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Original Article

Molecular and morphological characterization of *Hortaea werneckii*, an endophytic fungus on mangrove trees (*Avicennia marina*) in Iran

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ABSTRACT

Mangrove forests are resilient ecosystems inhabiting tropical coastlines and provide a habitat for species such as *Avicennia marina*, which exhibits notable tolerance to environmental stresses. These mangrove-associated habitats support the production of bioactive metabolites and host diverse endophytic fungi, highlighting their potential applications in agriculture and pharmaceutical development. Endophytic fungi are potent biological agents capable of suppressing plant pathogens, synthesizing medically relevant metabolites, and degrading environmental pollutants, thereby contributing substantially to sustainable agriculture and environmental health. The present study aimed to isolate and characterize endophytic fungal species associated with mangrove trees in the Qeshm region, Hormozgan Province, Iran. Root, stem, and leaf tissues were surface-sterilized in 70 % ethanol for 30 seconds (leaves) or 1 minute (roots and stems), followed by diluted 2% sodium hypochlorite solution for 1.5 minutes (leaves) or 3 minutes (roots and stems), and finally rinsed three times with sterile distilled water. The disinfected tissues were cultured on potato dextrose agar (PDA) and water agar (WA), and the emerging endophytic fungi were subsequently purified to obtain monoconidial isolates. Morphological identification was conducted using standard taxonomic references, while molecular characterization was based on sequencing the internal transcribed spacer (ITS) region of rDNA. Morphological and molecular characterization indicated that 9.8% of leaf isolates, 36.84% of stem isolates, and 7.14% of root isolates belong to the genus *Hortaea*, with phylogenetic analysis confirming these isolates as *H. werneckii*. Based on current knowledge, *H. werneckii* represents a new record for mangrove forests in Iran Fungi, with *Avicennia marina* serving as a plant host.

KEYWORDS
Mangrove forest, Molecular identification, Morphology, Pathogenicity.

INTRODUCTION

Avicennia marina (Forssk.) Vierh (*Lamiales*, *Acanthaceae*) is a mangrove species distributed across Australia, South and Southeast Asia, the Middle East, and Africa. In Iran, *A. marina* occurs along the Persian Gulf, extending up to approximately 400 km north of the Mand Protected Area in Bushehr Province. The binomial name is attributed to a renowned Iranian physician, as noted by ElDohaji et al. (2020). *Avicennia marina* exhibits high tolerance to extreme environmental conditions, including elevated salinity, high temperatures, strong winds, and anaerobic soil conditions (Das et al. 2016, Thatoi et al. 2016).

Endophyte fungi inhabit the internal tissues of apparently healthy and asymptomatic hosts. In fact, endophytes appear to be ubiquitous, with no reported cases of complete absence in any plant species (Nisa et al. 2015). Fungal endophytes are widely studied for their potential to maintain plant health and defend against abiotic and biotic stresses by increasing plant resistance, producing secondary metabolites, and promoting plant growth (Latz et al. 2018). The association of endophytic fungi with mangrove plants is thought to bolster tolerance to harsh environmental conditions and to confer a competitive advantage over saprophytic fungi that decompose senescent tissues (Kumaresan 2002).

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Hortaea werneckii is a halophilic black yeast that serves as a model organism for studying eukaryotic halotolerance (Zalar et al. 2019). The genus *Hortaea* and the species *H. werneckii* were first described by Nishimura and Miyaji in 1984. To date, only a single species has been listed in major taxonomic databases such as Index Fungorum, Species Fungorum, and MycoBank. *Hortaea werneckii* has been isolated as an endophytic fungus from diverse hosts in various regions, including *Aegiceras comiculatum* in the mangrove forest at the South China Sea (Chen et al. 2012), *Rhizophora apiculate* in Thailand (Doilom et al. 2017), *Avicennia marina* along the Red Sea coast of Saudi Arabia (Hodhod et al. 2020), and *Solanum nigrum* in Najran, Saudi Arabia (Attia et al. 2021). Beyond plant associations, *H. werneckii* has been linked to humans (Bonifaz et al. 2008, Kelarestaghi et al. 2022), seawater (Kogej et al. 2005, Marchetta et al. 2018), soil (Rizk and Magdy 2022), animals (Sharmin et al. 2002), and diving equipment (Cabañes et al. 2012).

This study presents the first identification and pathogenicity assessment of *H. werneckii* on *A. marina* leaves in the Qeshm region of Iran.

