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ENVIRONMENTAL MICROBIOLOGY - SHORT COMMUNICATION

Antifungal susceptibility of the endophytic fungus *Rhinocladiella similis* (URM 7800) isolated from the Caatinga dry forest in Brazil

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Abstract

The present study reports a new occurrence of *Rhinocladiella similis* isolated as an endophytic fungus in the Caatinga dry tropical forest in Brazil and describes its antifungal susceptibility. The isolate *R. similis* URM 7800 was obtained from leaves of the medicinal plant *Myracrodruon urundeuva*. Its morphological characterization was performed on potato dextrose agar medium and molecular analysis using the ITS rDNA sequence. The antifungal susceptibility profile was defined using the Clinical and Laboratory Standards Institute (CLSI) protocol M38-A2. The colony of isolate URM 7800 showed slow growth, with an olivaceous-gray color and powdery mycelium; in microculture, it showed the typical features of *R. similis*. In the antifungal susceptibility test, isolate URM 7800 showed high minimal inhibitory concentration (MIC) values for amphotericin B (>16 µg/mL), voriconazole (16 µg/mL), terbinafine (>0.5 µg/mL), and caspofungin (>8 µg/mL), among other antifungal drugs. Pathogenic melanized fungi are frequently isolated in environments where humans may be exposed, and these data show that it is essential to know if these isolates possess antifungal resistance.

Keywords Endophytes · Herpotrichiellaceae · Dry tropical forest · Antifungal susceptibility · DHN-melanin

Introduction

Rhinocladiella is a genus of melanized fungi associated with cases of chromoblastomycosis (CBM), mainly the species *R. similis* and *R. aquaspersa* [1–4]. CBM is a chronic granulomatous fungal infection prevalent in rural workers, which causes cutaneous and subcutaneous tissue hyperproliferation, forming verrucous lesions with murine fungal cells. It is one of the most prevalent implant mycoses in tropical and subtropical areas and is classified as a neglected tropical disease [5].

Although species of *Rhinocladiella* inhabit many natural environments, reports of isolation from the environment are few. The predominant sites are tropical forests, where they live in association with lichens [6], colonizing plant tissue under immersed decaying leaves [7], and inhabiting roots [8].

Rhinocladiella similis de Hoog & Calig. [9] is a rare etiologic agent of chromoblastomycosis (CBM) and is found mainly in tropical and subtropical countries. This species

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has previously been isolated from environmental samples, mainly from piped and underground water, and has also been recorded in dialysis water [10, 11]. In addition, *R. similis* has been found in different environments: in babassu coconut shells in Brazil [12], as a parasite of corals [13], in the exoskeleton of an ant [14], and as an endophytic fungus in desert plants [15, 16].

Antifungal susceptibility tests of clinical isolates of *Rhinocladiella* spp. are not performed frequently [2, 4, 17, 18], and analyses of these isolates from the natural environment, especially extreme environments such as dry tropical forests, are still infrequent. Melanized endophytic fungi are exposed to multiple stresses and may possess mechanisms to tolerate various stressful environmental conditions, such as UV radiation, high temperatures, and low water availability [19, 20]. Some melanized endophytes under stress conditions can become pathogenic [21, 22], and melanin is linked to the virulence of plant and human pathogenic fungi [23].

It is important to know the endophytic fungi that inhabit stressful environments and which may be opportunistic pathogens in humans, as well as to analyze their ability to resist antifungal agents. The present contribution reports a new occurrence of the endophytic *Rhinocladiella similis* URM 7800 from the leaves of a medicinal plant of the Caatinga dry forest in Brazil and defines its antifungal susceptibility profile.

Material and methods

Fungus isolation

The endophytic fungus was isolated from leaves of the medicinal plant *Myracrodruon urundeuva* Allemão in the Caatinga dry forest located in Triunfo municipality, Pernambuco state, Brazil (07°49′32.99″S 38°06′54.21″W). Information on the study area as well as the processing of leaves, isolation of endophytes, and authorizations for plant collection was provided by Pádua et al. (2019) [24]. The strain is deposited in the URM culture collection (Micoteca URM Profa. Maria Auxiliadora Cavalcanti – WDCM 604), located at the Universidade Federal de Pernambuco, Recife, Brazil.

