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Culture Medium-dependent Antifungal Activity of Endophytic *Deniquelata quercina* against *Sclerotinia sclerotiorum*

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ABSTRACT

Sclerotinia sclerotiorum is a destructive soilborne phytopathogen affecting agricultural crops. Endophytic fungi as valuable antimicrobial sources provide sustainable biological control strategies. The type of culture medium substantially affects the antifungal activity of endophytic fungi. For each production medium, we used a corresponding assay medium with the same basal composition. This study evaluated four liquid culture media for their effects on the antifungal activity of *Deniquelata quercina*, an endophytic fungus isolated from the roots of *Tamarix* sp., against *S. sclerotiorum*. *D. quercina* isolate 8B2B was recovered from healthy *Tamarix* roots in Khormoj, Bushehr Province, Iran. The antifungal activity of *D. quercina* was assessed in four liquid media (potato dextrose broth, tryptic soy broth, malt extract broth, and yeast extract broth) using the minimum inhibitory concentration (MIC) and the percentage of mycelial growth inhibition on days 4 and 7, following a completely randomized design with three replicates. The ITS sequence of *D. quercina* isolate 8B2B was deposited in GenBank under accession number OP710154. The MIC results showed that culture filtrates of *D. quercina* obtained from potato dextrose broth and tryptic soy broth media exhibited the greatest inhibitory activity against *S. sclerotiorum*, with a MIC of 3.12%, compared with 6.25% for malt extract broth and >50% for yeast extract broth. Culture filtrates obtained from PDB and TSB media exhibited the highest inhibitory activity, with an MIC of 3.12%. In contrast, the filtrate obtained from malt extract broth (MEB) medium had an MIC of 6.25% and ranked next. The culture filtrate produced in yeast extract broth (YEB) medium showed no observable inhibitory effect on the growth of *S. sclerotiorum* up to a concentration of >50%. In the mycelial growth inhibition assay, potato dextrose broth showed the highest inhibition on day 7, with a mean of 99.60%, followed by tryptic soy broth at 94.05%. MEB and YEB demonstrated inhibition rates of 19.05% and 10.71%, respectively. To the best of our knowledge, this study provides the first evidence of antifungal activity of *D. quercina* against *S. sclerotiorum*. Moreover, our findings reveal a marked dependence of this activity on the culture medium, with potato dextrose broth yielding the most pronounced inhibitory effects. These results emphasize that optimization of fermentation conditions is essential for the reliable screening and development of endophytic fungi as potential candidates for further biocontrol evaluation.

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Introduction

Sclerotinia species cause important diseases in a wide range of economically significant agricultural crops. Among them, *Sclerotinia sclerotiorum* (Lib.) de Bary is recognized as one of the most destructive, and widespread plant-pathogenic fungi. *Sclerotinia* species cause severe damage to agricultural crops largely due to the absence of effective genetic resistance, their broad host range, and the limited success of

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agronomic and chemical control strategies. This pathogen causes economic losses primarily through complete crop loss and unmarketability in vegetables, and through reduced seed number, weight, and quality in cereals and oilseed crops. Economically important crops commonly affected by *S. sclerotiorum* include oilseed crops (canola, sunflower) and legumes (soybean, peanut, chickpea, bean), in which infection often causes substantial yield reduction and production losses (O'Sullivan *et al*., 2021). Effective control of *S. sclerotiorum* remains difficult owing to its soilborne nature, the lack of safe and reliable soil disinfection strategies, and a distinctive life cycle centered on the formation of sclerotia, which are highly resistant to chemical and biological degradation. Furthermore, the absence of effective chemical compounds capable of eliminating sclerotia from the soil, combined with the adverse environmental consequences of excessive fungicide use, has directed research attention toward biological control methods. Accordingly, numerous studies have examined the potential of biological control agents for managing *Sclerotinia* species (Smolińska and Kowalska, 2018). Biological control agents, owing to their diverse modes of action, can act as alternatives or complements to chemical fungicides. Their use not only reduces dependence on chemical inputs but also helps preserve fungicide efficacy over time and limits the emergence and spread of resistant pathogen populations (O'Sullivan *et al*., 2021). In this context, endophytic fungi, as valuable and emerging sources of bioactive compounds, have attracted considerable research attention. These microorganisms, by producing a broad range of antimicrobial metabolites without exerting harmful effects on the host plant, represent a promising option for controlling plant pathogens. Endophytic fungi colonize the living internal tissues of plants without causing disease and establish a stable symbiotic relationship with their host (Eo *et al*., 2022; Senanayake *et al*., 2022). Endophytic fungi contribute to plant-microbe interactions in several important ways, including enhancing rhizosphere nutrient uptake, promoting plant growth and development, providing biological control against pests and pathogens, inducing systemic resistance to biotic and abiotic stresses, and biosynthesizing secondary metabolites (Jha *et al*., 2023). These fungi produce a wide array of secondary metabolites that confer diverse biological activities, including antimicrobial, anticancer, antioxidant, anti-inflammatory, and antidiabetic effects (Gupta *et al*., 2023). As a result, the high biodiversity and bioactive compound-producing capacity of endophytic fungi have positioned them as valuable resources for developing new, effective, and safe compounds.

