Diversity and Biocontrol Potential of Culturable Fungi Isolated from Deep-Sea Sediments

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Article

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Abstract

Deep-sea sediments harbor unique fungal communities adapted to extreme conditions, offering an untapped resource for novel bioactive compounds. However, systematic studies of their potential for agricultural biocontrol remain limited. This study assessed the diversity of culturable fungi from deep-sea sediments and screened their antagonistic activity against important phytopathogens. Using a multi-method approach (dilution plating and plate stamping), we isolated 159 fungal isolates from sediments at four sites. Internal transcribed spacer (ITS)-based phylogenetic analysis identified Ascomycota as the dominant phylum, along with 35 genera dominated by *Cladosporium*, *Penicillium*, and *Aspergillus*. ITS sequence similarities of 34 strains to known species were <95%, suggesting potentially novel taxa. Of 23 representative isolates tested, 82.6% inhibited at least one of the following pathogens: *Colletotrichum gloeosporioides*, *Ganoderma pseudoferreum*, or *Phellinus noxius*. Nine selected strains inhibited pathogen growth through both sterile culture filtrates and volatile organic compounds (VOCs), with effects varying by the strain-pathogen combination. Notably, VOCs from certain strains stimulated the growth of specific pathogens, demonstrating complex fungal chemical interactions. These results highlight the diversity and biocontrol potential of culturable deep-sea fungi and support their evaluation as environmentally friendly agricultural biocontrol agents.

Keywords: deep-sea fungi; fungal diversity; biological control; antagonistic activity; phylogenetic analysis

1. Introduction

The deep-sea, an environment characterized by extreme hydrostatic pressure, low temperature, perpetual darkness, and chronic nutrient limitation, is one of the most distinctive and least explored ecosystems on Earth [1]. Deep-sea microorganisms have evolved unique physiological and metabolic adaptations to survive under these conditions. Fungi are an important component of deep-sea eukaryotic microbial communities, and they have attracted increasing attention due to their remarkable potential for secondary metabolite production. Adaptation to high pressure and oligotrophic conditions has driven the diversification of fungal biosynthetic pathways, positioning deep-sea fungi as a promising and largely underexplored source of structurally diverse and biologically active natural products [2–4].

Studies employing both culture-dependent [5] and culture-independent approaches [6,7] have revealed diverse fungal communities in deep-sea sediments across multiple oceanic regions, including the Mariana Trench [8,9], the Indian Ocean basins [6,10], and the South China Sea [5,11,12].

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Notably, studies in the South China Sea have found abundant culturable fungal populations. Due to the paucity of comparable investigations across different deep-sea regions, the extent to which these findings can be generalized beyond the South China Sea remains unclear. Genera such as *Aspergillus*, *Penicillium*, and *Talaromyces* have been frequently isolated and shown to exhibit strong abilities for secondary metabolite production and broad adaptability. These taxa have been shown to produce novel metabolites with antimicrobial, antitumor, and other biological activities [13–18]. These findings suggest that deep-sea fungi are an important component of marine microbial ecosystems, extending beyond the South China Sea, and they represent a valuable reservoir of bioactive compounds with potential applications outside the marine environment.

Unlike deep-sea bacteria and archaea, the diversity and ecological functions—particularly the biocontrol potential—of culturable deep-sea fungi have not been systematically explored, even for frequently isolated genera. In particular, there is a lack of studies that integrate deep-sea fungal resource exploration with functional screening relevant to practical challenges, such as the control of phytopathogens including *Colletotrichum gloeosporioides* [19], *Ganoderma pseudoferreum* [20], and *Phellinus noxius* [21], which threaten crop production and ecosystem stability. Given the limitations and environmental concerns associated with intensive pesticide use [22,23], there is growing interest in identifying alternative biological control strategies.

Endophytic fungi from terrestrial plants have been widely studied as biocontrol agents [24,25]. However, extreme environments such as the deep sea, with its distinct selective pressures (high hydrostatic pressure, low temperature, and intense competition for limited nutrients) may promote the evolution of fungi producing antimicrobial metabolites with unique chemical structures and modes of action [26,27]. Deep-sea fungi produce a diverse set of bioactive metabolites [13–18]; however, research has predominantly focused on their pharmaceutical potential. To the best of our knowledge, the antagonistic activity of deep-sea fungi—particularly through diffusible metabolites and volatile organic compounds (VOCs)—against agriculturally important phytopathogens has been largely unexplored.

To evaluate the application potential of culturable deep-sea fungi, we employed a multi-method cultivation strategy to systematically isolate and phylogenetically characterize fungal strains from deep-sea sediments. In contrast to previous work primarily oriented toward drug discovery, the present study adopted a screening approach targeting fungi antagonistic to major phytopathogens. Metabolically active taxa, particularly *Aspergillus* and *Penicillium*, were further assessed for in vitro antagonistic activity against *C. gloeosporioides*, *G. pseudoferreum*, and *P. noxius*. Moreover, both culture filtrates and VOCs were evaluated to comprehensively assess antifungal potential. This study presents an integrated framework for investigating the diversity of deep-sea fungi and evaluating their biocontrol potential.

2. Materials and Methods

2.1. Sample Collection and Processing

Four deep-sea sediment samples were collected from different locations at depths ranging from 2,595 to 6,000 m (Figure 1 and Supplementary Table S1). To generate the sampling map, base vector maps were obtained from the Aliyun Map Service in GeoJSON format. The vector data were converted to shapefile (shp) format and imported into ArcMap v10.8 (Esri, Redlands, CA, USA) for visualization. Sampling locations, geographic coordinates, and a scale bar were annotated and finalized in ArcMap. The sediment samples, serially designated DS01 through DS04, were collected using a sterile push corer, immediately sealed in sterile bags after retrieval, and stored at 4°C during transport. All samples were kindly provided by the Institute of Deep-Sea Science and Engineering, Chinese Academy of Sciences.

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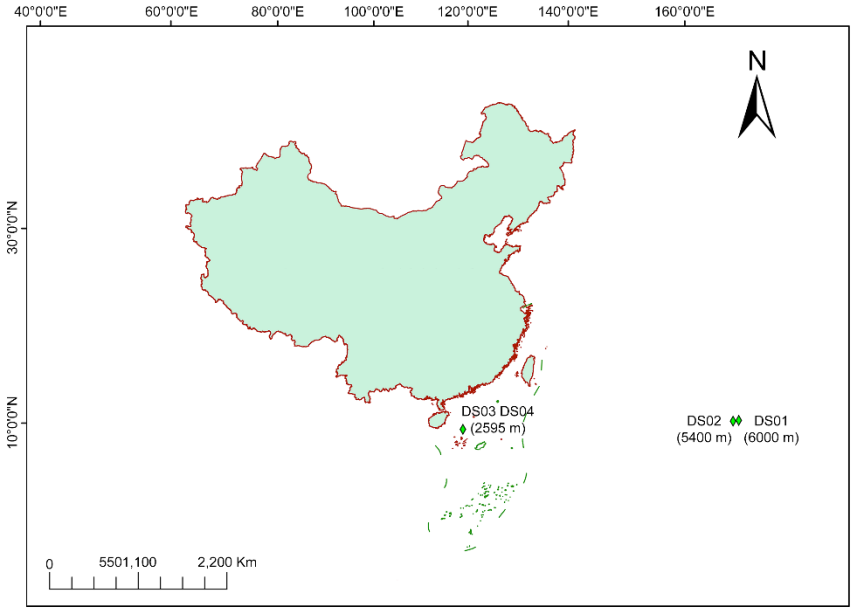

Figure 1. Sampling locations and corresponding water depths of deep-sea sediment samples.

