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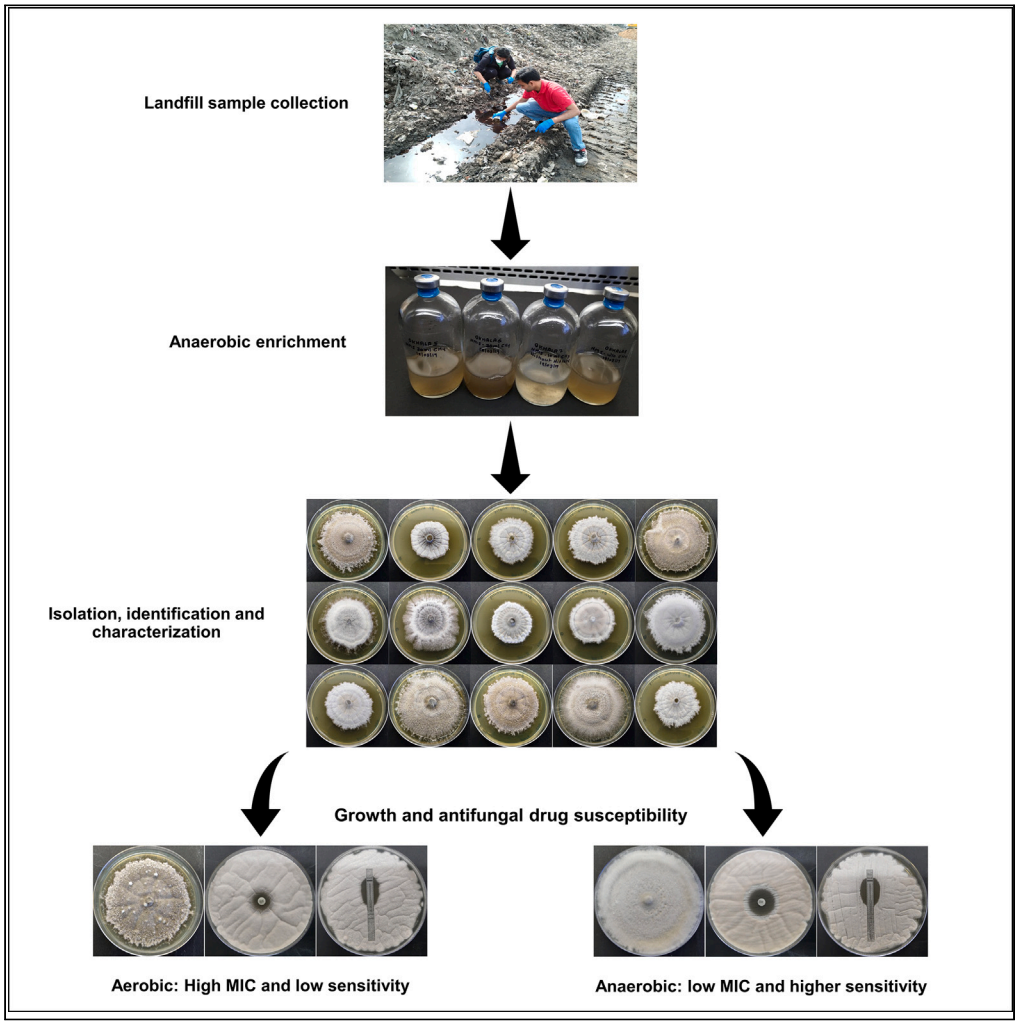

Krishna K. Yadav, Yogesh Nimonkar, Stefan J. Green, ..., Rohit Sharma, Vinay R. Rale, Om Prakash

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Highlights

Pathogenic fungi, *S. apiospermum*, can grow under strict anaerobic conditions

Drug sensitivity of *S. apiospermum* increases 2- to 4-fold under anaerobic conditions

Amphotericin-B is effective against *S. apiospermum* only under anaerobic conditions

Low sporulation and very light pigmentation under anaerobic conditions

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Anaerobic growth and drug susceptibility of versatile fungal pathogen *Scedosporium apiospermum*

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SUMMARY

Although severe cases of invasive mycoses of different hypoxic and anoxic body parts have been reported, growth and drug susceptibility of fungal pathogens under anaerobic conditions remains understudied. The current study evaluated anaerobic growth potential and drug susceptibility of environmental *Scedosporium apiospermum* isolates under aerobic and anaerobic conditions. All tested strains showed equivalent growth and higher sensitivity to tested antifungal drugs under anaerobic conditions with lower minimum inhibitory concentration (MIC) as compared to aerobic conditions. Antifungal azoles were effective against isolates under both aerobic and anaerobic conditions. Most strains were resistant to antifungal echinocandins and polyenes under aerobic conditions but exhibited sensitivity under anaerobic conditions. This study provides evidence that resistance of *S. apiospermum* to antifungal drugs varies with oxygen concentration and availability and suggests re-evaluating clinical breakpoints for antifungal compounds to treat invasive fungal infections more effectively.