Given the role of endophytic fungi in the biological control of plant pathogens without harming the host plant, humans, or the environment, screening and identifying the bioactive metabolites produced by these fungi is of particular importance. These fungi represent a sustainable approach for managing plant diseases, as they are able to colonize internal plant tissues without causing disease while producing diverse antimicrobial compounds. Despite extensive research on endophytic fungi across diverse plant species, little is known about the antagonistic potential against plant pathogens of those associated with plants adapted to Iran's arid and desert regions. Because of their adaptation to environmentally stressful conditions, such fungi are expected to possess distinctive metabolic pathways and to produce valuable bioactive metabolites. Alidadi *et al*. (2019) isolated *Deniquelata quercina* (family Didymosphaeriaceae) as an endophyte from the branches of Persian oak (*Quercus brantii*) in the Zagros forests of Iran and described it as a new species based on morphological and phylogenetic data. The antimicrobial activity of *D. quercina* has not been previously investigated. In the present study, this fungus was isolated as an endophyte from the desert plant *Tamarix* sp., a genus of trees and shrubs comprising about 23 species, roughly 10 of which grow in south-eastern Iran (Arianmanesh *et al*., 2016). Highly long-lived, it withstands harsh conditions such as heat, soil salinity, and drought, tolerating saline soils by excreting excess salt through its leaves (Long *et al*., 2021). The genus is valued for controlling erosion, serving as a windbreak, and providing a traditional herbal remedy (Xia *et al*., 2015). Its biological and environmental traits reflect strong adaptability and stress resistance (Long *et al*., 2021), enabling it to thrive in saline, drought-prone, hot environments and making it a

dominant species in arid and semi-arid regions (Xia *et al*., 2015; Rouhi-Moghaddam *et al*., 2015; Ohrtman *et al*., 2012).

Antifungal activity of endophyte filtrates is known to be culture medium-dependent and has been evaluated in other species, but this has never been tested for *D. quercina*. Therefore, the type of culture medium substantially affects the antifungal activity of endophytic fungi. Accordingly, the present study evaluated, for the first time, the effect of four different culture media on the antifungal activity of the endophytic strain *D. quercina*, isolated from *Tamarix* sp., against the important plant pathogen *S. sclerotiorum*.

Materials and Methods

Isolation of endophytic fungi

The endophytic fungal isolate 8B2B of *D. quercina* was collected and isolated from the roots of healthy, asymptomatic *Tamarix* sp. plants in the Khormoj region, Bushehr Province, Iran (geographic coordinates 524735.06 E, 3188075.42 N) in May 2018. Root samples were collected from healthy plants and immediately cut into 1.5 cm segments. Root segments were washed under gently running tap water for 20 min. Surface sterilization of the samples was then performed in three steps: 1) immersion in 70% ethanol for 1 min; 2) immersion in 2% sodium hypochlorite solution (40 mL sodium hypochlorite and 60 mL sterile distilled water) for 3 min; and 3) a second immersion in 70% ethanol for 30 s. After three rinses with sterile distilled water and drying on sterile paper towels, the root segments were cut with sterile scissors, and the outer surface was removed with a scalpel blade. The segments were then briefly flame-passed, and the internal root tissue was placed on potato dextrose agar (PDA) containing inhibitory compounds, 250 µL of the surfactant TERGITOL type TMN-6 (to reduce the growth of fast-growing fungi), 0.01 g rifampicin, and 0.5 g ampicillin per liter of medium (to prevent bacterial growth). Six plant segments were placed in each 8 cm-diameter Petri dish. Plates were incubated at 25°C in darkness for 1- 3 weeks. After fungal colonies appeared, fragments of approximately 5 mm showing fungal growth were transferred to 2% water agar (WA). Isolates were purified using the hyphal-tip technique, whereby hyphal tips were transferred to fresh PDA and incubated at 25°C for 7- 10 days (Bakhshi *et al*., 2023). To evaluate the efficiency of surface sterilization, two separate controls were included: in the first, surface-sterilized plant tissue segments were placed directly on PDA; in the second, an aliquot of the final rinse water (from the third washing step) was plated on PDA. The absence of fungal growth in these controls confirmed the effectiveness of the surface sterilization procedure. After identification, purified isolates were preserved on sterile filter paper at -20°C and as PDA slants at 4°C. Purified fungal isolates were deposited and maintained in the Mycological Culture Collection of Shiraz University.

Morphological identification

Isolate 8B2B was first grown on PDA (Potato dextrose agar) for one week at 25 °C in darkness. Agar plugs 5 mm in diameter were then cut from the actively growing colony margin and transferred to the center of plates containing PDA, Oatmeal agar (OA), and Malt extract agar (MEA). Morphological studies of isolate 8B2B were carried out at 27°C. Colony diameter and appearance- including color, texture, margin shape, and surface and reverse views - were recorded on days 7 and 14 after inoculation. To induce the formation of sexual structures, cultures were placed on water agar (WA) supplemented with pine needles and incubated for three months under a 12 h light/12 h dark photoperiod. After ascomata formation, the morphological characteristics of the sexual structures - ascomata, asci, and ascospores were examined.

For microscopic (Light microscope; Carl Zeiss Axiophot, Germany) studies, samples were taken from 7- to 10-day-old colonies grown on MEA as well as from ascomata formed on WA, and microscope slides were prepared. All microscopic observations and measurements were carried out accordingly. Morphological characteristics recorded included, for ascomata: shape, size, color, ostiole type, and peridium structure; for asci: shape, size, number of ascospores, wall type, and stipe characteristics; and for ascospores: shape, size, color, number, and arrangement of transverse and longitudinal septa. No asexual structures were observed in the studied isolate.