2.2. Isolation of Cultivable Fungi

Sediment samples were aseptically air-dried in a laminar flow cabinet and gently homogenized using a sterile mortar and pestle. To maximize fungal recovery, our isolation strategy combined dilution plating (with and without pretreatment) and plate stamping onto glucose-peptone-yeast extract (GPY) [28], malt extract agar [29], Fungus No. II medium [30], and Martin's agar [31], i.e., 12 distinct isolation protocols (T01-T12, detailed in Supplementary Table S2). All media were supplemented with a filter-sterilized antibiotic cocktail (chloramphenicol, streptomycin, and ampicillin, each at 100 μg/mL) to inhibit bacterial growth.

2.2.1. Dilution Plate Technique

Each sample was diluted to disperse fungal propagules using a standard method consisting of suspending 2 g of sediment in 18 mL of sterile artificial seawater and shaking at 180 rpm for 3 h at room temperature [32,33]; and a pretreatment method, consisting of suspending the sediment in 18 mL of sterile artificial seawater containing 10% (v/v) ethylenediaminetetraacetic acid (EDTA) prior to shaking [34]. After shaking, samples were serially diluted up to 10^{-2} in sterile artificial seawater. Aliquots (0.2 mL) from the dilution tubes of both dilution methods were spread onto the surfaces of the four types of agar media in three independent replicates. Plates were incubated at 28°C and monitored for 1–3 weeks.

2.2.2. Plate Stamping Technique

The plate stamping protocol was adopted from Jensen et al. [35]. Briefly, 1 g of sediment was air-dried in a biosafety cabinet for 1 h, and any aggregates were gently dispersed. A sterile foam plug (2 cm diameter) was pressed firmly onto the sediment and then stamped methodically in a clockwise pattern onto the surface of Fungus No. II and GPY agar plates to create a dilution gradient. This operation was performed in three independent replicates for each medium. Plates were incubated at 28°C for up to 3 weeks.

2.3. Purification, Morphological Grouping, and Preservation

Emerging fungal colonies from all plates were examined daily and grouped based on colony texture, color of aerial and substrate mycelia, production of soluble pigments, and growth rate [36].

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Representative colonies from each distinct morphotype were carefully picked and repeatedly subcultured on fresh Potato Dextrose Agar (PDA; Hope Bio-Technology, Qingdao, China) plates until pure cultures were obtained. All purified strains were assigned unique labels (e.g., DSF001) and preserved on PDA slants at 4°C for short-term use and as 20% (v/v) glycerol stock suspensions stored at −80°C for long-term maintenance.

2.4. Molecular Identification and Phylogenetic Analysis

Molecular identification and phylogenetic analysis were performed following established methodologies [37]. Genomic DNA was extracted from mycelia using a commercial Fungal Genomic DNA Extraction Kit. The internal transcribed spacer (ITS) region was amplified using universal primers ITS1 and ITS4 and then purified and sequenced bidirectionally. Sequences were assembled and manually corrected. Preliminary taxonomic assignments of the ITS sequences were based on searches in the NCBI GenBank database using the BLASTn algorithm (summarized in Supplementary Table S3). Phylogenetic relationships among a subset of representative isolates were determined by first aligning the ITS sequences with reference sequences from closely related type species using ClustalW. A phylogenetic tree was constructed using the Neighbor-Joining method in MEGA v7.0, with bootstrap analysis based on 1000 replicates.

2.5. In Vitro Antagonism Assay: Dual Culture

Based on the results of phylogenetic analysis and the recognized biocontrol potential of genera such as Penicillium [14,16] and Aspergillus [15,18], we selected 23 representative fungal isolates for primary antagonism screening. The assay targeted five phytopathogens: three strains of Colletotrichum gloeosporioides (CH008 from Stylosanthes, 171-1 from mango, and RC178 from rubber tree), Ganoderma pseudoferreum (GP030), and Phellinus noxius (Pn006), kindly provided by the Chinese Academy of Tropical Agricultural Sciences.

The dual-culture assay was performed as described by Al-Rashdi et al. [38] with modifications. Briefly, a 5-mm mycelial plug of a pathogen was placed at the center of a PDA plate. All plates were supplemented with streptomycin sulfate at a final concentration of 100 mg/L. Then, a plug of a deep-sea fungal isolate was inoculated at four points equidistant (2.5 cm) from the center. Control plates contained only the pathogen. Plates were incubated at 28°C until the mycelial growth of the pathogen in the control plate either reached the plate edge or stopped advancing for three consecutive days. The percentage of mycelial growth inhibition (MGI) was calculated as: MGI (%) = [(Rc - Rt) / Rc] × 100, where Rc is the radial growth in the control and Rt is the radial growth in the treatment [24]. Each test was performed in three independent replicates.

2.6. Antifungal Activity of Culture Filtrates

Nine of the isolates exhibiting notable antagonism were selected for the culture filtrate antagonism assay. Each isolate was cultured in 50 mL of potato dextrose broth in a 250-mL flask at 28°C with shaking at 180 rpm for 7 days [39]. The culture broth was centrifuged, and the supernatant was filter-sterilized through a 0.22 μm membrane. This filtrate was mixed with PDA medium at a 4:1 (v/v) ratio. PDA mixed with sterile water served as the negative control. All media were supplemented with streptomycin sulfate at a final concentration of 100 mg/L. A 5-mm mycelial plug of the target pathogen was placed at the center of each prepared plate, which were then incubated at 28°C until the mycelial growth of the pathogen in the control plate either reached the plate edge or stopped advancing for three consecutive days. The percentage MGI was calculated as described in Section 2.5, with three independent replicates per treatment.

2.7. Antifungal Activity of Fungal Volatile Organic Compounds

The antifungal activities of VOCs produced by the nine selected isolates were evaluated using a dual-compartment Petri dish (90 mm diameter) method adapted from previous studies [40]. This

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setup physically separates two types of agar media while sharing a common headspace, enabling interaction solely through gaseous metabolites. For each test, two PDA plates supplemented with streptomycin sulfate (100 mg/L) were prepared. One plate was centrally inoculated with a 5-mm mycelial plug of the target phytopathogen. The companion plate was either centrally inoculated with a plug of a deep-sea fungal antagonist or left uninoculated to serve as the control. Immediately after inoculation, the two plates were joined base-to-base, and the junction was tightly sealed with three layers of Parafilm® [41] to prevent air exchange. All paired assemblies were incubated at 28°C until the mycelial growth of the pathogen in the control setup (paired with an uninoculated plate) either reached the plate edge or stopped advancing for three consecutive days. The radial growth of the pathogen was then measured, and the MGI was calculated using the formula described in Section 2.5. Each antagonist-pathogen combination was tested with three independent replicates.

2.8. Data and Statistical Analysis

All statistical analyses were performed using specialized software and online platforms. Bar plots and heatmaps were generated using ImageGP 2 (https://www.bic.ac.cn/ImageGP/) [42]. Culturable fungi diversity was measured by calculating alpha-diversity indices (including observed taxa, Shannon, Simpson, Pielou's evenness, and Chao1) and beta-diversity based on the Bray-Curtis distance matrix using PAST software (version 4.17) [43]. Principal coordinate analysis (PCoA) for beta-diversity visualization was performed using the online tools of the Majorbio Cloud Platform (https://www.majorbio.com) [44].