MATERIALS AND METHODS

Sampling, fungal isolation, and purification

In spring and autumn 2024, we collected one hundred samples from leaves, roots, and branches of healthy *A. marina* trees from multiple sites in the Qeshm region of Hormozgan Province, Iran (N, 55.764908° E 26.786977°). Endophytic fungi were isolated by surface-sterilizing the plant tissues to remove epiphytic microbes. Tissues were first washed under running water and then cut into small sections (~1 cm × 1 cm). For leaves, the surface-sterilization sequence was: (1) immersion in 70% ethanol for 30 s; (2) immersion in 2% sodium hypochlorite for 90 s; and (3) rinsing three times in sterile distilled water. For branches and roots, the sequence was: (1) immersion in 70% ethanol for 60 s; (2) immersion in 2% sodium hypochlorite for 3 min; and (3) rinsing three times in sterile distilled water (Wulandari et al., 2022). Sterilized tissues were placed on potato dextrose agar (PDA) supplemented with 500 ppm chloramphenicol and incubated at 25°C. Fungal isolates were purified by hyphal-tip transfer onto Water Agar and subsequently stored at −20°C.

Morphological and molecular characterization

To evaluate morphological features, fungal isolates were incubated on PDA and Sabouraud Dextrose Agar (SDA; 40 g Dextrose, 10 g Peptone, 15 g Agar, 1 L distilled water) at 25°C in the darkness, with morphological and cultural characteristics recorded at 7, 14, and 21 days. Fungal structures were investigated in mounting lactophenol medium using an Olympus VS2 microscope. Measurements of fungal structure were taken from at least 30 dimensions. For molecular identification, DNA was extracted using the Jank method (Zhang et al. 2010). The internal transcribed spacer (ITS1-5.8S-ITS2) region of ribosomal DNA was amplified and sequenced with primers ITS1 and ITS4 (White et al., 1990). The PCR mix (total volume = 20 µL) contained 10 µL of Taq DNA Polymerase Master Mix RED, 6 µL of ddH2O, 1 µL of each primer (10 pmol), and 2 µL of template DNA. PCR cycling consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 7 min. The PCR products were purified and sequenced by Codon Genetics Group (Tehran, Iran). The obtained sequence in this research (IRAN 5472C), was aligned with ex-type strain sequences retrieved from GenBank using MAFFT v7 (online server) (Katoh and Standley 2013). Phylogenetic analysis included Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI). MP and BI analyses were conducted via the CIPRES Science Gateway (Miller et al., 2012) using PAUP* 4.0b10 (Swofford, 2003) and MrBayes 3.2.6 (Ronquist et al. 2012, Huelsenbeck and Ronquist 2001), respectively. ML analysis was performed with IQ-TREE 2.2.2 (Nguyen et al. 2015), with the most suitable model identified by ModelFinder (Kalyaanamoorthy et al. 2017) and ultrafast bootstrap (UFBoot) with 1000 replicates (Hoang et al. 2018), both integrated in IQ-TREE. Phylogenetic trees were visualized and edited with FigTree v1.4.3 and Adobe Illustrator CS2. Newly generated sequences were deposited in GenBank. Isolates characterized in this study were deposited in the IRAN culture collection at the Iranian Research Institute of Plant Protection (Tehran, Iran).

Pathogenicity tests

To confirm non-pathogenicity, a pathogenicity test was done on the wounded leaves of *A. marina* in Petri plates under laboratory conditions. Sterile PDA plugs were placed on leaves as a negative control. The pathogenicity activity of the fungal isolate was assessed by inoculating leaves and incubating them at 25°C for 72 hours. Subsequently, symptoms on the inoculated leaves were recorded.

Salinity and temperature stress

To assess salinity tolerance, PDA plates were amended with sodium chloride (NaCl) at 10, 20, 30, and 40 percent. Fungal discs (5 mm in diameter) were placed at the center of each plate and incubated at 25°C for 28 days. For the temperature-tolerance test, PDA plates containing the isolate were incubated at 25°C, 35°C, and 45°C for 28 days. Radial growth (colony diameter) was measured on each plate and compared with a control plate to evaluate salt and temperature tolerance.

RESULTS

Sampling, fungal isolation, and purification

In the present study, approximately 210 isolates were obtained, of which 30, determined by morphological characterization, belonged to the genus *Hortaea* and were isolated from leaves, stems, and roots (Fig. 1).

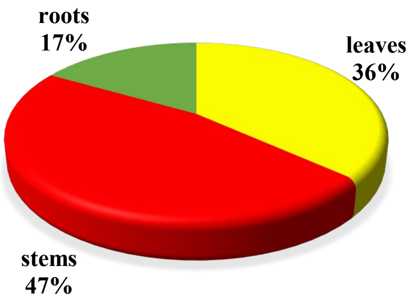

Fig. 1. The isolation rate of *Hortaea* from various tissue samples of *Avicennia marina* in the Qeshm region.