Molecular identification and phylogenetic analysis

Genomic DNA was extracted from lyophilized fungal mycelium. Briefly, 400 µL of extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 150 mM NaCl; pH 7.5- 8) was added to the mycelium in a porcelain mortar and thoroughly ground. The resulting mixture was transferred to a microtube, vortexed for 10 s, and incubated at 65°C in a water bath for 15 min. Following incubation, 400 µL of chloroform: isoamyl alcohol (24:1) was added, and the mixture was held on ice for 10 min. Samples were then centrifuged at 10,000 rpm for 10 min at 4°C. The upper aqueous phase was transferred to a new microtube, and an equal volume of isopropanol was added. After centrifugation at 12000 rpm for 10 min, the DNA pellet was washed with 70% ethanol, air-dried, and dissolved in 30 µL of sterile deionized water. The quality and quantity of the extracted DNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA) at 260 nm. The internal transcribed spacer (ITS) region was amplified using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990). PCR reactions were carried out in a final volume of 25 µL, containing 10 µL of Master Mix RED (Amplicon, Denmark; containing Taq DNA polymerase, buffer, 1.5 mM $MgCl_2$, and dNTPs), 1 µL of each primer (10 pmol), 2 µL of template DNA (~20 ng), and 11 µL of sterile deionized water. The PCR thermal program consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 40 s, primer annealing at 58°C for 40 s, and extension at 72°C for 40 s, with a final extension step at 72°C for 10 min. PCR reactions were carried out using a CG1-96 thermocycler (Corbett Research, Australia). After confirming amplicon size on a 1% agarose gel, PCR products were sent to Cardiogenetic Company (Tehran, Iran) for purification and sequencing. After receipt of the raw sequence files (ab1 format), chromatogram quality was checked using Chromas software ver. 2.33, and sequences were edited and corrected using Geneious software ver. 4.8.5. Nucleotide sequence similarity of the ITS region of the studied isolate was assessed using the BLASTn tool against the GenBank database. The ITS sequence of *D. quercina* isolate 8B2B was deposited in GenBank under accession number OP710154. Multiple sequence alignment was performed using the MUSCLE algorithm in MEGA software (version 11). A phylogenetic tree was constructed using the Neighbor-Joining method with a bootstrap test of 100 replicates, using CLC Genomics Workbench software (ver. 3.6.5). *Didymosphaeriaceae* sp. (accession number KJ780753.1) was selected as outgroup of rooting the phylogenetic tree.

Preparation of culture filtrate from endophytic fungi

To evaluate antifungal activity, the endophytic isolate *D. quercina* 8B2B was cultured separately in four liquid media - potato dextrose broth (PDB), tryptic soy broth (TSB), malt extract broth (MEB), and yeast extract broth (YEB). We measured/adjust of the media pH 7.0. A 5 mm mycelial plug taken from the actively growing margin of 7-day-old colonies was transferred to 250 mL Erlenmeyer flasks containing 100 mL of each liquid medium. Flasks were incubated for 14 days at 26°C on a rotary shaker incubator at 90 rpm in darkness. After the fermentation period, the culture containing the mycelial mass was first centrifuged at high speed (7341× g; 2236R, LABOGENE, South Korea) for 20 min and then vacuum-filtered through Whatman No:1 filter paper (Whatman, UK). To ensure complete removal of cellular debris and fungal fragments, the filtrate was further sterile-filtered through a 0.45 µm Minisart NML syringe filter (Sartorius, Germany). The resulting solution was used as the fungal culture filtrate in subsequent bioassays.

Inhibition of *S. sclerotiorum* mycelial growth

The antifungal activity of endophytic isolate 8B2B *D. quercina* against the phytopathogenic fungus *S. sclerotiorum* (KACC 40457, obtained from the Korean Agricultural Culture Collection) was evaluated. For the bioassay, 2 mL of the endophyte culture filtrate was mixed with 18 mL of autoclaved medium (prior to solidification) and poured into 9 cm petri dishes. A 6 mm mycelial plug of *S. sclerotiorum*, taken from an actively growing culture on PDA, was placed at the center of the solidified medium containing the endophyte culture filtrate. The assay was performed in four different culture media: tryptic

soy agar (TSA), PDA, MEA, and yeast extract agar (YEA). Control petri dishes without addition of the endophyte culture filtrate served as controls. Controls consisted of the corresponding uninoculated assay media (PDA, TSA, MEA, and YEA) without fungal filtrate.

All treatments and controls were performed in three replicates (three separate Petri dishes per treatment). Plates were incubated for 7 days at 26°C in darkness.

The colony diameter of the pathogen was measured on days 4 and 7 after incubation. The percentage of mycelial growth inhibition of *S. sclerotiorum* was calculated using the following formula (Chen *et al*., 2011): Mycelial growth inhibition (%)= [(Dck - D)/ (Dck- 6)] × 100 (Equation 1); where Dck represents the mean colony diameter of the control, D represents the mean colony diameter of the treatment, and 6 represents the diameter of the inoculum plug (in millimeters).

Determination of MIC

The MIC assay was performed using the broth microdilution method in 96-well plates. To prepare the culture filtrate, endophytic isolate *D. quercina* 8B2B was grown in the different liquid media - TSB, PDB, MEB, and YEB. Two-fold serial dilutions of the culture filtrate were prepared using the corresponding sterile medium. The final concentrations tested were 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78%, with the undiluted culture filtrate considered as the 100% concentration. The inoculum of *S. sclerotiorum* KACC 40457 was prepared by growing the fungus in liquid PDB medium at 26°C with shaking at 100 rpm for 3- 5 days. The resulting culture containing mycelial fragments was used as the mycelial suspension. This suspension was diluted 1:5 in sterile PDB medium and thoroughly vortexed to achieve a uniform suspension of mycelial fragments.