Morphological characterization

The endophytic isolate was maintained in slanted tubes with malt extract agar (MEA). For macromorphological analysis, the fungus was inoculated at a central point on potato dextrose agar medium (PDA) at the center of Petri dishes, and the dishes were incubated in the dark at 25 °C. After 28 days, the diameter of the colonies was measured and the morphological characteristics was analyzed (e.g., shape, texture, presence of soluble pigments, exudates, and colony colors). Colony color was analyzed using the Rayner color chart [25].

Micromorphological analyses were performed on PDA using the microculture technique [9, 26]. For this analysis, a humid chamber was constructed from culture dishes containing sterilized water with a U-shaped glass rod on the water surface. A sterile slide with a block of freshly grown fungal colony (ca. 1 cm^2) was placed on this structure, together with a sterile cover slip. Fungal sporulation was monitored over time and, when sporulation reached a maximum, lactophenol cotton blue was used to mount slides. The fungal structure and microscopic characters (e.g., size, shape, and pigmentation of somatic and reproductive cells) were analyzed under a light microscope.

Molecular analysis

Molecular analysis was performed by sequencing the ITS rDNA region, using the primer pairs ITS5/ITS4 as described by Pádua et al. (2019) [24]. The ITS sequence was compared to the type strain or reference sequences using the BLASTn tool in the GenBank database of the NCBI. The maximum likelihood (ML) analysis was performed using ITS rDNA sequences from our isolate, ex-type, and reference strains of *Rhinocladiella* species retrieved from GenBank, and following methods described by Bezerra et al. (2017) [27]. *Cladophialophora carrionii* (CBS 108.97 and CBS 161.54) was used as the outgroup.

Antifungal susceptibility testing

Minimum inhibitory concentrations (MICs) were determined using the 96-well plate microdilution technique, following the Clinical and Laboratory Standards Institute (CLSI) protocol M38-A2 [28]. The fungus was cultured on Sabouraud dextrose agar at 35 °C for 48 h and the inoculum was prepared in saline solution (0.85%), with quantification of the conidia in a Neubauer chamber. The final concentration in each well was 2.5×10^4 CFU/mL.

The antifungal agents amphotericin B, itraconazole, ketoconazole, voriconazole, posaconazole, terbinafine, and caspofungin (all from Sigma-Aldrich, USA) were evaluated in the final concentration range of 0.03–16 µg/mL [28]. The incubation temperature was 35 °C for up to 5 days [29]. Minimum inhibitory concentrations (MICs) were determined when there was 100% visual inhibition, comparing the concentration of antifungal with growth in wells without antifungals (growth control) [28]. The test was performed in triplicate.

Results and discussion

Our ITS sequence showed 100% identity with the sequence of the type strain *R. similis* (CBS 111763) (GenBank NR_166008.1) and other sequences deposited as *R. similis* (e.g., CBS 127588, GenBank MH864632.1). The sequence from our isolate is deposited in GenBank under the access no. MG870428 [24], and the isolate was termed *R. similis* URM 7800.

The ITS sequence from *R. similis* URM 7800 was compared to the ex-type strain and reference sequences using the BLASTn tool in the GenBank database of the NCBI, obtaining 100% identity with the sequence of the ex-type strain *R. similis* (CBS 111763) (GenBank NR_166008.1) and other sequences deposited as *R. similis* (e.g., CBS 127588, GenBank MH864632.1). The ML tree using ITS rDNA sequences placed our isolate *R. similis* URM 7800 in a well-supported clade (ML-BS = 100%) including the ex-type strain and other isolates of *R. similis* (Fig. 1).