Molecular identification

To determine the taxonomic position of the *Hortaea* isolate IRAN 5472C, we analyzed its internal transcribed spacer (ITS) sequence using three phylogenetic approaches: Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI) on the ITS alignment. The dataset comprised 551 characters, of which 355 were constant, 70 were variable but parsimony-uninformative, and 126 were parsimony-informative. MP analysis of the 408 parsimony-informative characters yielded 45 most parsimonious trees (TL = 507, CI = 0.59, RI = 0.53, HI = 0.40). IQ-TREE identified the best ML tree (log-likelihood = -2998.405) after 20 iterations, with ModelFinder selecting TIM2+F+G4 as the best evolutionary model. BI analysis generated 1,432 trees, of which 358 were discarded as burn-in. The consensus tree and posterior probabilities were calculated from the remaining 1,074 trees, and the average standard deviation of split frequencies at the end of the analysis was 0.009717. Phylogenetic analyses placed isolate IRAN 5472C (GenBank accession no.: PX467158) in a well-supported clade with the ex-type strain of *Hortaea werneckii* (Fig. 2).

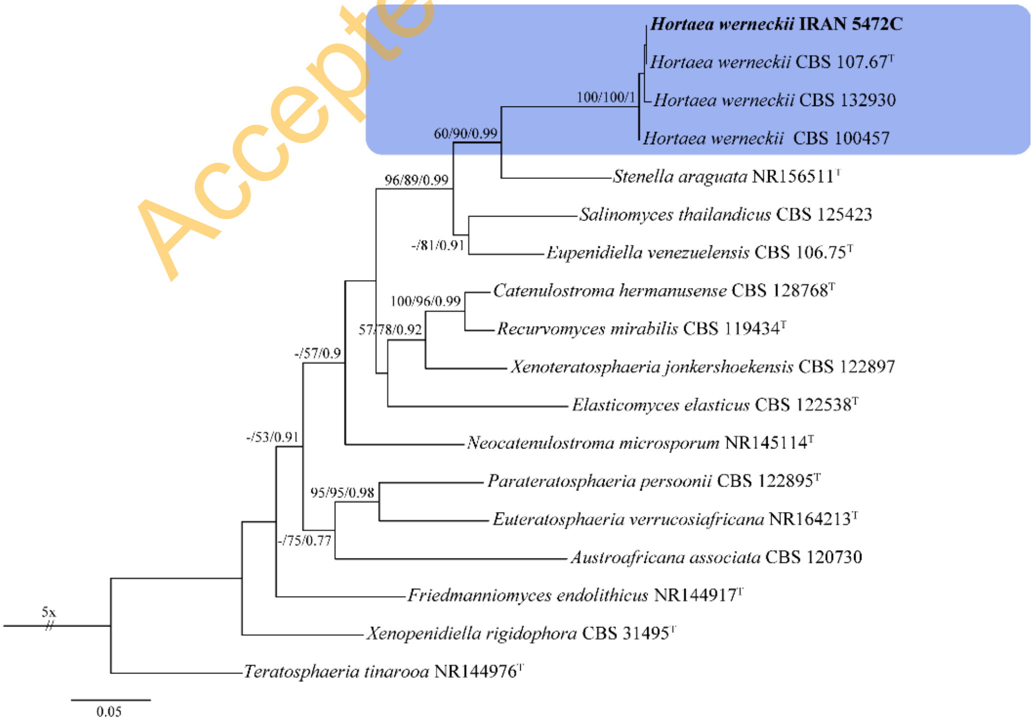

Fig. 2. Maximum likelihood phylogenetic tree of *Hortaea werneckii* inferred using IQ-TREE under the TIM2+F+G4 model. The tree is rooted to *Teratosphaeria tinarooa* (NR144976). The scale bar indicates the expected number of changes per site. Node support values are shown as Maximum Parsimony (MP) bootstrap / Maximum Likelihood (ML) bootstrap / Bayesian Inference (BI) posterior probabilities (MP-BS/ML-BS/BI-PP). T = Ex-type.

Morphological Characteristics

Hortaea werneckii (Horta) Nishim. & Miyaji, Jap. J. Med. Mycol. 26 (2): 145 (1984). Fig. 3.

Colonies on PDA and SDA are slow-growing. After 21 days at 25ºC, reaching a diameter of 34 and 20 mm. Initially, on both media, colonies are yeast-like and shiny black. On PDA, after 21 days, turn to velvety aerial mycelium, olive or dark gray color. On SDA, after 21 days, turn to flat mycelium, shiny olive color. Conidia produced abundantly in annellidic, cylindrical to ellipsoidal, 1-2 cellular, unseptate conidia (3–)5–9(–10) × (2–)3–5(–6) µm (av. ± S.D. = 6.67 ± 0.08 × 3.7 ± 0.06 µm) and one-septate (5–)7–12(–16) × (2–)3–6(–7) µm (av. ± S.D. = 9.57 ± 0.12 × 4.34 ± 0.07 µm), smooth, prominent dark septum in the middle of the conidia, light brown to olivaceous.