To standardize the mycelial inoculum, the mycelial culture was homogenized using a sterile tissue grinder to obtain a uniform suspension of mycelial fragments. The suspension was filtered through sterile gauze and adjusted visually to a turbidity of approximately McFarland standard 0.5, then diluted 1:5 in sterile PDB medium. The final diluted suspension was used as the pathogen inoculum. Subsequently, 100 µL of the pathogen suspension was added to each well of a 96-well plate. Then, 100 µL of the various dilutions of the endophyte culture filtrate were added to the wells, giving a final volume of 200 µL per well. Plates were incubated for three days at 26°C. After the incubation period, the lowest concentration of the endophyte culture filtrate that resulted in no visible growth of the pathogen, compared with the negative control, was recorded as the MIC value (Tahmasebi *et al*., 2025).

Growth was assessed visually by comparing each well against the negative control (pathogen inoculum without filtrate). The following controls were included in each 96-well plate: 1) negative control (pathogen inoculum in sterile medium without filtrate) to confirm pathogen viability and growth; 2) sterility control (sterile medium only) to confirm medium sterility; 3) filtrate sterility control (filtrate dilutions without pathogen) to confirm that the culture filtrate was free of viable fungal cells; and 4) medium blank control (sterile medium only) for visual comparison. All controls were tested in triplicate.

Statistical analysis

All experiments were conducted in a completely randomized design (CRD) with three independent biological replicates. Data on the percentage of mycelial growth inhibition were statistically analyzed using SPSS software (version 27; IBM, USA). Data on the percentage of mycelial growth inhibition were statistically analyzed using SPSS software (version 27; IBM, USA). Prior to one-way analysis of variance (ANOVA), the assumptions of normality and homogeneity of variances were verified. Normality was assessed using the Shapiro-Wilk test ($p > 0.05$ for all groups), and homogeneity of variances was confirmed using Levene's test ($p > 0.05$ for all groups).

Once these assumptions were met, one-way ANOVA was performed. When significant differences among treatment means were detected, multiple comparisons were carried out using Tukey's honestly significant difference (HSD) test, and statistically homogeneous groups were denoted by similar letters. The significance level for all statistical tests was set at $p < 0.05$. Results are reported as mean± standard deviation (SD).

Results

Isolation, identification, and morphological characteristics of the endophyte

In this study, endophytic fungal isolate 8B2B was recovered from the roots of *Tamarix* sp. in the Khormoj region, Bushehr Province. *Tamarix* grows as a shrub, typically occurring naturally in hot, arid desert regions. The absence of any fungal colony growth in control plates confirmed the effectiveness of the surface sterilization procedure. Based on combined morphological and molecular data, isolate 8B2B was identified as *D. quercina* (order Pleosporales, family Didymosphaeriaceae). The corresponding sequence was deposited in GenBank under accession number OP710154. BLASTn analysis against the NCBI GenBank database (accession number OP710154) revealed 99.71% nucleotide identity with isolate 20SA *Deniquelata* sp. (GenBank accession number MH316153.1) that was shown in figure 1. To the best of our knowledge, this study represents the first report of *D. quercina* occurring as an endophytic fungus in the host plant *Tamarix* sp.

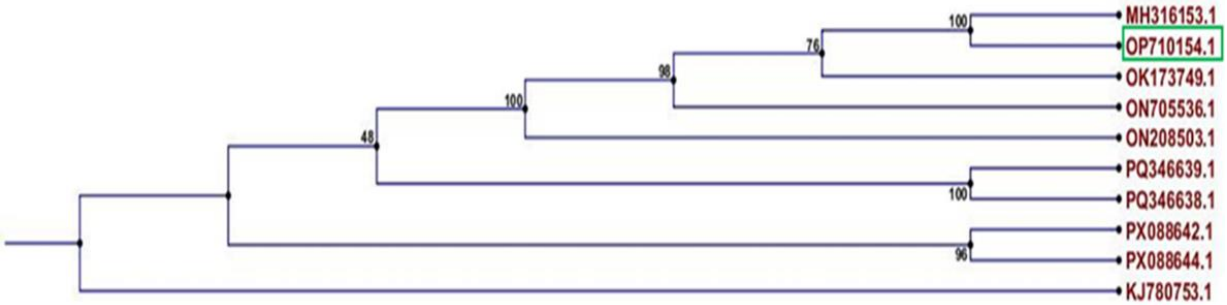

Fig. 1. Phylogenetic tree based on the nucleotide sequence of the ITS region of the fungal isolate *D. quercina* 8B2B (accession number OP710154). Nucleotide sequences were retrieved from the NCBI database. The phylogenetic tree was constructed using the Neighbor-Joining method with a bootstrap test of 100 replicates, using CLC Genomics Workbench software (version 3.6.5). Numbers at the tree nodes indicate bootstrap support values for the corresponding branches.