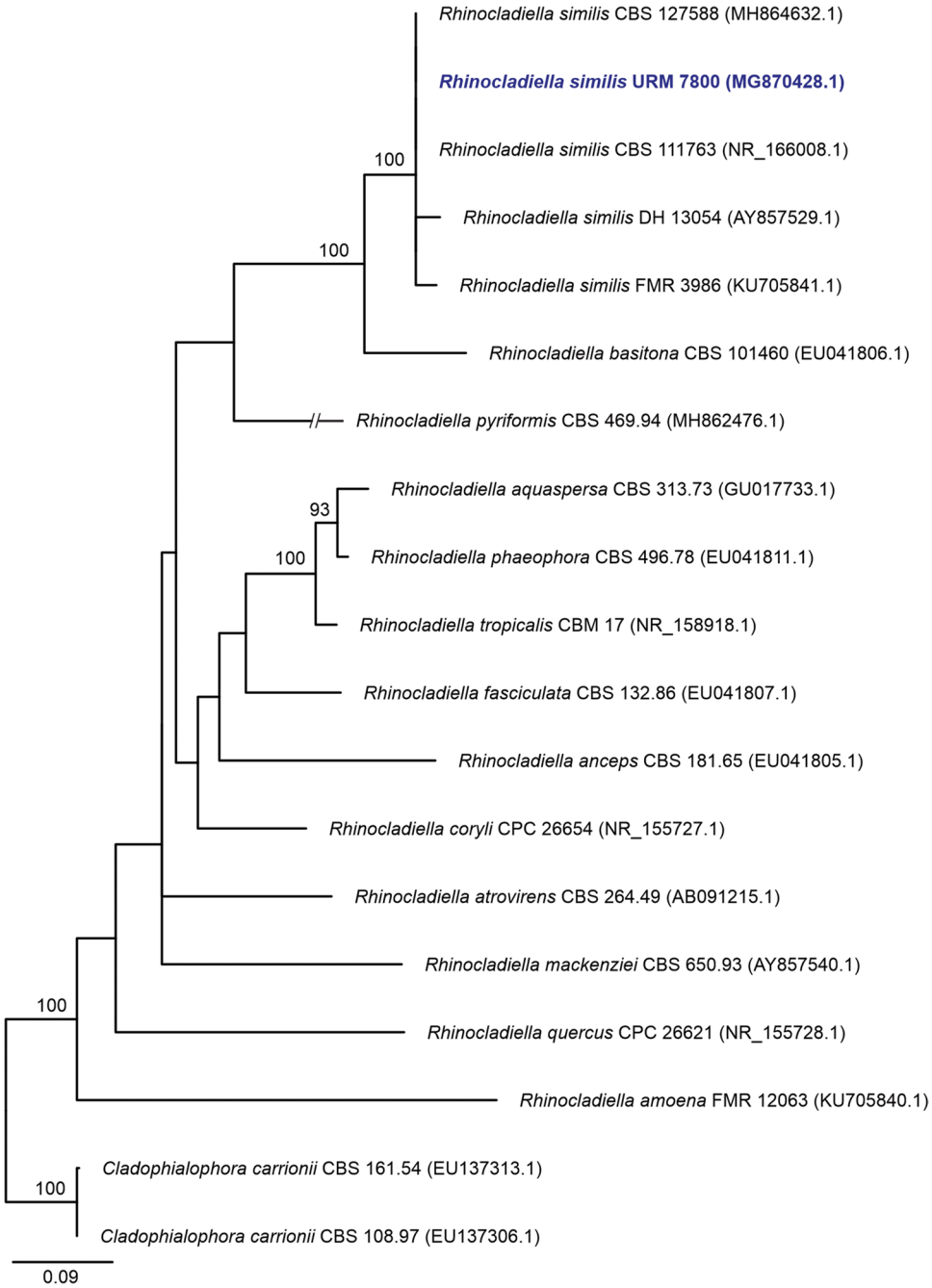

Fig. 1 Maximum likelihood tree constructed with ITS rDNA sequences from ex-type and reference strains of *Rhinocladiellla* species. The isolate *Rhinocladiellla similis* URM 7800 is in bold and blue. The ML-BS values ≥ 70% are placed near nodes. *Cladophialophora carrionii* (CBS 108.97 and CBS 161.54) was used as the outgroup. The bar represents expected number of substitutions per site