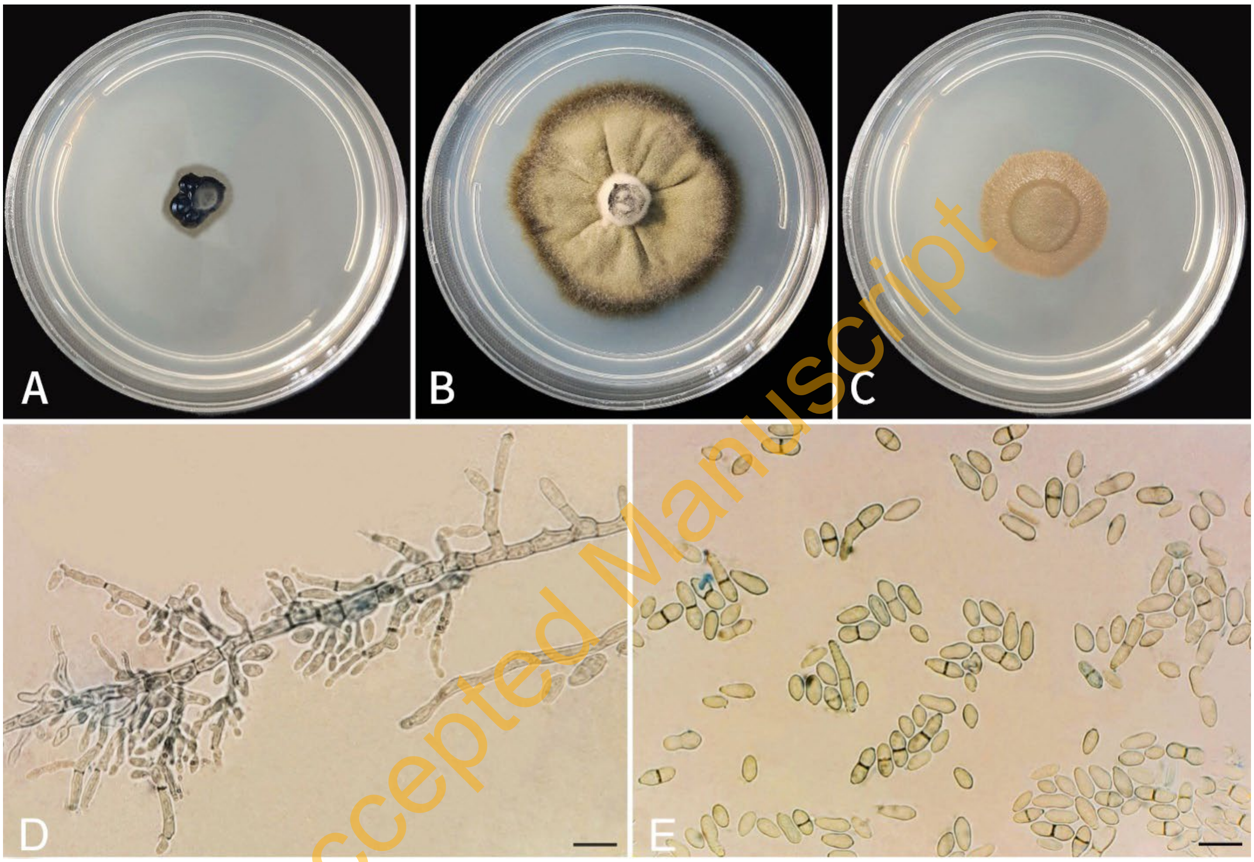

Fig. 3. *Hortaea werneckii* (IRAN 5472C). (A) Colony on PDA (7 days old), (B) Colony on PDA (21 days), (C) Colony on SDA (21 days) at 25ºC, (D) Conidiophores, (E) Conidia. Scale bars: (D, E) 10 µm.

Pathogenicity

The pathogenicity test showed that *H. werneckii* did not induce disease symptoms—such as necrosis or leaf spots—on *Avicennia marina* leaves (Fig. 4). These results confirmed that it is an endophyte fungus.