Statistical analyses for the in vitro antagonism assays (dual culture, culture filtrate, and VOC) were conducted using IBM SPSS Statistics v26.0 [45]. MGI data are presented as the mean ± standard deviation of three biological replicates. Differences in MGI among different deep-sea fungal isolates against a specific pathogen or for one isolate against different pathogens were analyzed using one-way ANOVA. P-values <0.05 were considered statistically significant. Tukey's honestly significant difference post hoc test was applied for multiple comparisons.

3. Results

3.1. Phylogenetic and Diversity Analysis of Culturable Fungal Strains

A total of 159 culturable fungal strains were isolated from four deep-sea sediment samples (Supplementary Table S3) using 12 distinct isolation protocols (Supplementary Table S2). Taxonomic status was determined by sequencing the ITS region, which identified 128, 30, and 1 strains as Ascomycota, Basidiomycota, and Mucoromycota, respectively. The ITS sequences of 125 strains were highly similar (95%–100%) to known species belonging to 34 genera. However, similarity matches of 34 strains (listed in Supplementary Table S3) were <95%, a threshold commonly used to identify species, indicating their potential as novel taxonomic units.

We selected 76 representative isolates for phylogenetic analysis based on combined ITS sequence and phenotypic data, generating the neighbor-joining tree shown in Figure 2. The 76 isolates belonged to the following 35 genera: *Alfaria*, *Aspergillus*, *Botryosporium*, *Cladosporium*, *Coprinellus*, *Curvularia*, *Diaporthe*, *Epicoccum*, *Exobasidiomycetidae*, *Fusarium*, *Gliomastix*, *Hortaea*, *Hypoxylon*, *Leptospora*, *Letendraea*, *Meyerozyma*, *Montagnula*, *Neodevriesia*, *Neodidymella*, *Neogyrothrix*, *Neovaginatispora*, *Nigrograna*, *Nigrospora*, *Nodulisporium*, *Odontoefibula*, *Penicillium*, *Perenniporia*, *Peroneutypa*, *Phlebiopsis*, *Pseudocercospora*, *Pyrenochaetopsis*, *Setophaeosphaeria*, *Talaromyces*, *Torula*, and *Ustilaginoidea*. These were distributed across 17 orders: Agaricales, Amplistromatales, Chaetothyriales, Cladosporiales, Diaporthales, Eurotiales, Helicobasidiales, Hymenochaetales, Hypocreales, Mycosphaerellales, Mucorales, Pleosporales, Polyporales, Russulales, Serinales, Xylariales, and Phomatosporales. Additionally, two, one, and one strains belonged to the class Dothideomycetes (order uncertain), class Exobasidiomycetes (order uncertain), and x Ascomycota (class and order uncertain), respectively. The dominant genera were *Cladosporium* (41 strains, 25.79%), followed by *Penicillium* (19 strains, 11.95%), and *Aspergillus* (15 strains, 9.43%) (Table 1).

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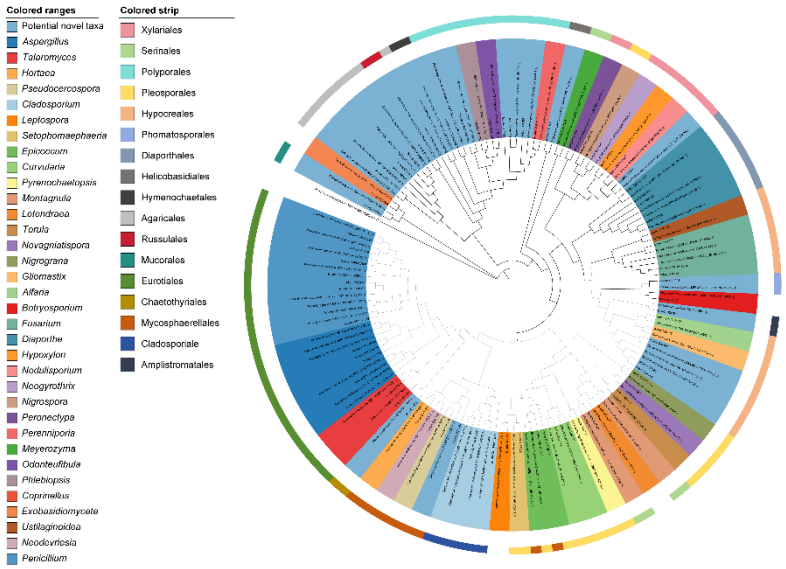

Figure 2. Neighbor-joining phylogenetic tree based on ITS sequences of representative fungal isolates obtained from deep-sea sediments.

Table 1. Taxonomic composition of culturable fungi isolated from deep-sea sediments.

Phylum	Class	Order	Genus	Number of isolates	Relative abundance (%)
Ascomycota	Sordariomycetes	Hypocreales	*Alfaria*	2	1.26
Ascomycota	Eurotiomycetes	Eurotiales	*Aspergillus*	15	9.43
Ascomycota	Dothideomycetes	Botryosporiales	*Botryosporium*	1	0.63
Ascomycota	Dothideomycetes	Cladosporiales	*Cladosporium*	41	25.79
Basidiomycota	Agaricomycetes	Agaricales	*Coprinellus*	1	0.63
Ascomycota	Dothideomycetes	Pleosporales	*Curvularia*	2	1.26
Ascomycota	Sordariomycetes	Diaporthales	*Diaporthe*	5	3.14
Ascomycota	Dothideomycetes	Pleosporales	*Epicoccum*	2	1.26
Basidiomycota	Exobasidiomycetes	Exobasidiales	*Exobasidiomycetidae*	1	0.63
Ascomycota	Sordariomycetes	Hypocreales	*Fusarium*	5	3.14
Ascomycota	Sordariomycetes	Hypocreales	*Gliomastix*	1	0.63

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Ascomycota	Dothideomycetes	Capnodiales	*Hortaea*	1	0.63
Ascomycota	Sordariomycetes	Xylariales	*Hypoxylon*	1	0.63
Ascomycota	Dothideomycetes	Pleosporales	*Leptosphora*	1	0.63
Ascomycota	Dothideomycetes	Pleosporales	*Letendraea*	1	0.63
Ascomycota	Saccharomycetes	Saccharomycetales	*Meyerozyma*	2	1.26
Ascomycota	Dothideomycetes	Pleosporales	*Montagnula*	1	0.63
Ascomycota	Dothideomycetes	Capnodiales	*Neodevriesia*	1	0.63
Ascomycota	Dothideomycetes	Pleosporales	*Neodidymella*	2	1.26
Ascomycota	Sordariomycetes	Xylariales	*Neogyrothrix*	1	0.63
Ascomycota	Dothideomycetes	Pleosporales	*Neovaginatispora*	1	0.63
Ascomycota	Dothideomycetes	Pleosporales	*Nigrograna*	2	1.26
Ascomycota	Sordariomycetes	Hypocreales	*Nigrospora*	2	1.26
Ascomycota	Sordariomycetes	Xylariales	*Nodulisporium*	2	1.26
Basidiomycota	Agaricomycetes	Polyporales	*Odontoefibula*	1	0.63
Ascomycota	Eurotiomycetes	Eurotiales	*Penicillium*	19	11.95
Basidiomycota	Agaricomycetes	Polyporales	*Perennporia*	2	1.26
Ascomycota	Sordariomycetes	Xylariales	*Peroneutypa*	1	0.63
Basidiomycota	Agaricomycetes	Polyporales	*Phlebiopsis*	1	0.63
Ascomycota	Dothideomycetes	Capnodiales	*Pseudocercospora*	1	0.63
Ascomycota	Dothideomycetes	Pleosporales	*Pyrenochaetopsis*	1	0.63