After growing on PDA for 4 weeks at 25 °C, the colonies of *R. similis* URM 7800 reached 5 cm in diameter, with an entire and irregular margin, powdery mycelium, becoming hairy in the center with an elevation, presence of streaks that started at the center and extended to the edge of the colony, olivaceous-gray color at the edge and dark olivaceous-gray at the center of the colony, and without exudate (Fig. 2A). In microculture, widely branched conidiophores and pale-brown pigmentation of the mycelium were observed, which are typical characteristics of the genus *Rhinocladiella* (Fig. 2B). The conidiophores are basitonously branched, with triangular conidia [9].

This is the first study to describe the occurrence of *R. similis* as an endophytic fungus from leaves of a medicinal plant in Caatinga dry forest in Brazil. Human pathogenic endophytes are rarely reported in the Caatinga; recently, an endophytic fungus *Cladophialophora bromeliacearum* was isolated and described from a species of bromeliad endemic to the Caatinga [30]. *R. similis* has also been isolated from the plant *Agriophyllum squarrosum* in the Tengger Desert of China [15], a stressful environment similar to the Caatinga forest. This study focused on the production, by *R. similis*, of seven resorcylic acid lactones (RALs), polyketides that have different biological activities, with antimicrobial, cytotoxic, and anti-inflammatory properties. This shows that *R. similis*, as an endophytic fungus, can play important ecological roles in these environments, helping plants to adapt to adverse conditions [15, 20]. Compounds isolated from endophytic fungi can also be useful in biotechnological processes, such as the production of enzymes and drugs [21].

Regarding its pathogenicity, *R. similis* has previously been reported as an etiologic agent of chromoblastomycosis in a patient in the state of Amazônia, Brazil [31]; in a rural worker in Mexico [3]; and in a case in Catalonia, Spain [32]. All these cases occurred in areas favorable to transmission, where the inhabitants have wide contact with vegetation and river sediments. However, *R. similis* has been frequently reported in urban environments, such as freshwater, and in polluted environments, which may indicate a problem with the dissemination of this opportunistic pathogen in urban populations [10, 11, 33]. This may contribute to more frequent cases of diseases caused by unusual fungal species, which are inadequately studied in relation to their antifungal susceptibility.

R. similis is a producer of 1,8-dihydroxynaphtalene (DHN)-melanin [34, 35], which gives the colonies a pale olivaceous-gray color (Fig. 2A). In a study that quantified the melanin produced by 77 strains of chromoblastomycosis agents distributed within five genera, *R. similis* had the smallest amount of melanin extracted [17]. DHN-melanin is a polymer responsible for protecting fungal cells against environmental adversities. Melanin is also a protective factor against the action of antifungal agents and against oxidative stress [17, 36–38], making it more difficult to treat mycoses caused by melanized fungi. Therefore, it is essential to perform in vitro susceptibility tests, also considering the growing problem of antifungal resistance, especially in species of *Candida* but also in melanized filamentous fungi [39].

CLSI antifungal susceptibility protocols do not have breakpoints (BPs) for species of *Rhinocladiella*, which define a particular isolate as sensitive or resistant to an antifungal agent and can predict the clinical response to that agent [28, 40]. Therefore, it is not possible to establish these parameters for isolates of *R. similis*; it is only possible to compare the MIC values obtained.