Effects of salinity and temperature stress

The diameter growth of *H. werneckii* colonies was measured across NaCl concentrations from 0 to 40% over 7, 14, and 28 days (Fig. 5). The results showed a salinity-dependent effect on mycelial growth. At 10% NaCl, a short lag phase was observed, with growth on days 7 and 14 lower than in the control, yet the fungus adapted and reached the control size by day 28. At 20% NaCl, growth was zero on day 7, indicating strong osmotic stress; growth resumed after day 7 but reached only 44.1% of the control by day 28, suggesting the fungus did not die but entered a longer adaptation phase. At higher salinities (30–40%), strong inhibition was evident. Despite these stresses, *H. werneckii* maintained its mycelial growth form across all salinity levels, including the hypersaline 20% NaCl condition (Fig. 6).

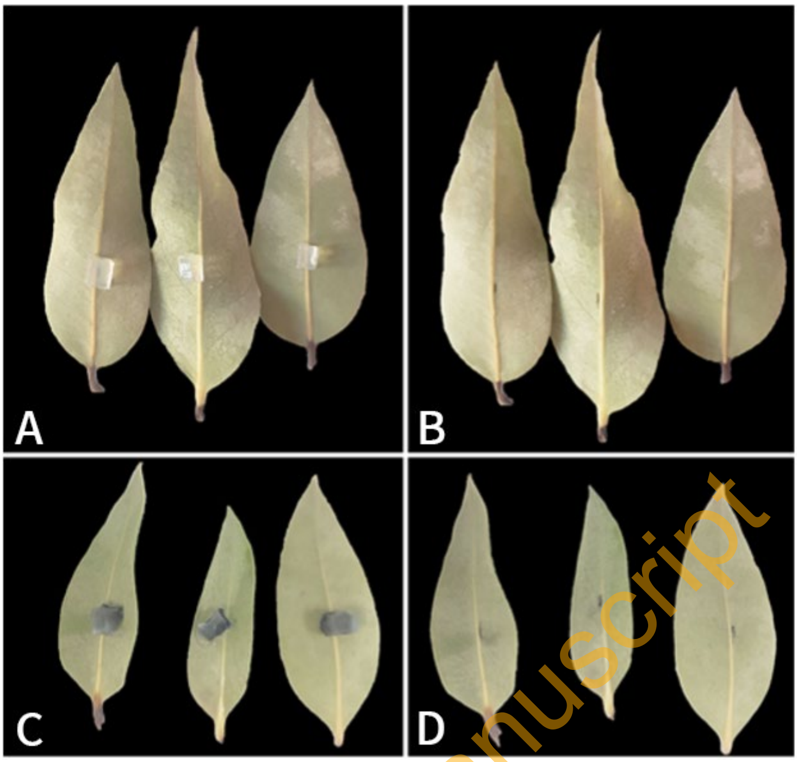

Fig. 4. The pathogenicity test of *Hortaea werneckii* on *Avicennia marina* leaves under in vitro conditions. Panels: (A–B) control leaves. (C–D) inoculated leaves. No disease symptoms were observed in inoculated leaves.

Thermal responses of the *H. werneckii* isolate were tested at 25°C, 35°C, and 45°C (Fig. 7). Growth was optimal at 25°C, with a maximal colony diameter of 43 mm observed by the end of the experiment. At 35°C, heat stress markedly reduced growth, yielding a colony diameter of 10.5 mm, a decrease of 75.5% from the 25°C maximum. At 45°C, growth was not observed (approximately 5 mm), indicating that this temperature exceeds the isolate's physiological tolerance range (Fig. 8).

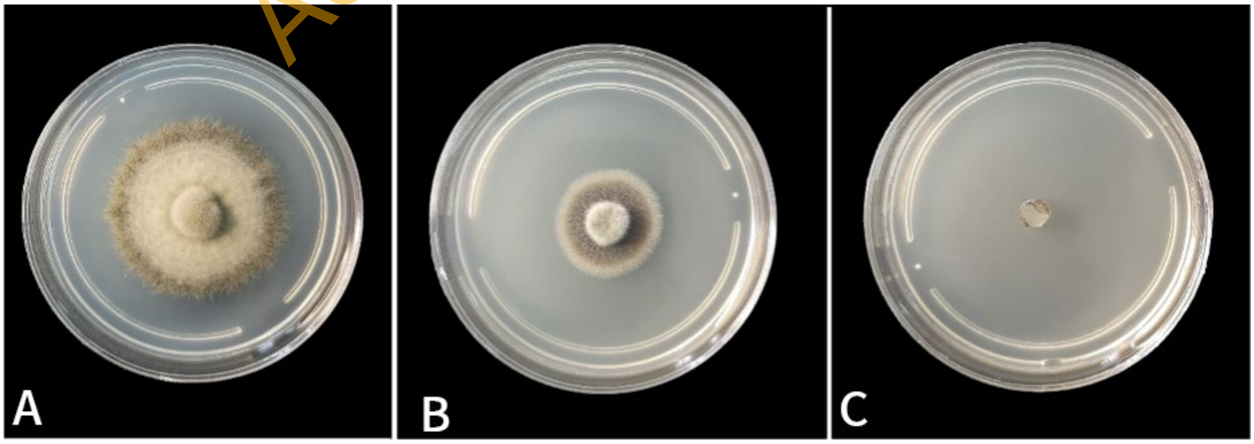

Fig. 5. Mycelial growth (mm) of *Hortaea werneckii* isolate across NaCl concentrations. (A) 10%, (B) 20%, (C) 30%.