Morphological characteristics of isolate 8B2B of *D. quercina*

After 7 days of incubation at 27°C, colonies on PDA, MEA, and OA media reached diameters of 60, 72, and 55 mm, respectively. After 14 days, colonies on the different media differed in color and texture. On PDA, colonies were circular, cottony to floccose, with a white center and a gray to light brown margin; the colony reverse was yellow to light brown. On MEA, colonies had a velvety to floccose texture and were grayish-olive to olive-brown in color, with a light brown reverse. On OA, colonies were white to very pale gray, with a brown center and a white margin. The sexual stage formed after approximately three months on pine needles under a 12 h light/12 h dark photoperiod. Ascomata were solitary, globose to subglobose, dark brown to black, covered with mycelial hyphae, and possessed a central ostiole, measuring 155-335× 130-320 µm (n= 20). At maturity, a dark mass of ascospores was extruded through the ostiole. The peridium was multilayered (more than 10 layers), 30-50 µm thick at the lateral wall and up to 75 µm thick at the apical region (n= 20), with an outer layer composed of thick-walled, brown to black cells of the textura angularis type, and an inner layer composed of hyaline, thin-walled cells. The hamathecium consisted of dense, hyaline, septate pseudoparaphyses embedded in a gelatinous matrix, 1-2 µm in diameter (n= 20). Asci were eight-spored, bitunicate and fissitunicate, cylindrical to cylindrical-clavate, with a rounded apex, measuring 80-160× 13-20 µm (mean 115.3× 16.5 µm, n= 30). Ascospores were muriform, rectangular, ellipsoidal to ovoid or fusiform, straight or slightly asymmetric, measuring 17-23× 10-14 µm (mean 20 × 11.2 µm, n= 30); they were initially hyaline, then yellow to yellowish-brown, and finally reddish-brown to black at maturity. Ascospores typically had three transverse septa (rarely four) and one to three longitudinal septa, with slight constriction at the septa. No asexual morph was observed under the conditions of this study (Fig. 2).

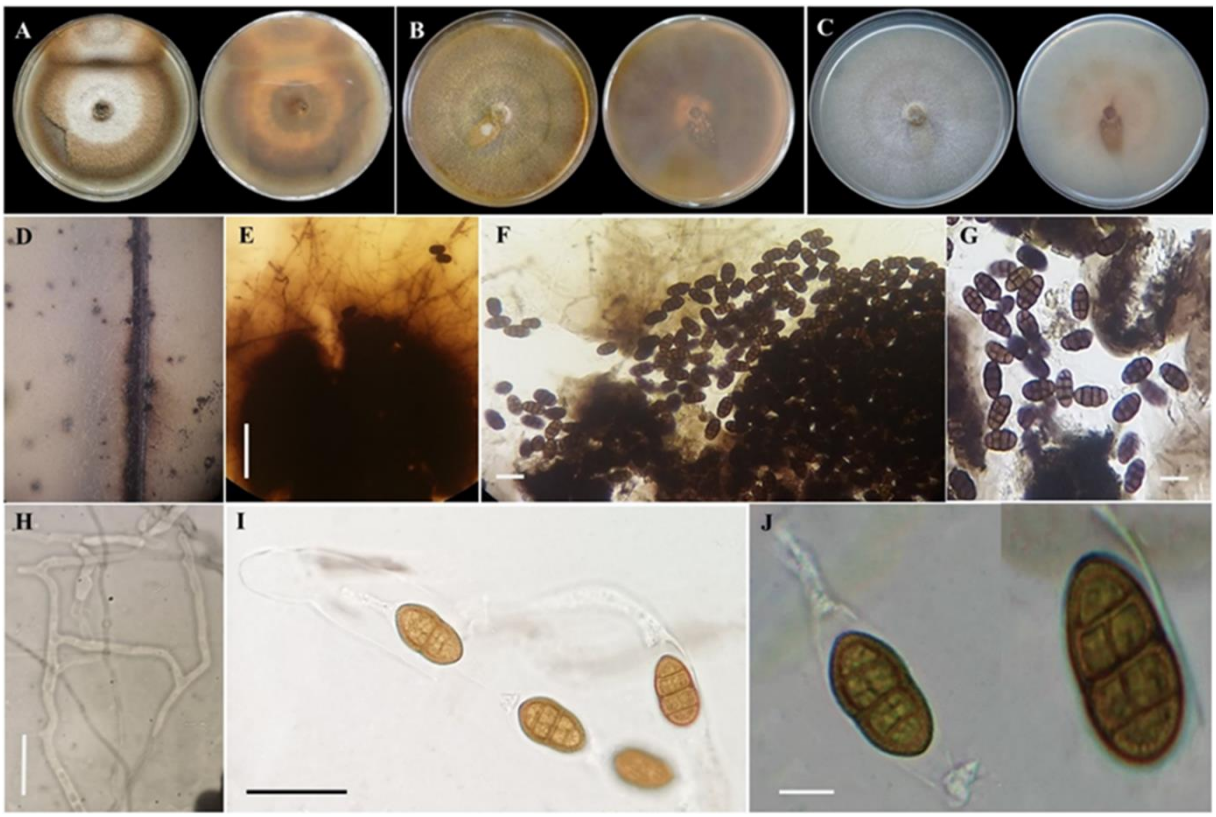

Fig. 2. Morphological characteristics of *D. quercina* isolate 8B2B: A- C) Surface and reverse views of 14-day-old colonies on PDA, MEA, and OA, respectively, incubated at 27°C in darkness; D) Ascomata formed on pine needles after 3 months under a 12 h light/12 h dark photoperiod;.E) Ascoma; F) Extruding ascospores; G) Section of the peridium with muriform ascospores; H) Hyaline pseudoparaphyses; I) Ascus with mature ascospores; J) Muriform ascospores. Scale bars: E)= 100 µm; F and G)= 5 µm; H)= 50 µm; I)= 20 µm; J)= 10 µm.