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Ascomycota	Dothideomycetes	Pleosporales	*Setophaeosphaeria*	1	0.63
Ascomycota	Eurotiomycetes	Eurotiales	*Talaromyces*	2	1.26
Ascomycota	Dothideomycetes	Pleosporales	*Torula*	1	0.63
Ascomycota	Sordariomycetes	Ustilaginoideales	*Ustilaginoidea*	1	0.63
Potential novel taxa				34	21.38

The distribution of the 159 isolates across the four sampling sites is shown in Figures 3A and 3B. Site DS04 yielded the highest number and diversity of isolates (48 strains, 15 genera, 30.19%), followed by DS02 (41 strains, 11 genera, 25.79%), DS03 (36 strains, 15 genera, 22.64%), and DS01 (34 strains, 11 genera, 21.38%). Heatmap analysis (Supplementary Figure S1A) showed *Cladosporium* as the dominant (20.83%–31.71% of isolates) genus across all sites, followed by *Penicillium* (5.56%–17.65%). *Aspergillus* was isolated from all sites except DS01 and was particularly abundant at DS02, constituting 24.39% of its community. We identified 20 rare genera (defined as comprising <1% of total abundance), which were mostly detected at DS03 (7 genera), and least detected at DS02 (2 genera). Sites DS02 and DS04 had the fewest and highest proportion of potentially novel taxa, respectively.

Fungal recovery varied significantly with the isolation protocol, as shown in Figures 3C and 3D. Among the 12 treatment combinations, T12 yielded the highest number and diversity of isolates, accounting for 35.85% of the total (57 strains, 13 genera), followed by protocol T11 (42 strains, 14 genera, 26.42%). Specifically, the "stamping method" combined with either GPY or Fungi No. II medium was the most effective strategy, collectively accounting for 99 strains (62.26% of the total). The 10^{-1} dilution (T1-T4) yielded 32 strains (20.13%, 14 genera), whereas the 10^{-2} dilution (T5-T8) yielded 15 strains (9.43%, 12 genera), consistent with the dilution effect. The 10^{-1} dilution on MEA (T2) yielded the highest number of isolates (10 strains, 6 genera); however, the differences among the different media were not significant. In contrast, at the 10^{-2} dilution, Fungus No. II medium (T7) and Martin's agar (T8) grew significantly more isolates (6 strains each) than GPY (T5) or MEA (T6). Pretreatment with EDTA (T9 and T10) yielded slightly more isolates, but this improvement was not statistically significant ($P > 0.05$) (Supplementary Figure S1B).

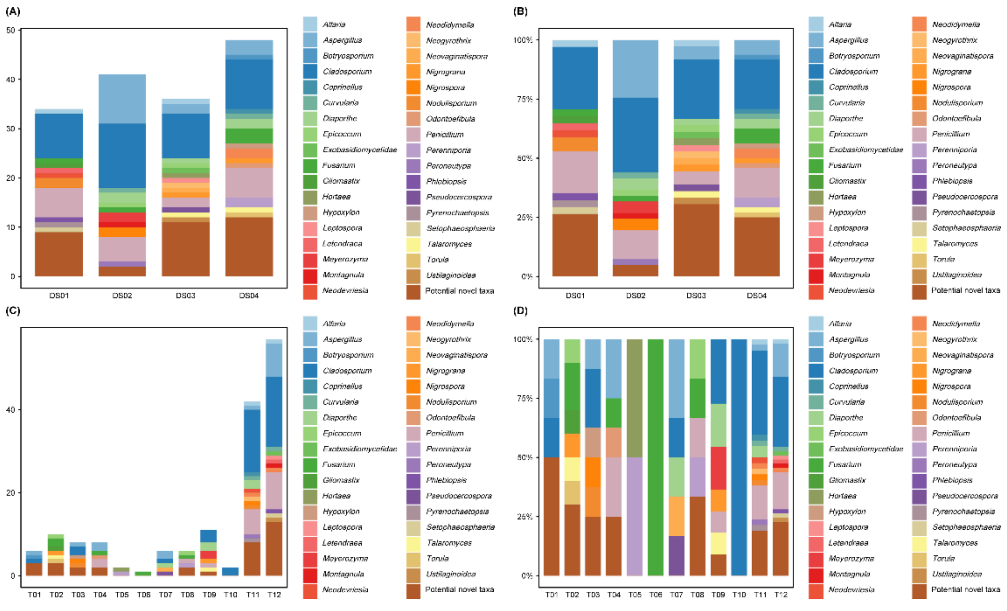

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Figure 3. Distribution of culturable fungi in four deep-sea sediment samples. (A, B) Bar charts showing taxonomic composition at the genus level; (C, D) bar charts showing genus-level taxonomic distributions using different isolation methods.

3.2. Alpha and Beta Diversity of Fungal Communities

Comparison of alpha and beta diversity indices of fungal communities across different sites and isolation methods revealed significant differences in alpha diversity among the four sampling sites (Table 2). The values of Shannon and Simpson indices indicate the highest species richness and diversity (H′ = 3.589, 1–D = 0.9699) at site DS04, followed by DS03 (H′ = 3.066, 1–D = 0.9302). The evenness index values for DS01, DS03, and DS04 are high (>0.97), with a value of 1.131 for DS04, indicating a relatively balanced abundance distribution, whereas the evenness at DS02 was lower (0.8143). Values of all indices consistently suggest lower community diversity at DS02. Notably, the Chao1 richness estimator was substantially higher than the observed species count for all sites. Results of PCoA based on Bray-Curtis dissimilarity reveal clear differences in community composition (beta diversity) among sites (Supplementary Figure S2A). The first two PCoA axes explain 71.45% of the total variation. The plot shows four distinct, nonoverlapping clusters corresponding to each site. Sites DS01 and DS03 show a similar clustering pattern, while DS02 and DS04 form distinct clusters separated from the others.

Table 2. Alpha diversity indices of culturable fungal communities from four deep-sea sediment samples.