INTRODUCTION

Scedosporium apiospermum and *Pseudallescheria boydii* are ubiquitous environmental molds known to cause severe invasive fungal diseases called scedosporiosis and pseudallescheriasis.[1] Although frequently reported as opportunistic pathogens,[2] they appear to have intrinsic resistance against the traditional polyene class of antifungal drugs like amphotericin-B. Scedosporiosis cases are rare in healthy individuals but prevalent in immunocompromised patients.[3] Thus, individuals with diabetes mellitus, cystic fibrosis, renal failure, acquired immune deficiency syndrome (AIDS), undergoing hematopoietic stem cell, solid organ transplant or chemotherapy, long-term corticosteroid or immunosuppressive therapies, and peritoneal dialysis are at increased risk of developing *S. apiospermum* infection. Systemic fungal infections like invasive scedosporiosis can be severe and life-threatening, with high mortality and morbidity (~50%).[4,5] The high cost of treatment and long term of therapy makes the situation even more precarious in developing countries. Antifungal agents are the primary therapy for systemic fungal infections, followed by surgical removal of infected tissue.[4,6–8] Although this approach is generally successful in cases of superficial fungal infections, the rate of failure increases in cases of invasive fungal infections of hypoxic or anoxic areas including abscesses of lungs, brain, and gastrointestinal tracts.[9]

Like most other fungal pathogens, *S. apiospermum* can cause a severe infection of different deep internal organs like the heart, lungs, brain, kidneys, sinuses, eyes, bones, gastrointestinal tract, and central nervous system.[3] Even in healthy individuals, the oxygen concentration or partial pressure (pO_2) in different organs varies greatly, ranging from less than 2% (15 mmHg) to a maximum of 14% (100 mmHg) in the alveoli of healthy lungs, which is far less than that of normal atmospheric oxygen pressure (21% or 160 mmHg) in which fungal pathogens usually reside.[10,11] In patients, infection-induced hypoxia or anoxia has been reported, and can result in a drop in oxygen concentration at infection sites to below 2% (0–1%).[11] Thus, fungal pathogens experience drastic changes in oxygen concentration when infecting hosts; and must have mechanisms to overcome this stress condition (hypoxia and anoxia) for growth and replication.[11]

Although frequently reported from hypoxic or anoxic host infections, the anaerobic nature of fungal pathogens remains understudied. In part, this is due to the general misconception that fungi, being eukaryotes, cannot grow anaerobically. However, several studies have demonstrated that some clinically relevant fungi can tolerate or grow under hypoxic or anoxic conditions, and the response of antifungal compounds

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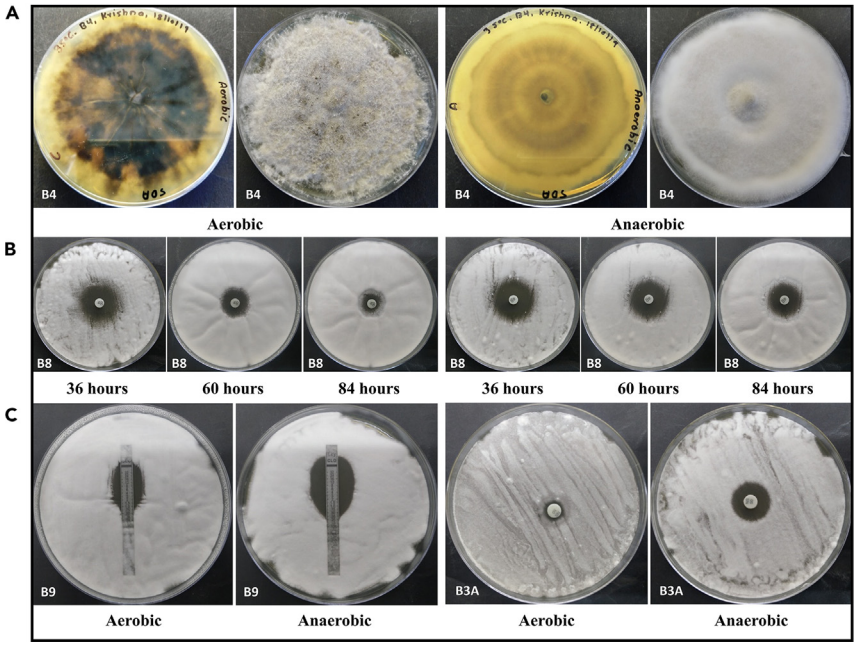

Figure 1. Growth and antifungal drug susceptibility of *S. apiospermum* strains under aerobic and anaerobic conditions

(A) Morphological details of fungal strain B4 on SDA medium at 35°C after 30 days of incubation. Under aerobic conditions, more spores and black pigment were observed, while fungal strains grown under anaerobic conditions did not produce visible spores or pigmentation.