Table 1 lists all studies that have evaluated the antifungal susceptibility of species of *Rhinocladiella*, including the results of the present study. In general, the *Rhinocladiella* species analyzed (*R. similis*, *R. aquaspersa*, *R. mackenziei*, *R. basitona*, *R. phaeophora*, and *R. tropicalis*) have similar susceptibility profiles. Comparing the antifungal susceptibility of isolate *R. similis* URM 7800 from the present study with the results from the literature shows that this isolate has higher MIC values for amphotericin B, terbinafine, and

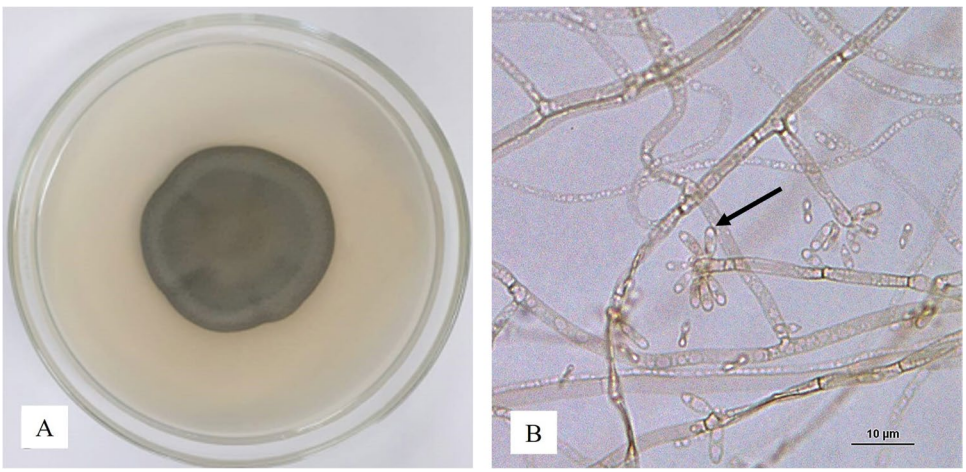

Fig. 2 **A** Colony of *R. similis* URM 7800 on PDA after incubation for 28 days at 25 °C. **B** Micromorphology of *R. similis* URM 7800 showing the presence of basitonously branched conidiophores (arrow) and conidia at the tip

Table 1 Minimal inhibitory concentrations (MICs) of *Rhinocladiella* species

Source	Protocol	*n*	MIC (μg/mL)																		Ref.
			ITC	AMB	TRB	VRC	POS	CAS	KTC	FLC	ISA	AFG	MFG	5FC	LZN	LLCZ	NM	EZ	BIZ	NYS	
R. similis																					
Nature	CLSI M38-A2	1	0.25	>16	>0.5	16	0.5	>8	2	-	-	-	-	-	-	-	-	-	-	-	Present study
BAL fluid	EUCAST E.Def 9.3.1	9 (MIC range)	0.25	1	-	0.5	0.015–0.03	1-4	-	-	-	0.5–1	0.06–0.25	-	-	-	-	-	-	-	[41]
Clinical	EUCAST E.Def 9.3	2 (MIC range)	0.06	1–2	0.06–0.12	0.06–0.25	-	-	-	-	-	-	-	4–8	-	-	-	-	-	-	[31]
Clinical	CLSI M38-A2	1	1	8	0.5	2	0.5	-	2	-	-	-	-	-	-	-	-	-	-	-	[38]
R. aquaspersa																					
Clinical	CLSI M38-A2	1	0.125	2	0.06	1	0.25	-	-	-	-	-	-	-	-	-	-	-	-	-	[2]
Clinical	CLSI M27-P modified	1	0.8	0.8	0.8	-	-	-	1.6	1.6	-	-	-	1.25	-	-	-	-	-	-	[4]
Clinical	CLSI M38-A2	2 (MIC GM)	0.25	4	0.177	2.828	-	-	1.414	-	-	-	-	-	-	-	-	-	-	-	[29]
Clinical	CLSI M38-A2	3 (MIC range)	0.063–0.125	1–2	-	2	0.063–0.125	8	-	32–64	2	1	-	-	-	-	-	-	-	-	[1]
Clinical	CLSI M38-A2	1	2	1	0.5	2	1	-	2	-	-	-	-	-	-	-	-	-	-	-	[38]
R. mackenziei																					
Clinical	CLSI M38-A2	1	0.125	16	-	0.25	0.063	-	-	-	-	-	-	-	0.002	0.002	-	-	-	-	[42]
Clinical	CLSI M38-A2	1	≤0.03	16	-	≤0.03	≤0.03	1	-	-	-	4	1	-	-	-	-	-	-	-	[43]
Clinical	CLSI M38-A2	1	0.063	16	-	0.5	0.031	-	-	32	0.5	-	-	-	-	-	-	-	-	-	[44]
Clinical	CLSI M38-A2	10 (MIC range)	0.063–0.25	2–16	-	0.25–2	0.016–0.063	4-8	-	16–64	0.25–1	1–8	-	-	-	-	-	-	-	-	[45]
R. basitona																					
Clinical	CLSI M38-A2	1	0.5	0.5	-	0.25	-	-	1	64	-	-	-	-	-	-	0.5	-	-	-	[46]
Clinical	CLSI M38-A2	1	0.5	-	0.03	1	-	-	1	>64	-	-	2	-	-	-	-	0.125	0.5	>64	[47]