MIC of *S. sclerotiorum*

The MIC results showed that the type of culture medium used to prepare the endophyte culture filtrate affected antifungal activity against *S. sclerotiorum*. Culture filtrates obtained from PDB and TSB media exhibited the highest inhibitory activity, with an MIC of 3.12%. In contrast, the filtrate obtained from MEB medium had an MIC of 6.25% and ranked next. The culture filtrate produced in YEB medium showed no observable inhibitory effect on the growth of *S. sclerotiorum* up to a concentration of >50% (Table 1).

Table 1. MIC of endophyte culture filtrates (8B2B) against *S. sclerotiorum* in different culture media.

Culture medium (Ingredients)	MIC of 8B2B (%)
Potato dextrose broth (Dextrose, 20 g/L, Potato Extract, 4 g/L; Merck)	3.12
Malt extract broth (Malt extract 17g/L; Merck)	6.25
Yeast extract broth (6 g/L Tryptone, 3 g/L Yeast; Merck)	> 50%
Tryptic soy broth (Peptone from casein 17.0g/L; peptone from soymeal 3.0g/L; D (+) glucose monohydrate 2.5g/L; sodium chloride 5.0g/L; di-potassium hydrogen phosphate 2.5g/L; Merck)	3.12

Percentage of mycelial growth inhibition

Results of the mycelial growth inhibition assay on day 4 showed that culture filtrates obtained from PDB and TSB media, with a mean inhibition of 100%, exhibited the greatest antifungal activity against *S. sclerotiorum*. These were followed by MEB and YEB media, with mean inhibition values of 83.33% and 78.57%, respectively. On day 7, PDB medium showed the highest inhibition of pathogen growth, with a mean of 99.60%, followed by TSB medium at 94.05%. No significant difference was observed between the PDB and TSB treatments, but both differed

significantly from MEB (19.05%) and YEB (10.71%) media (Tukey's HSD, $p < 0.05$). Images corresponding to the extent of mycelial growth inhibition on days 4 and 7 are presented in figures 3, 4 and 5. As shown, treatments containing the culture filtrate of isolate 8B2B of *D. quercina* produced in PDB and TSB media resulted in the highest suppression of pathogen mycelial growth (Fig. 4 and 5). On day 4, growth inhibition in the PDB and TSB treatments was nearly complete, whereas by day 7 only slight regrowth was observed.

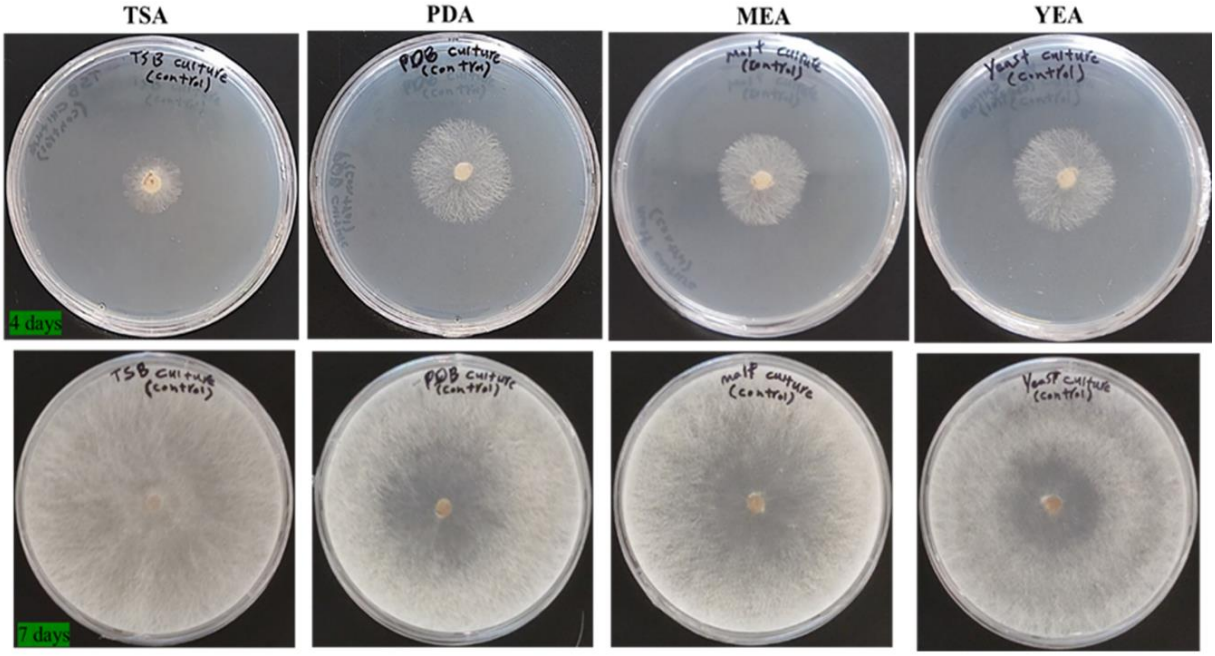

Fig. 3. Growth of the fungus *S. sclerotiorum* on various culture media at 4 and 7 days after inoculation (TSA= tryptic soy agar; PDA= potato dextrose agar; MEA= malt extract agar; YEA= yeast extract agar).