(B) Representative images of *S. apiospermum* (strain B8) longitudinal antifungal susceptibility tests (AFST) performed with clotrimazole (10 μg/disc) under aerobic and anaerobic conditions. Increased incubation time led to decreased inhibition zones under aerobic conditions, while no similar effect was observed under anaerobic conditions.

(C) Representative images from antifungal susceptibility tests and minimum inhibitory concentration (MIC) assays performed on *S. apiospermum* strains (B3A and B9) under aerobic and anaerobic conditions. Selected strains showing lower MICs and larger zone of clearance under anaerobic conditions for clotrimazole (strain B9), and nystatin (strain B3A) are shown. Statistical analyses are shown in Table 2 for all strains and tested compounds.

is affected by oxygen concentrations.[11] Clinical breakpoints of antifungals have been primarily derived from aerobic minimum inhibitory concentration (MIC) data, leading to a knowledge gap in appropriate dosages for hypoxic or anoxic infections. Inappropriate dosages can lead to the development of conditional drug tolerance and resistance with failure of antifungal therapy and concomitant high morbidity and mortality rates.

This knowledge gap, coupled with an outbreak of mucormycosis after the second wave of COVID-19 in India and the failure of antifungal therapy,[12] led us to explore anaerobic growth and drug susceptibility of invasive fungal pathogens from the species *S. apiospermum*. This study aims to characterize the growth response and susceptibility of versatile fungal pathogen *S. apiospermum* against different classes of antifungal drugs under aerobic and anaerobic conditions. The result of the study can be used to develop new clinical breakpoints and treatments for antifungal therapy.

RESULTS

Enrichment, purification, and phylogenetic analysis

Fifteen strains of *S. apiospermum* were isolated from Okhala landfill samples and purified; strains were differentiated by growth patterns and pigmentation behaviors (Figures 1 and S1–S5). All strains were identified through sequencing of the internal transcribed spacer (ITS) region, and all strains had greater than 98.2% similarity over at least 550 bases relative to *S. apiospermum* strain CBS 117407 (Table S1). All strains clustered with *S. apiospermum* strain CBS 117407 and were distinct from other species of the genus *Scedosporium* and *Pseudallescheria* (Figure S9). Strains grew most rapidly on Czapek Dox agar (CDA), with less rapid but still robust growth on oatmeal agar (OA) and sabouraud dextrose agar (SDA). As growth characteristics of strains were most distinct and consistent on SDA, and growth was robust, SDA was employed for all further analyses (Figure S10). All strains grew at temperatures from 15°C to 40°C with optimal growth between 30°C and 35°C; growth rates decreased substantially at 40°C (Figure S11). Growth was seen at all NaCl concentrations tested (0–4%) but was optimal without NaCl (Figure S12). Growth was observed at all tested pH levels (5–11), but optimal growth was obtained at pH 9 and 11 (Figure S13).

Growth under aerobic and anaerobic conditions

Except for strains B6A and B8, the growth rate of all strains was faster under anaerobic condition than aerobic condition (Figures 2 and S1–S4). Initially pigmentation was absent in fungal strains grown under anaerobic conditions (~15 days), while dark greyish colored mycelial growth and

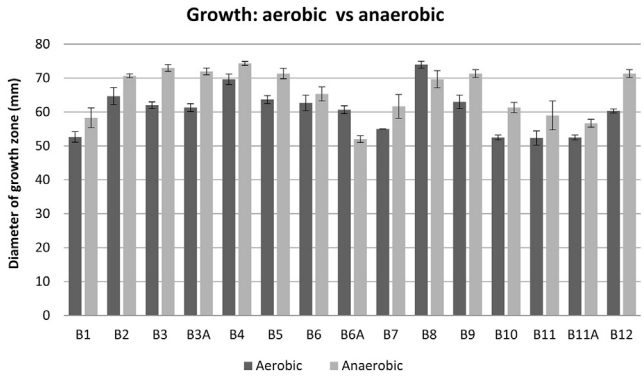

Figure 2. Comparative growth of *S. apiospermum* strains under aerobic and anaerobic conditions on SDA agar plates at 35°C

Triplicate growth zone measurements were taken after 15 days of incubation, and the mean diameter of growth is plotted with standard deviation.

pigmentation was observed for strains grown under aerobic conditions (Figures 1 and S1–S5). Longer incubations (>30 days) produced dark pigmentation in these strains under both aerobic (black and brown) and anaerobic (yellowish brown) growth conditions (Figures 1 and S5).