Table 1 (continued)

Source	Protocol	*n*	MIC (μg/mL)															Ref.
			ITC	AMB	TRB	VRC	POS	CAS	KTC	FLC	ISA	AFG	MFG	5FC	LZN	LLCZ	NM	EZ	BIZ	NYS	
R. phaeophora																					
Nature	CLSI M38-A2	1	0.5	2	-	2	0.25	8	-	>64	2	8	-	-	-	-	-	-	-	-	[1]
R. tropicalis																					
Clinical	CLSI M38-A2	2 (MIC GM)	2	1	0.5	0.188	0.125	-	2	-	-	-	-	-	-	-	-	-	-	-	[38]

MIC, minimal inhibitory concentration; *ITC*, itraconazole; *AMB*, amphotericin B; *TRB*, terbinafine; *VRC*, voriconazole; *POS*, posaconazole; *CAS*, caspofungin; *KTC*, ketoconazole; *FLC*, fluconazole; *ISA*, isavuconazole; *AFG*, anidulafungin; *MFG*, micafungin; *5FC*, flucytosine; *LZN*, lanoconazole; *LLCZ*, luliconazole; *NM*, natamycin; *EZ*, econazole; *BIZ*, bifonazole; *NYS*, nystatin; *BAL*, bronchoalveolar lavage

voriconazole (Table 1). However, the antifungal susceptibility of this species is inadequately known, and the present study is the first to evaluate an environmental isolate.

Only one study has evaluated the MIC values of *R. phaeophora* as an environmental isolate obtained from soil in a corn field [1]. Isolate *R. similis* URM 7800 showed higher MIC values for amphotericin B, voriconazole, posaconazole, and caspofungin. Fungicides are used to control plant diseases of crops, and this exposure can lead to the development of resistance in fungal isolates present in farming areas [48]. Even so, isolate *R. similis* URM 7800 showed higher MIC values for four antifungal agents, showing that fungi from stressful environments, such as the Caatinga forest, are adapted to different stress conditions. All other studies evaluated clinical *Rhinocladiella* isolates (Table 1).

Considering that pathogenic melanized fungi are frequently isolated in environments related to humans, it is essential to know their antifungal susceptibility profiles. Even though in vitro assays do not necessarily have a correlation with in vivo antifungal resistance, these tests remain important as long as there are no alternative assays that better simulate in vivo growth conditions and that allow a better evaluation of these etiological agents [39].

The present results highlight the importance of increasing knowledge of the environments where human opportunistic pathogenic fungi occur, especially those fungi that produce melanin and have wide pathogenicity profiles. The results also emphasize the importance of performing antifungal susceptibility assays with these fungal isolates, to determine their potential for antifungal resistance. It would be useful to perform antifungal susceptibility tests with other fungal species isolated in the Caatinga forest, in order to determine if the profile found in our study occurs in other fungal isolates.

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Author contribution All authors made substantial contributions to the design of the study; for data acquisition and analysis; for the writing of the manuscript and/or its critical review; approved the version to be published; and agree to be responsible for all aspects of the work.

Declarations

Conflict of interest The authors declare no competing interests.

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