	Taxa	**Shannon**	**Simpson**	**Evenness**	**Chao1**
DS01	18	2.871	0.9323	0.9809	39.35
DS02	19	2.739	0.8951	0.8143	31.88
DS03	22	3.066	0.9302	0.975	66.07
DS04	32	3.589	0.9699	1.131	86.05

Alpha diversity also varied with the different isolation methods (Table 3). The observed number of genera ranged from 1 (T06) to 30 (T12), with the latter yielding the highest diversity (Taxa = 30, H′ = 3.242, 1–D = 0.9292, Evenness = 0.8526, Chao1 = 71.26). Methods using 10^{-2} diluted soil suspensions yielded the lowest diversity. The evenness index values are close to or slightly above 1 for most dilution-based methods (T01-T10), reflecting an extremely even distribution of recovered taxa, likely a consequence of the limited number of isolates per method. In contrast, we observed lower evenness in T11 and T12 (0.7411 and 0.8526, respectively), suggesting the dominance of a few taxa. Notably, we found higher Chao1 values than the observed count for all methods, most dramatically for T11 (229.1 Chao1 vs. 24 observed), indicating that even the most effective isolation strategy captured only a fraction of the presumed fungal diversity. Results of PCoA of beta diversity reveal that the isolation method was the primary factor shaping the recoverable fungal community composition (Supplementary Figure S2B). The first two axes explained 55.22% of the variation, with the plot showing two distinct clusters: T01-T10, i.e., samples that underwent dilution-based methods forming a relatively tight cluster, whereas those from stamping methods (T11 and T12) were clearly separated and distant from the former. This result indicates that the stamping and dilution methods accessed distinct subsets of the fungal community.

Table 3. Alpha diversity indices of culturable fungal communities obtained using different isolation methods.

	Taxa	Shannon	Simpson	Evenness	Chao1
T01	6	2.208	1	1.517	18.5
T02	8	2.432	0.9722	1.423	17.33
T03	8	2.517	1	1.549	32.5

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T04	7	2.281	0.9643	1.398	13.56
T05	2	0.9431	1	1.284	2.5
T06	1	0	0	1	1
T07	5	1.894	0.9333	1.329	7.5
T08	6	2.208	1	1.517	18.5
T09	7	2.119	0.9091	1.189	8.818
T10	2	0.9431	1	1.284	2.5
T11	24	2.878	0.8738	0.7411	229.1
T12	30	3.242	0.9292	0.8526	71.26

3.3. In Vitro Antagonism Assessed by Dual-Culture Assay

The 23 representative fungal isolates screened for antagonism against phytopathogenes were selected based on a dereplication analysis of ITS sequences to capture phylogenetic diversity. We focused on *Penicillium* and *Aspergillus* because they are well-documented as prolific producers of a broad spectrum of antimicrobial metabolites [2,15,16]. Nineteen of these isolates (82.6%) exhibited antagonistic activity against at least one of the five phytopathogens tested, confirming our hypothesis that deep-sea fungi are a reservoir of potential biocontrol agents with a broad spectrum of antifungal activity (Table 4, Figure 4).

Table 4. In vitro antagonistic activity of 23 representative deep-sea fungal isolates against five phytopathogens assessed by dual-culture assay.

Isolates	Maximum similarity strain	Maximum similarity (%)	Mycelial Growth Inhibition Rate (%)				
			CH008	Pn006	171-1	RC178	GP030
DSF005	*A. brunneoviolaceus* NRRL 4912 (NR_138279.1)	99.44	69.8 ± 0.68a	78.82 ± 2.04a	81.57 ± 0.68a	69.17 ± 0.72abc	68.46 ± 1.68def
DSF007	*A. brunneoviolaceus* NRRL 4912 (NR_138279.1)	98.08	66.67 ± 1.36ab	72.55 ± 1.36ab	71.37 ± 1.36b	76.25 ± 0a	77.2 ± 0.84bcd
DSF012	*P. citrinum* NRRL 1841 (NR_121224.1)	100	62.35 ± 3.11bcd	63.92 ± 0.68cde	63.92 ± 0.68cd	66.88 ± 1.65abcd	77.44 ± 1.26bcd
DSF024	*P. coffeae* NRRL 35363 (NR_121312.1)	99.06	51.37 ± 0.68g	59.61 ± 1.8efgh	49.02 ± 0.68fg	56.25 ± 2.72cde	69.19 ± 5.16cdef
DSF025	*P. coffeae* NRRL 35363 (NR_121312.1)	99.06	53.33 ± 0.68fg	52.75 ± 1.22h	52.75 ± 0.9ef	55.42 ± 1.91cde	64.58 ± 2.22f
DSF034	*P. sclerotiorum* FRR 2074 (NR_077157.1)	99.27	54.51 ± 0.68efg	56.08 ± 1.8gh	54.9 ± 1.8e	57.5 ± 4.51bcde	70.4 ± 7.98cdef
DSF036	*P. coffeae* NRRL 35363 (NR_121312.1)	99.06	54.12 ± 1.56fg	52.94 ± 2.35h	54.51 ± 1.8e	55.83 ± 2.6cde	61.18 ± 0.84f
DSF044	*A. assiutensis* AUMC 5748 (NR_151787.1)	100	69.8 ± 0.68a	69.02 ± 1.36bcd	34.51 ± 0.68h	69.17 ± 0.72abc	92.72 ± 0a

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DSF047	*A. brunneoviolaceus* NRRL 4912 (NR_138279.1)	99.44	54.12 ± 6.11fg	63.92 ± 0.68cde	46.67 ± 3.4g	27.5 ± 19.49g	69.43 ± 7.7cdef
DSF054	*P. macrosclerotiorum* CBS 116871 (NR_156585.1)	99.44	50.2 ± 5.43g	19.61 ± 3.4i	0 ± 0i	35 ± 2.17fg	83.5 ± 0.84ab
DSF059	*P. macrosclerotiorum* CBS 116871 (NR_156585.1)	91.57	65.49 ± 0.68abc	70.2 ± 0.68bc	65.1 ± 0.68cd	79.17 ± 9.02a	83.99 ± 3.17ab
DSF069	*T. beijingensis* CBS 140617 (NR_172251)	98.88	59.61 ± 0.68cdef	69.41 ± 0bcd	52.94 ± 0ef	47.5 ± 1.25ef	61.67 ± 0.84f
DSF073	*A. brunneoviolaceus* NRRL 4912 (NR_138279.1)	98.90	70.59 ± 0a	63.53 ± 8.15cdef	81.96 ± 3.4a	70 ± 2.17abc	34.98 ± 2.22g
DSF078	*P. mallochii* DAOM 239917 (NR_111674.1)	99.06	62.35 ± 1.18bcd	64.71 ± 1.18cde	63.92 ± 1.36cd	59.58 ± 3.15bcde	81.56 ± 0.84b
DSF094	*P. paxilli* CBS 360.48 (NR_111483.1)	98.81	58.63 ± 1.48def	62.75 ± 1.36defg	62.75 ± 1.8d	64.38 ± 1.88abcd	65.31 ± 1.52f
DSF095	*P. sclerotiorum* FRR 2074 (NR_077157.1)	99.44	60.78 ± 2.45bcde	59.22 ± 1.8efgh	61.96 ± 0.68d	63.13 ± 4.88abcde	79.14 ± 1.68bc
DSF103	*P. paxilli* CBS 360.48 (NR_111483.1)	98.87	61.96 ± 2.96bcd	56.86 ± 1.8fgh	62.75 ± 1.36d	58.75 ± 5.96bcde	76.71 ± 1.46bcde
DSF109	*P. oxalicum* NRRL 787 (NR_121232.1)	99.64	69.2 ± 0.68a	71.76 ± 2.04b	67.84 ± 0.68bc	72.71 ± 3.44ab	81.08 ± 1.46b
DSF137	*A. tennesseensis* NRRL 13150 (NR_135447.1)	98.49	0 ± 0	5.1 ± 0.68j	0 ± 0i	−6.25 ± 0h	34.98 ± 4.45g
DSF138	*A. tennesseensis* NRRL 13150 (NR_135447.1)	99.25	0 ± 0h	0.78 ± 1.36j	0 ± 0i	3.75 ± 4.33h	31.1 ± 4.68g
DSF148	A. jensenii NRRL 58600 (NR_135444.1)	97.77	0 ± 0h	1.18 ± 0j	0 ± 0i	−6.25 ± 0h	3.93 ± 0h
DSF149	*T. Resinae* (NR_190238.1)	96.04	65.1 ± 1.36abcd	56.86 ± 0.68fgh	63.92 ± 0.68cd	52.92 ± 1.44de	67.01 ± 2.22ef
DSF160	A. sydowii CBS 593.65 (NR_131259.1)	99.01	0 ± 0h	0.39 ± 0.68j	0 ± 0i	−6.25 ± 0h	37.41 ± 1.46g

Note: Values in the table are expressed as mean ± standard deviation. Different lowercase letters following the values indicate significant differences at the 0.05 level.