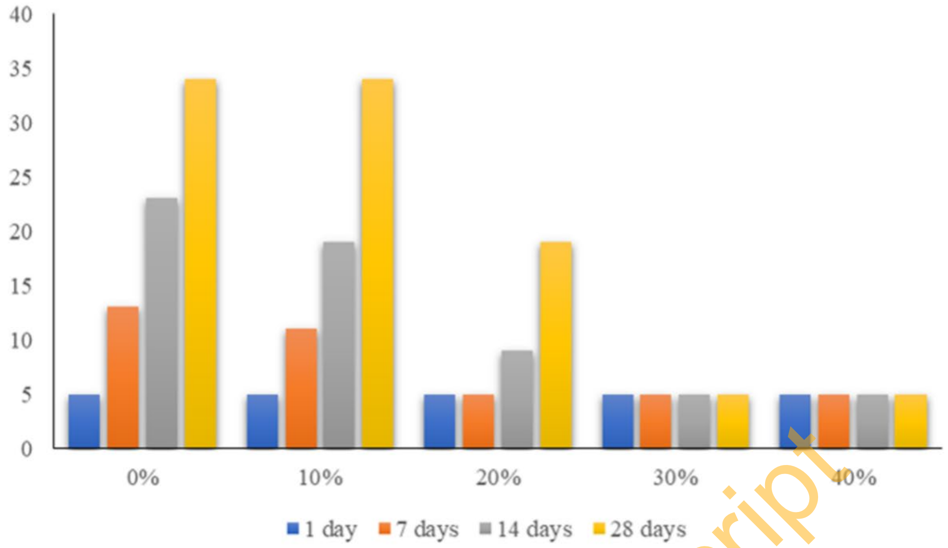

Fig. 6. Bar graph of fungal growth on different NaCl concentrations.

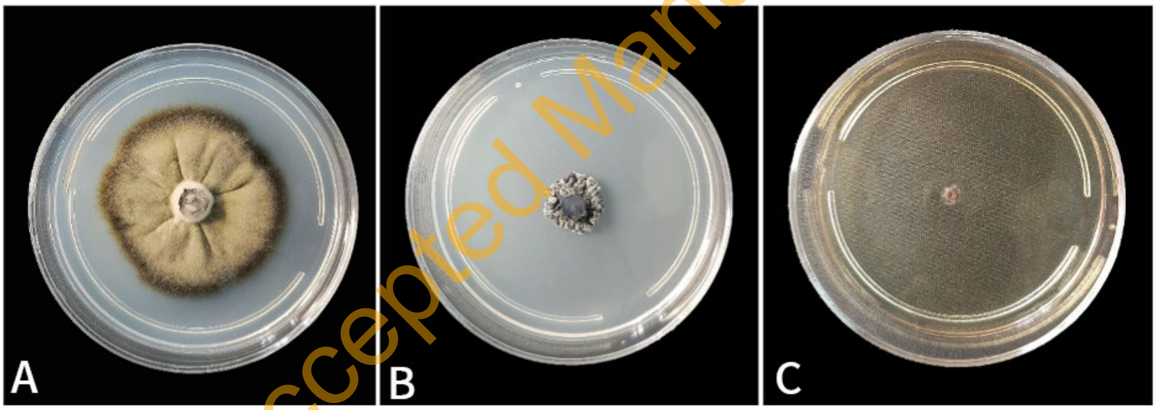

Fig. 7. Mycelial growth (mm) of *Hortaea werneckii* isolate at various incubation temperatures. (A) 25°C, (B) 35°C, (C) 45°C.

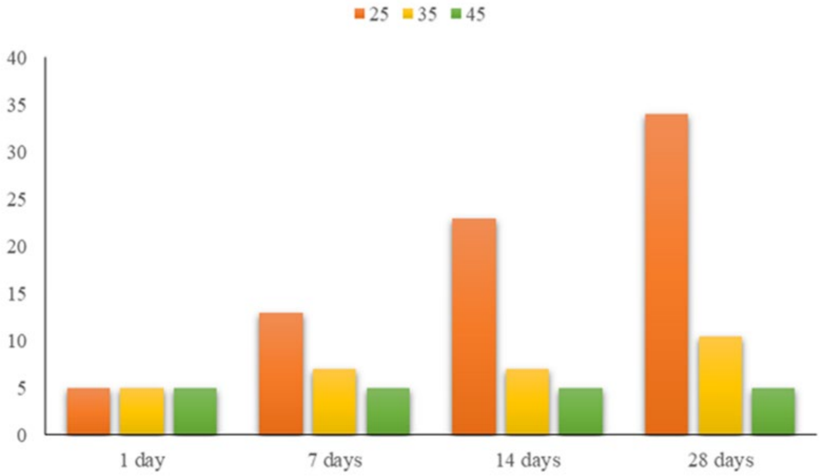

Fig. 8. Bar graph of fungal growth at different temperatures.