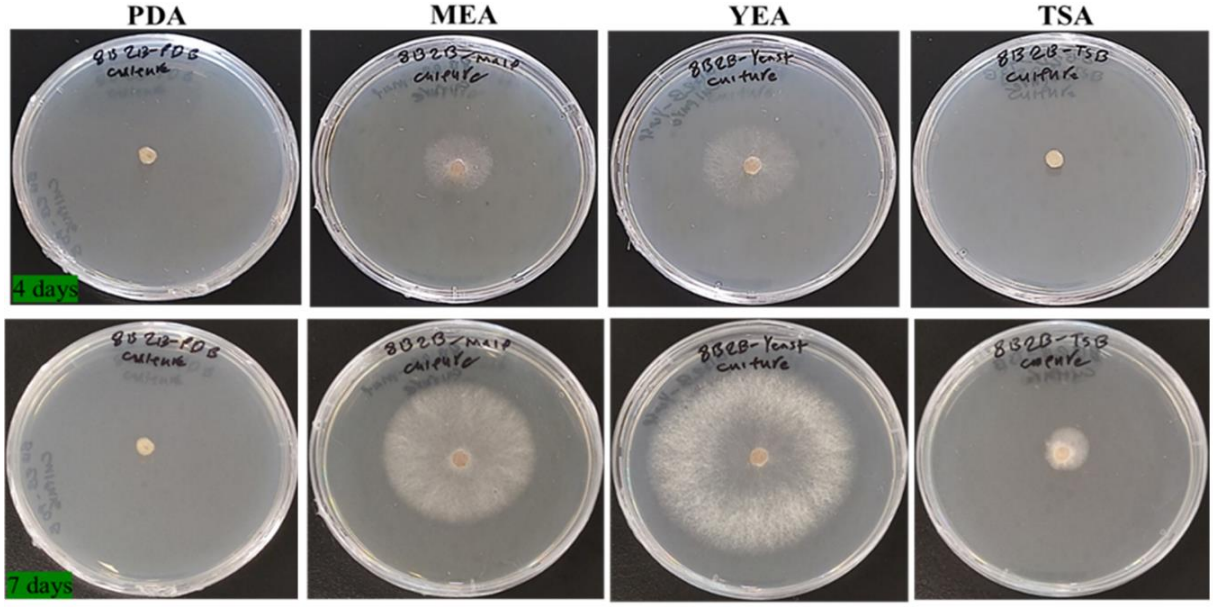

Fig. 4. Growth of the fungus *S. sclerotiorum* on various culture media containing the culture filtrate of *D. quercina* isolate 8B2B, at 4 and 7 days after inoculation: TSA= tryptic soy agar; PDA= potato dextrose agar; MEA= malt extract agar; YEA= yeast extract agar.

Discussion

The results of this study showed that the endophytic isolate *D. quercina* 8B2B possesses notable antifungal activity against *S. sclerotiorum*, and that culture medium composition plays an important role in the magnitude of this activity. Among the media tested, PDB and TSB were identified as the media most conducive to the antifungal activity of this isolate's culture filtrates. MIC results showed that PDB and TSB media had the lowest MIC values (3.12%) and exhibited the highest inhibitory activity. In contrast, MEB and YEB media had MIC values of 6.25% and > 50%, respectively, indicating a weaker antifungal effect of the culture filtrates obtained in these media. Likewise, results of the mycelial growth inhibition assay on day 7 after inoculation showed that PDB and TSB treatments, with mean inhibition of 99.60% and 94.05%, respectively, outperformed MEB (19.05%) and YEB (10.71%) media. The consistency between the two independent assays indicates that the observed antifungal activity is attributable to a genuine effect of culture medium composition, which strengthens the reliability of the present findings.

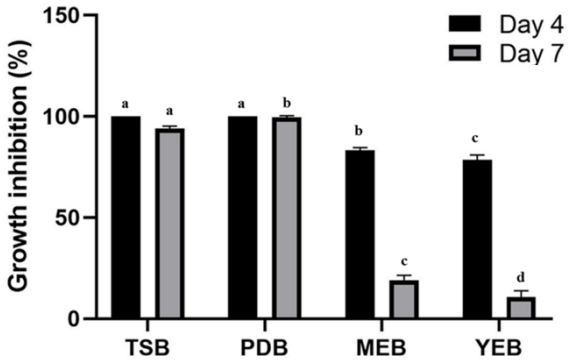

Fig. 5. Inhibition of *S. sclerotiorum* mycelial growth by the culture filtrate of endophyte 8B2B in various culture media (4- and 7-days post-inoculation): To evaluate the effect of the culture medium on the percentage of inhibition, data from days 4 and 7 were analyzed independently using one-way ANOVA followed by Tukey's post-hoc test (at $p < 0.05$). Different letters above the bars within each daily group indicate a statistically significant difference between the various culture media on the same day. Error bars represent the standard deviation of three technical replicates (n= 3). PDB, potato dextrose broth; TSB, tryptic soy broth; MEB, malt extract broth; YEB, yeast extract broth.