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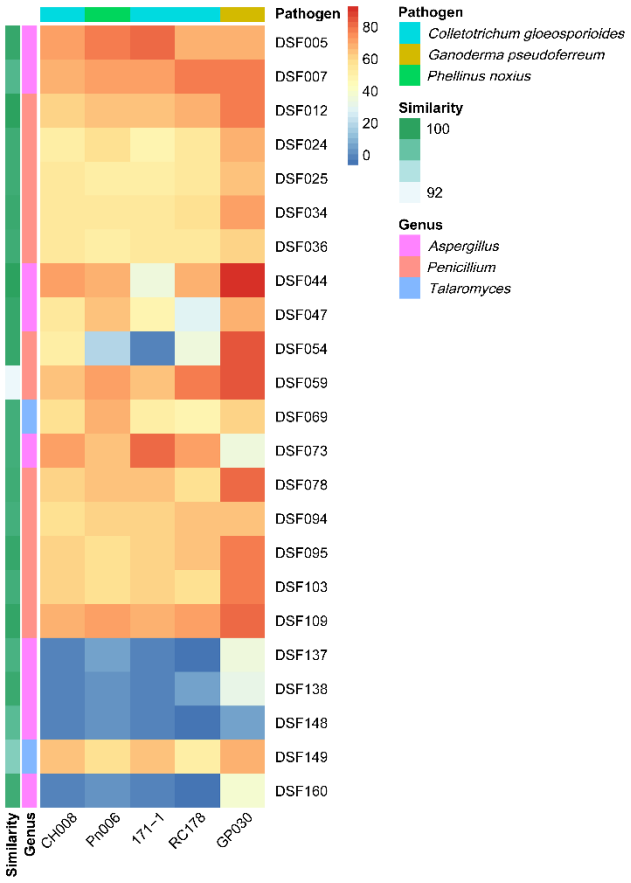

Figure 4. Heatmap illustrating the antagonistic activity of 23 representative deep-sea fungal isolates against five phytopathogenic fungi, as determined by the dual-culture assay.

The antagonistic activity of isolates varied considerably according to the pathogen. The three strains of *C. gloeosporioides* were inhibited at rates that ranged from 50.2 ± 5.43% to 70.59 ± 0% for CH008; from 34.51 ± 0.68% to 81.96 ± 3.4% for 171-1; and from 3.75 ± 4.33% to 79.17 ± 9.02% for RC178. The basidiomycete pathogens were inhibited at rates that ranged from 0.39 ± 0.68% to 78.82 ± 2.04% against *P. noxius* Pn006, and from 3.93 ± 0% to a maximum inhibition of 92.72 ± 0% against *G. pseudoferreum* GP030.

We identified a subset of highly effective antagonists: Isolates DSF005, DSF007, DSF044, DSF059, DSF073, and DSF109 consistently performed well against multiple pathogens. For instance, DSF073 showed the highest inhibition（81.96% ± 3.4%) against *C. gloeosporioides* 171-1, whereas DSF044 exhibited the strongest overall activity (92.72% ± 0%) against *G. pseudoferreum* GP030. DSF005 demonstrated potent broad-spectrum activity, achieving high inhibition rates against *P. noxius* Pn006 (78.82% ± 2.04%) and several *C. gloeosporioides* strains (Figure 5A). These isolates form a robust subset of promising candidates for subsequent, mode-of-action-focused assays.

3.4. Antifungal Activity of Fungal Culture Filtrates

To determine the contribution of diffusible metabolites in the inhibition of phytopathogens, we evaluated the antifungal activities of sterile culture filtrates from nine fungal strains (DSF005, DSF007, DSF012, DSF044, DSF059, DSF073, DSF078, DSF109, and DSF149). These strains were selected based on the antagonistic spectrum observed in the dual-culture assay and their taxonomic classification. The results (Table 5, Figure 5B) reveal pronounced heterogeneity in inhibition mediated by diffusible metabolites.

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Table 5. Antifungal activity of sterile culture filtrates from nine selected deep-sea fungal isolates.

Isolates	Maximum similarity strain	Maximum similarity (%)	Mycelial Growth Inhibition Rate (%)				
			CH008	Pn006	171-1	RC178	GP030
DSF005	*A. brunneoviolaceus* NRRL 4912 (NR_138279.1)	99.44	36.95 ± 1.39a	0 ± 0c	40.83 ± 22.09a	0 ± 0c	18.82 ± 4.06bc
DSF007	*A. brunneoviolaceus* NRRL 4912 (NR_138279.1)	98.08	23.29 ± 0.7bc	21.96 ± 1.36b	0 ± 1.25b	0 ± 0c	12.37 ± 2.46c
DSF012	*P. citrinum* NRRL 1841 (NR_121224.1)	100	22.49 ± 2.51bc	63.53 ± 4.24a	25.63 ± 4.38ab	12.75 ± 4.17ab	46.77 ± 4.27a
DSF044	*A. assiutensis* AUMC 5748 (NR_151787.1)	100	30.92 ± 1.39ab	74.12 ± 4.08a	11.67 ± 0.72ab	0 ± 0c	−4.3 ± 0.93de
DSF059	*P. macrosclerotiorum* CBS 116871 (NR_156585.1)	91.57	6.43 ± 3.03d	3.92 ± 4.13c	2.08 ± 1.91b	7.45 ± 1.36bc	−11.56 ± 5.25e
DSF073	*A. brunneoviolaceus* NRRL 4912 (NR_138279.1)	98.90	−2.41 ± 0d	0 ± 0c	25.83 ± 20.97ab	0 ± 0c	24.73 ± 6.72b
DSF078	*P. mallochii* DAOM 239917 (NR_111674.1)	99.06	16.06 ± 8.13c	3.92 ± 1.8c	4.58 ± 1.91b	7.84 ± 2.45b	0 ± 0d
DSF109	*P. oxalicum* NRRL 787 (NR_121232.1)	99.64	17.67 ± 2.51c	70.2 ± 11.18a	10.42 ± 1.91b	18.43 ± 4.75a	−0.81 ± 2.91de
DSF149	*T. Resinae* (NR_190238.1)	96.04	24.9 ± 3.03bc	70.59 ± 1.18a	3.75 ± 2.5b	10.59 ± 4.08b	22.04 ± 2.46bc

Note: Values in the table are expressed as mean ± standard deviation. Different lowercase letters following the values indicate significant differences at the 0.05 level.