DISCUSSION

This study reports *H. werneckii* for the first time as an endophytic fungus associated with *Avicennia marina* obtained from the Qeshm region of Iran. The discovery expands the known range of the halophilic black yeast recorded from mangrove habitats in the South China Sea (Chen et al. 2012) and Saudi Arabia (Hodhod et al. 2020).

The dominance of *H. werneckii* in stem tissues (36.84%) indicates a high degree of tissue specificity. Recent work by Plemenitaš (2021) highlights that *H. werneckii* employs unique molecular strategies, including the High-Osmolarity Glycerol (HOG) signaling pathway, to regulate osmotic balance. By infecting the host from within, the fungus may help *A. marina* to tolerate the high salinity of the Persian Gulf through the synthesis of compatible solutes like glycerol and erythritol, which have been shown to alleviate osmotic stress in plants (Zalar et al. 2019, Wulandari et al. 2022).

The absence of pathogenic symptoms in our *in vitro* tests further supports the endophytic status of the Iranian *H. werneckii* strains. This association aligns with patterns observed among mangrove fungi, which have adapted to withstand extreme intertidal ecosystems. As Suryanarayanan and Ravishankar (2023) note, halotolerant endophytes in mangrove ecosystems are active participants rather than passive associates, contributing to the broader concept of ecosystem services by enhancing host resistance to abiotic stress.

The high level of halotolerance observed in our *H. werneckii* isolates, which maintained viability at 20% NaCl concentrations, supports its status as one of the most halotolerant eukaryotes recorded to date. This extreme tolerance not only enables survival in hypersaline conditions but also represents an evolutionary advantage in the hypersaline ecosystems of the Persian Gulf. Plemenitaš et al. (2014) describe a “strategy of flexibility” in *H. werneckii*, whereby adaptability in cell wall composition and ion transport mechanisms counteracts osmoregulatory challenges. In the mangrove endosphere, it is reasonable to hypothesize that such a fungal symbiont could play a role in salt exclusion from the host environment. However, recent evidence suggests that halotolerant endophytes in plants such as *Avicennia marina* may exhibit ABA-mediated signaling pathway modifications that enhance physiological adaptation to salinity within plant tissues. The successful adaptation of *H. werneckii* to 10–20% NaCl concentrations implies a sophisticated co-evolutionary relationship, potentially providing the plant with a biological buffer against sodium toxicity (Plemenitaš 2021, Verma et al. 2022).

The data show a growth optimum at 25°C, strong inhibition at 35°C, and complete inhibition at 45°C. The mesophilic nature of *H. werneckii* in a region with such high ambient temperatures in southern Iran suggests the existence of an ecological refuge, a refuge strategy that enables mesophilic fungi to maintain metabolic activity within the host even when external conditions exceed their physiological tolerance. Studies indicate that the interior of mangroves provides a microhabitat shielding its inhabitants from heat and UV radiation. The pronounced suppression of 75.5% at 35°C underscores the susceptibility of this symbiosis to global warming. Consequently, a continued rise in ambient temperature could impair the host plant’s ability to sustain cool internal conditions and, in turn, disrupt the fungal–plant interactions that are vital for mangrove health and stability (Suryanarayanan and Shaanker 2021).

CONCLUSION

The present study reports, for the first time, the documented presence of *Hortaea werneckii* as an endophytic fungus associated with *Avicennia marina* in the mangrove forest ecosystem of Iran. The results indicate that this black yeast is non-pathogenic and exhibits high salt tolerance, occupying a specialized ecological niche in the endosphere of mangrove forests in the Qeshm region. The observed high abundance of *H. werneckii* in stem tissues, together with its ability to grow in very high salinity conditions (up to 20% NaCl), suggests a strong evolutionary adaptation to the hypersaline environments of the Persian Gulf. In addition, the fungus’s optimal growth at 25°C and the suppression of growth at higher temperatures imply that the host plant may provide a protective thermal microclimate, enabling the fungus to persist despite extreme environmental temperatures.

