S5) *Staphylococcus epidermidis* (S6) *Escherichia coli* – ESBL (S7)
LGMF694 *D. helianthi*	*Staphylococcus saprophyticus* (ATCC 15305) *Escherichia coli* (ATCC 25922)	*Staphylococcus epidermidis* (S6)
LGMF658 *D. terebinthifolii*	*Staphylococcus saprophyticus* (ATCC 15305) *Klebsiella pneumoniae* (NEWP 0083)	-
LGMF701 *D. infecunda*	*Staphylococcus saprophyticus* (ATCC 15305) *Klebsiella pneumoniae* (NEWP 0083)	-

Source: Resource data.

The biological activity against the standard pathogenic strains were distinct from the biological activity against clinical pathogens. Two endophytes, although controlling the growth of standard strain bacteria, were not effective in preventing the growth of clinical isolates and are therefore considered to have low biotechnological potential. *Diaporthe endophytica* LGMF653 was the only endophyte capable of inhibiting Gram-negative bacteria obtained from patients with clinical infectious processes with antimicrobial drug resistance, including an extended spectrum beta-lactamase (ESBL) producer isolate.

Diaporthe is the most isolated fungal genus as an endophyte and has production of metabolites with investigated bioactivity to serve as a source for exploration of new secondary metabolites with biotechnological potential (UDAYANGA *et al*., 2011; SAVI; ALUIZIO; GLIENKE, 2019). The results obtained in this work show that antagonism is not species-specific, but an individual characteristic of the microbial isolate, highlighting the relevance of microorganism screening work.

Furthermore, the result highlight the importance of isolating and testing pathogens from clinical infections in addition to standard strains. The insertion in the bioactivity assays of microorganisms obtained from clinical samples, including bacteria with antimicrobial drug resistance characteristics, allowed to identify among the ten prospected endophytes the one with the highest applicable biotechnological potential against isolates that were causing infectious processes in patients. This study model allows, among a wide diversity of microorganisms to be prospected, to identify the one with the greatest potential of pharmaceutical industrial interest for the demand required by the community.

The importance of the clinical isolates in support to the selection process in the drug discovery pipeline was demonstrated in a study that aimed at improving the drug discovery process for anti-leishmanials (HEFNAWY *et al*., 2018). The authors report that out of 120 compounds active on a *Leishmania* standard strain (LdBOB) just 45% were also active on the clinical isolates, independently of their pentavalent antimonials susceptibility. They also discussed the importance to evaluate the activity of strains with diverse susceptibility, and that the screening should at least cover all strains of a particular geographical population (HEFNAWY *et al*., 2018). In particular, in the context of antibacterial chemotherapy, there is much more value in a drug candidate that covers clinical representatives of the selected group of antibiotic resistant bacteria of international concern panel. We agree with Hefnawy *et al*. (2018), that the screening should involve recent clinical isolates, with different susceptibility to existing drugs and from different geographical areas.

4 Conclusion

Diaporthe endophytica endophyte LGMF653 inhibits the growth of Gram-positive and Gram-negative bacterial from standard strains and clinical isolates resistant to antibiotics, which demonstrates biotechnological potential. This study provided a stimulus to investigate the bioactivity of compounds produced by endophytic fungi LGMF653 as an alternative for drug-resistant organisms control. Furthermore, the introduction of bacteria isolated from clinical samples in the prospecting trial allowed selecting the endophyte with the best potential for the control of bacteria with antimicrobial drug resistance characteristics, ensuring greater efficiency in the selection of microorganisms for bioprospecting. Therefore, the authors suggest, based on the results obtained in this study that, in antimicrobial prospection assays, clinical and environmental isolates that characterize the problem, as well as standard strain bacteria, should be evaluated.

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