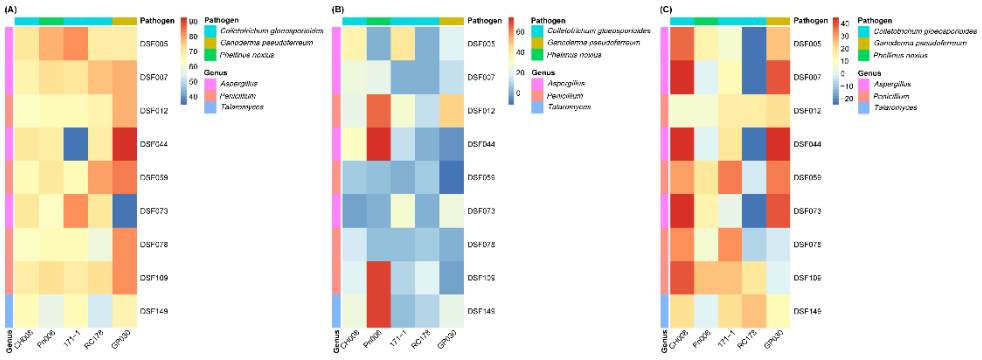

Figure 5. Heatmap comparison of antifungal activity mediated by (A) dual-culture assays, (B) culture filtrates, and (C) volatile organic compounds (VOCs).

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C. gloeosporioides CH008 was most strongly inhibited by the culture filtrates of DSF005 and DSF044 at inhibition rates of 36.95 ± 1.39% and 30.92±1.39%, respectively. Several isolates, including DSF149, DSF007, and DSF012, showed moderate inhibition （22.49 ± 2.51%-24.9±3.03%), whereas DSF059, DSF078, and DSF109 displayed weak inhibition (<18%). Notably, the culture filtrate of DSF073 did not inhibit the mycelial growth of CH008.

P. noxius Pn006 was strongly inhibited by the culture filtrates of multiple isolates. Strong inhibition (>70%) was observed for DSF044 (74.12% ± 4.08%), DSF149 (70.59% ± 1.18%), and DSF109 (70.20% ± 11.18%), and DSF012 (63.53% ± 4.24%). Other isolates exhibited weak or no inhibitory effects against this pathogen.

C. gloeosporioides 171-1 was most strongly inhibited by DSF005 (40.83% ± 22.09%), with DSF012 and DSF073 showing moderate activity (~25%). *C. gloeosporioides* RC178 was inhibited by DSF109 at a rate of 18.43% ± 4.75%, followed by DSF012 and DSF149.

Among the tested isolates, DSF012 displayed the highest inhibitory activity against *G. pseudoferreum* GP030 (46.77% ± 4.27%), whereas DSF073 and DSF149 showed moderate inhibition. The remaining isolates exhibited little to no inhibitory effect and in some cases appeared to promote mycelial growth.

Overall, these results demonstrate that antifungal activity mediated by culture filtrates was highly pathogen-dependent and did not necessarily correlate with antagonistic performance observed in the dual-culture assay. These findings suggest that antifungal activity of the isolates is not fully explained by diffusible metabolite production.

3.5. Antifungal Activity of Fungal Volatile Organic Compounds

The antifungal activity of VOCs produced by the nine selected deep-sea fungal isolates varied according to both isolate and pathogen (Table 6, Figure 5C). Overall, VOC-mediated effects differed substantially from those observed in both dual-culture and culture filtrate assays, indicating a distinct and independent mode of antagonism.

Table 6. Antifungal activity mediated by volatile organic compounds (VOCs) produced by nine deep-sea fungal isolates.

Isolates	Maximum similarity strain	Maximum similarity (%)	Mycelial Growth Inhibition Rate (%)				
			CH008	Pn006	171-1	RC178	GP030
DSF005	*A. brunneoviolaceus* NRRL 4912 (NR_138279.1)	99.44	36.14 ± 0ab	13.33 ± 2.72bc	6.91 ± 1.41b	−25 ± 0d	25.42 ± 4.02bc
DSF007	*A. brunneoviolaceus* NRRL 4912 (NR_138279.1)	98.08	44.18 ± 1.39a	0.39 ± 0.68d	10.16 ± 0.7ab	−25 ± 0d	40 ± 4.51ab
DSF012	*P. citrinum* NRRL 1841 (NR_121224.1)	100	6.02 ± 2.41c	6.67 ± 7.09cd	16.67 ± 14.75ab	14.71 ± 19.91abc	20.83 ± 5.64bc
DSF044	*A. assiutensis* AUMC 5748 (NR_151787.1)	100	44.18 ± 1.39a	0 ± 0d	19.11 ± 1.41ab	−25 ± 0d	45 ± 14.74a
DSF059	*P. macrosclerotiorum* CBS 116871 (NR_156585.1)	91.57	30.12 ± 9.39ab	18.04 ± 4.13ab	33.74 ± 8.99a	−2.94 ± 7.35bcd	34.79 ± 1.57ab

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DSF073	*A. brunneoviolaceus* NRRL 4912 (NR_138279.1)	98.90	44.98 ± 0.7a	13.73 ± 3.4bc	2.44 ± 2.11b	−25 ± 0d	40 ± 4.51ab
DSF078	*P. mallochii* DAOM 239917 (NR_111674.1)	99.06	32.53 ± 14.77ab	6.67 ± 1.36cd	32.52 ± 7.62a	−7.35 ± 4.41cd	−2.71 ± 0.95d
DSF109	*P. oxalicum* NRRL 787 (NR_121232.1)	99.64	39.76 ± 4.78a	24.31 ± 4.45a	24.39 ± 16.91ab	17.89 ± 7.65ab	−0.63 ± 3.13d
DSF149	*T. Resinae* (NR_190238.1)	96.04	20.48 ± 3.61bc	0 ± 0d	19.51 ± 7.32ab	24.51 ± 5.94a	10.42 ± 9.38cd

Note: Values in the table are expressed as mean ± standard deviation. Different lowercase letters following the values indicate significant differences at the 0.05 level.

C. gloeosporioides CH008 was most strongly inhibited by VOCs from DSF007, DSF044, and DSF073 at rates of 44.18 ± 1.39%, 44.18 ± 1.39%, and 44.98 ± 0.70%, respectively. Isolates DSF005, DSF059, DSF078, and DSF109 exhibited notable inhibition rates ranging from 30.12 ± 9.39% to 39.76 ± 4.78%. VOCs from DSF012 and DSF149 were weakly inhibitory at rates of 6.02 ± 2.41% and 20.48 ± 3.61%, respectively.

P. noxius Pn006 was modestly inhibited by VOCs of DSF109 at a rate of 24.31 ± 4.45%, followed by DSF059 at a rate of 18.04 ± 4.13%. VOCs of isolates DSF005, DSF012, DSF073, and DSF078 were weakly inhibitory at rates ranging from 6.67 ± 1.36% to 13.73 ± 3.40%, whereas VOCs of DSF007, DSF044, and DSF149 failed to inhibit phytopathogens under the tested conditions.

A distinct inhibition pattern was observed for *C. gloeosporioides* 171-1: VOCs from DSF059 and DSF078 suppressed mycelial growth at the highest rates of 33.74 ± 8.99% and 32.52 ± 7.62%, respectively. Moderate inhibition was observed for DSF109 at a rate of 24.39 ± 16.91%, whereas DSF012, DSF044, and DSF149 exhibited intermediate inhibition rates ranging from 16.67 ± 14.75% to 19.51 ± 7.32%. The remaining isolates displayed limited inhibition.