In conclusion, the above association makes evident the robust nature of the mangrove mycobiome. Future studies should highlight the secondary chemical compounds produced by the Iranian isolate, as they have great potential for biotechnology applications, specifically to improve salt and heat resistance in important crop yields in the wake of global climate change.

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AUTHOR CONTRIBUTION

Zahra Cheraghi: Performed all *in Vitro* assays, Phylogenetic analyses, and Data visualization, and wrote the original draft manuscript; Khalil-Berdi Fotouhifar: Provided scientific comments and revised the manuscript; Vahid Etemad: Provided scientific comments and revised the manuscript; Samaneh Bashiri: Provided scientific comments, assisted in data analysis and revised the manuscript; Mohammad Javan-Nikkhah: Conceptualized and designed the study, coordinated the research, interpreted the results, and revised the manuscript. All authors reviewed the manuscript and agreed to the submission.

DATA AVAILABILITY

All datasets generated during this study are available from the corresponding author upon request.

FUNDING

This study was financially supported by the University of Tehran.

DECLARATION

In this manuscript, there is nothing to declare and the authors declare no conflicts of interest.

ETHICAL APPROVAL

Not applicable.

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مقاله کامل

ویژگیهای ریخت‌شناختی و مولکولی گونه *Hortaea werneckii*، یک قارچ اندوفیت روی درختان حَرّا (*Avicennia marina*) در ایران

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چکیده

جنگل‌های حَرّا اکوسیستم‌های پایداری هستند که در سواحل مناطق گرمسیری رشد می‌کنند و زیستگاهی مناسب برای گونه‌هایی مانند *Avicennia marina* فراهم می‌آورند، گونه‌ای که تحمل چشمگیری نسبت به تنش‌های محیطی دارد. این زیستگاه‌های وابسته به حَرّا از تولید متابولیت‌های زیست‌فعال پشتیبانی کرده و میزبان طیف متنوعی از قارچ‌های اندوفیت هستند که کاربردهای بالقوه‌ای در کشاورزی و توسعه دارویی دارند. قارچ‌های اندوفیت عوامل زیستی توانمندی هستند که می‌توانند عوامل بیماری‌زای گیاهی را مهار کنند، متابولیت‌های دارای اهمیت پزشکی تولید نمایند و آلاینده‌های محیطی را تجزیه کنند. از این رو، این قارچها نقش مهمی در کشاورزی پایدار و سلامت محیط زیست ایفا می‌کنند. هدف از پژوهش حاضر، جداسازی و شناسایی گونه‌های قارچی اندوفیت مرتبط با درختان حَرّا در منطقه قشم، استان هرمزگان، ایران بود. بافت‌های ریشه، ساقه و برگ ابتدا به‌صورت سطحی ضدعفونی شدند، به این ترتیب که در اتانول ۷۰ درصد به مدت ۳۰ ثانیه (برای برگ‌ها) یا یک دقیقه (برای ریشه‌ها و ساقه‌ها) قرار گرفتند، سپس در محلول رقیق‌شده هیپوکلریت سدیم رقیق شده دو درصد به مدت ۱/۵ دقیقه (برای برگ‌ها) یا ۳ دقیقه (برای ریشه‌ها و ساقه‌ها) تیمار شدند و در نهایت سه بار با آب مقطر استریل شستشو داده شدند. بافت‌های ضدعفونی‌شده روی محیط‌های کشت سیب‌زمینی دکستروز آگار (PDA) و آب آگار (WA) کشت شدند و قارچ‌های اندوفیت ظاهرشده پس از آن خالص‌سازی شدند تا جدایه‌های تک‌کنیدیومی به دست آید. شناسایی ریخت‌شناختی با استفاده از منابع استاندارد رده‌بندی انجام شد و شناسایی مولکولی بر اساس تعیین توالی ناحیه فاصله‌انداز رونویسی‌شده داخلی (ITS) در DNA ریبوزومی (rDNA) صورت گرفت. بررسی‌های ریخت‌شناختی و مولکولی نشان داد که ۹/۸ درصد از جدایه‌های برگ، ۳۶/۸۴ درصد از جدایه‌های ساقه و ۷/۱۴ درصد از جدایه‌های ریشه به جنس *Hortaea* تعلق دارند. همچنین، تحلیل فیلوژنتیکی تأیید کرد که این جدایه‌ها متعلق به گونه *Hortaea werneckii* هستند. بر اساس اطلاعات موجود، *H. werneckii* به‌عنوان یک گزارش جدید از جنگل‌های حَرّای ایران شناخته می‌شود و درخت حَرّا به‌عنوان گیاه میزبان آن معرفی می‌گردد.

واژگان کلیدی: بیماریزایی، تشخیص مولکولی، جنگلهای حَرّا، ریخت‌شناسی.

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