The differences in antifungal activity observed among the culture media are likely related to differences in nutrient composition, carbon and nitrogen sources, and other factors known to influence fungal metabolism (Ruiz *et al*., 2010; Sánchez *et al*., 2010; Chen *et al*., 2011). The observed differences cannot be attributed solely to the production medium because the pathogen was simultaneously exposed to different basal agar media. Also, no metabolite was extracted, quantified, or chemically characterized in this study, these results reflect differences in the antifungal activity of the crude culture filtrates rather than direct evidence of differential production of specific antimicrobial compounds. The comparatively higher activity of *D. quercina* 8B2B filtrates from PDB and TSB media is therefore consistent with, but does not directly confirm, more favorable nutritional conditions for antifungal metabolite biosynthesis in this isolate.

Changes in the type or concentration of nutrients in the culture medium can alter the regulatory pathways associated with the production of bioactive compounds. Different carbon sources such as glucose, glycerol, maltose, mannose, sucrose, and xylose have been reported to affect the production of antimicrobial compounds (Ruiz *et al*., 2010; Sánchez *et al*., 2010; Majerčíková *et al*., 2015). In the present study, MEB medium (containing glucose and maltose) and YEB medium yielded the lowest antifungal activity of endophyte *D. quercina* 8B2B against *S. sclerotiorum*. In one study, replacing glucose with fructose or galactose induced or enhanced antimicrobial activity against nearly all pathogens tested, although the magnitude of this effect depended on the actinomycete strain and target species; in contrast, no antimicrobial activity was observed when glucose was the sole carbon source (Ait Assou *et al*., 2025). This pattern is not fully consistent with our results, however, since PDB and TSB - the media associated with the highest antifungal activity in the present study - are also formulated with a glucose-based sugar (dextrose). We therefore do not attribute the observed differences specifically to glucose or dextrose content; the pattern more likely reflects the combined influence of several nutritional factors (*e.g.*, carbon and nitrogen source type and concentration) rather than any single component, and clarifying its precise chemical basis will

require future studies involving extraction and identification of the active metabolites.

The genus *Deniquelata* has so far received relatively little attention with regard to its biological activities; this study represents, to the best of our knowledge, the first report of antifungal activity of *D. quercina* against *S. sclerotiorum*, as well as the first report of this species occurring as an endophyte in *Tamarix* sp. Members of the order Pleosporales are among the most diverse fungi within the phylum Ascomycota and are capable of producing a wide range of secondary metabolites (Cao *et al*., 2017; Lai *et al*., 2020). The results of this study can therefore serve as a basis for further investigation of the biotechnological potential of this fungus and other members of the family Didymosphaeriaceae. Furthermore, the studied isolate was obtained from the roots of *Tamarix* sp., a plant well adapted to saline and arid conditions. Endophytes associated with desert plants are typically exposed to severe environmental stresses and may therefore possess distinctive metabolic pathways and the capacity to produce unique bioactive compounds. This finding may contribute to a broader understanding of the diversity of endophytic fungi associated with plants adapted to saline and arid conditions. In this study, the effect of different culture media on the antifungal activity of *D. quercina* 8B2B was investigated; however, further studies are needed to identify and analyze the secondary metabolites produced in each culture medium in order to optimize antimicrobial metabolite production. Despite these promising results, the present study has certain limitations. In particular, both bioassays used the assay/dilution medium matched to each production medium rather than a single common assay medium for all treatments; consequently, the possibility that the assay medium itself contributed to the observed differences in inhibition, independent of the endophyte culture filtrate, cannot be entirely excluded and should be addressed in future work using a standardized assay medium across all filtrate treatments. PDB filtrate was assayed in PDA, TSB filtrate in TSA, MEB filtrate in MEA, and YEB filtrate in YEA, therefore, production and assay medium are not independently varied. The contribution of the assay medium itself cannot be excluded; and further studies are required for a factorial design (crossing production medium× standardized assay medium) as a priority for future work. The compounds responsible for the observed antifungal activity have not yet been isolated or identified, and their precise mode of action against the pathogen remains unknown. Moreover, all evaluations were conducted under laboratory conditions; therefore, assessing the efficacy of this isolate and its metabolites under greenhouse and field conditions, as well as elucidating the chemical structure of the active compounds, warrants attention in future studies. Overall, the results of this study indicate that *D. quercina* may be considered a promising source for the development of biological control agents. Standardized bioassays (addressing the production/assay-medium), chemical characterization of the active compounds, and greenhouse/field evaluation are required for further studies.

Conclusion

To the best of our knowledge, this study offers, for the first time, the native Iranian endophyte species *D. quercina* (isolated from *Tamarix* sp.). *D. quercina* culture filtrates showed promising *in vitro* antifungal activity, particularly when produced in PDB and TSB, while further standardized bioassays, chemical characterization, and greenhouse/field studies are required. The results showed that the antifungal activity of this isolate is strongly influenced by culture medium type, with PDB and TSB media demonstrating greater inhibitory effects against the pathogen compared with the other media tested. These differences are likely related to the role of nutrient composition and carbon sources in the culture medium in regulating fungal growth and the production of bioactive compounds. This finding indicates that optimizing culture medium composition can play a decisive role in enhancing the production of bioactive compounds by endophytic fungi. The findings of this study underscore the importance of investigating endophytes associated with plants of Iran's arid and desert regions as valuable, understudied resources for discovering novel bioactive compounds and developing biological control agents. Nevertheless, precise identification of the compounds responsible for the antifungal activity

of *D. quercina* 8B2B will require further studies, including extraction, purification, and chemical structure determination of the active metabolites, as well as evaluation of their efficacy under greenhouse and field conditions.

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Conflicts of interest

The authors declare no conflict of interest.

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