Notably, VOCs exerted both inhibitory and stimulatory effects, depending on the pathogen. For example, *C. gloeosporioides* RC178 was inhibited by VOCs from DSF149 at a rate of 24.51 ± 5.94%, whereas VOCs from DSF005, DSF007, DSF044, and DSF073 significantly promoted mycelial growth by approximately 25%. Similarly, the growth of *G. pseudoferreum* GP030 was weakly promoted by VOCs from DSF078 and DSF109. Conversely, VOCs from DSF044 strongly inhibited *G. pseudoferreum* GP030, achieving a maximum inhibition of 45.00 ± 14.74%.

Collectively, these results demonstrate that fungal VOCs can both inhibit and stimulate growth of phytopathogens, underscoring the ecological complexity of VOC-driven fungal antagonism. Therefore, it is necessary to evaluate multiple antagonistic mechanisms when screening deep-sea fungi for biocontrol applications.

4. Discussion

We systematically assessed culturable fungal diversity from deep-sea sediments and evaluated their potential as agricultural biocontrol agents. Using a multifaceted isolation strategy, we isolated 159 fungal strains representing 35 genera. Our approach was highly effective at accessing a broad spectrum of culturable taxa by combining different dilution and stamping methods. ITS sequence analysis identified 34 isolates with similarities below 95%, indicating novel species. Importantly, this potential novelty was observed across sampling sites from different deep-sea regions, rather than being restricted to a single geographic area. These findings are consistent with the growing consensus that deep-sea ecosystems are underexplored biodiversity hotspots with significant potential for biotechnological and agricultural bioprospecting [3,46]. Ascomycota was the dominant phylum of

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culturable fungi, with *Cladosporium*, *Penicillium*, and *Aspergillus* as the most prevalent genera. This taxonomic profile aligns with reports from diverse marine environments and reflects the well-documented metabolic versatility and secondary metabolite–producing capacity of these taxa [47,48].

The results of in vitro antagonism assays provide compelling preliminary evidence supporting the biocontrol potential of deep-sea-derived fungi. Among the 23 representative isolates screened, 82.6% inhibited at least one of the tested phytopathogens (*C. gloeosporioides*, G. pseudoferreum, and *P. noxius*), which are responsible for economically important plant diseases worldwide. Isolates DSF005, DSF007, DSF044, DSF059, DSF073, and DSF109 consistently exhibited strong and broad-spectrum antagonistic activity in dual-culture assays. Notably, DSF044 inhibited the mycelial growth of *G. pseudoferreum* GP030 at an exceptionally high level (92.72% ± 0.00%), whereas DSF073 suppressed *C. gloeosporioides* 171-1 by 81.96 ± 3.40%. DSF005 strongly inhibited *P. noxius* Pn006 at a rate of 78.82 ± 2.04%. These inhibition levels are comparable to, and in some cases exceed, those reported for well-characterized terrestrial biocontrol fungi [49], suggesting that deep-sea fungi may have evolved potent antifungal strategies in response to long-term exposure to extreme conditions of high hydrostatic pressure, low temperature, and chronic nutrient limitation.

Analyses of culture filtrates and VOCs revealed that deep-sea fungi employ multiple and distinct antagonistic mechanisms. In several cases, the culture filtrates were more inhibitory against phytopathogens than direct fungal exposure, as exemplified by the DSF044 filtrate, which suppressed *P. noxius* Pn006 by 74.12%, highlighting the important role of diffusible secondary metabolites. In addition, VOCs produced by certain isolates inhibited only specific pathogens independently; for instance, VOCs from DSF044 inhibited *G. pseudoferreum* GP030 by 45%. Because of the volatile nature of these compounds, no direct physical contact is required, indicating biotechnological applications such as disease suppression in enclosed environments as environmentally benign alternatives to chemical fumigants [50]. Notably, we also observed that VOCs from some deep-sea fungi promoted the growth of specific pathogens, such as *C. gloeosporioides* RC178. This bidirectional effect underscores the complexity of VOC-mediated fungal interactions and suggests the involvement of nuanced chemical ecological mechanisms. These findings emphasize the need for a nuanced approach (i.e., considering both inhibitory and stimulatory effects) when evaluating fungal VOCs for biocontrol applications [51].

We acknowledge several limitations of the present study. First, our culture-dependent approach likely underestimates the true diversity of deep-sea fungal communities, given the prevalence of unculturable taxa in extreme environments. Future studies should incorporate culture-independent techniques, such as metagenomics and metatranscriptomics, to provide a more comprehensive understanding of community structure and functional potential [11]. Second, all antagonism assays were conducted in vitro, which cannot fully replicate the complexity of natural or agricultural ecosystems, where microbial interactions, host plant responses, and environmental variables interact dynamically [52]. Consequently, applying our results to field conditions should be undertaken with caution. This limitation should be addressed by performing greenhouse and field trials to assess biocontrol efficacy, environmental persistence, and ecological safety. Finally, the development and optimization of deep-sea fungi–based biocontrol strategies will require efforts to characterize both the chemical nature of the active metabolites and their molecular mechanisms of action.

5. Conclusion

This study employed a targeted screening approach to isolate and characterize culturable fungi from deep-sea sediments and evaluate their antagonistic activity against phytopathogens. The resulting diverse collection of isolates includes several strains with low ITS sequence similarity to known species, underscoring the view that the deep-sea environment is a reservoir of previously undescribed fungal diversity. The fungal community was dominated by genera such as *Penicillium* and *Aspergillus*, and included strains that are exhibited both specific and broad-spectrum inhibition against phytopathogens. Antagonistic activity was mediated by a combination of direct mycelial interaction, diffusible metabolites, and VOCs, highlighting multiple means of

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pathogen suppression. Notably, we also observed opposing (i.e., inhibitory and stimulatory) VOC-mediated effects, reflecting the complex chemical ecology of fungal interactions, which underscores the necessity of comprehensive screening in biocontrol agent selection. Results of our in vitro assays suggest that deep-sea fungi may be a promising source of novel biocontrol strategies. Our findings provide a foundation for future studies focusing on metabolite identification, mode-of-action analyses, and validation under controlled and field conditions.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org. Supplementary Table S1. Sampling details of deep-sea sediments, including site coordinates, depth, and sample type. Supplementary Table S2. Comparison of different pretreatment and culture methods for the isolation of culturable fungi from deep-sea sediments. Supplementary Table S3. Identification and BLAST results of fungal isolates from deep-sea sediments. Figure S1. Heatmap of genus-level distribution across sampling sites (A) and isolation methods (B). Figure S2. Beta diversity analysis of culturable fungal communities, (A) by sampling site (B) by isolation method.

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Data Availability Statement: The data generated in this study have been deposited in the GenBank database. The high-quality raw genome sequence data are available in the NCBI database under accession numbers PQ302328-PQ302480, PX857774-PX857779.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

GPY	Glucose-peptone-yeast
ITS	Internal transcribed spacer
MGI	Mycelial growth inhibition
PCoA	Principal coordinate analysis
PDA	Potato Dextrose Agar
VOC	Volatile organic compounds

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