Fungal spores were observed visually under aerobic conditions after 15 days of incubation while no spores were visually observed under anaerobic conditions, even after 30 days of incubation. Although not visually detected, spores could be detected under anaerobic growth conditions using microscopy, though spore density was much lower under anaerobic conditions (Figures 3A and 3B). The observed spores and mycelial structures of the isolated strains were typical of *S. apiospermum* (Figures 3A and 3B). All strains produced three types of spores (globose, slightly elongated [ovoid], and elongated [oblong/cylindrical]) under aerobic condition, whereas under anaerobic conditions (except strain B4), all strains produced globose and ovoid spores in greater number than elongated spores (Figures 3A and 3B). Strain B4 produced only elongated (oblong/cylindrical) spores under anaerobic condition. Amylase, cellulase, and pectinase were produced under aerobic and anaerobic conditions, while polyphenol oxidase was produced only under aerobic conditions (Figures S14 and S15).

Antifungal susceptibility testing (AFST) and MIC

Isolated strains of *S. apiospermum* were tested against a variety of antifungal agents, under aerobic and anaerobic conditions. Strains were more sensitive to antifungal agents (i.e., lower MICs) under anaerobic conditions relative to aerobic conditions for most tested antifungal agents (Tables 1 and 2; Table S2; Figures 1 and S6–S8). Variation in susceptibility to antifungal agents was observed among the different isolated strains of *S. apiospermum*. Under anaerobic conditions, larger (1.5X to 2.5X) inhibition zones were observed as compared to aerobic conditions. All strains were sensitive to most azole antifungal compounds under both aerobic and anaerobic conditions (Table 1). Resistance to fluconazole (25 μg/mL) under aerobic conditions was observed for strains B1, B7, B9, B10, B11, B11A, and B12, while these strains were sensitive to fluconazole under anaerobic conditions (Table 1). Similar results were observed for nystatin (50 μg/mL) and amphotericin-B (50 μg/mL) in the AFST (Table 1; Figures 1 and S6). In the case of nystatin, strains B10 and B11A showed complete resistance under aerobic conditions but sensitivity under anaerobic conditions (Table 1; Figures 1 and S6). Except for strain B5, all isolated strains showed complete resistance toward amphotericin-B under aerobic conditions and sensitivity under anaerobic conditions (Table 1; Figures 1 and S6).

In antifungal susceptibility tests (AFST) we observed more prominent inhibition zones (i.e., higher susceptibility) under anaerobic conditions relative to aerobic conditions. Similarly, in MIC assays we observed lower MIC values under anaerobic conditions than aerobic (Table 2; Figures 1 and S7). MIC values were, on average, approximately 2- to 3-fold lower under anaerobic conditions than aerobic conditions, indicating that azoles, echinocandins, and amphotericin-B were more effective against *S. apiospermum* under anaerobic conditions (Table 2; Figures S6–S8). All tested strains were resistant to amphotericin-B under aerobic conditions but sensitive under anaerobic conditions except strains B6, B6A, and B8 (Table 2). Strains B6 and B6A were also resistant to fluconazole and itraconazole under aerobic conditions. Under anaerobic conditions, all strains were sensitive to the echinocandin class of antifungal drugs, but under aerobic conditions, many showed complete resistance (Table 2; Figures 1 and S7). All fungal strains showed complete resistance to the antifungal compound's nystatin, griseofulvin, flucytosine, and terbinafine at tested concentrations (0.002–32 μg/mL) in MIC assays under aerobic and anaerobic conditions (Table S2). In AFST, we observed that the size of inhibition zones decreased over time under aerobic conditions while remaining unchanged under anaerobic conditions (Figure S8). These data suggest that selected antibiotics remained active for a longer duration under anaerobic conditions relative to aerobic conditions.

DISCUSSION

The phenotypic and phylogenetic analyses conducted in this study confirmed that all examined isolates are strains of the opportunistic fungal pathogen species *S. apiospermum*. *S. apiospermum* can cause localized infections of the skin, sinuses, bones, joints, thyroid abscess, mycetoma, diabetic ulcer, erythema, nodular infarction, eumycetoma, and corneal keratitis as well as invasive infections of deep internal organs such as the heart, central nervous system (CNS), lungs, upper urinary tract, liver, gastrointestinal tract, and kidneys.[3] Many of these infection

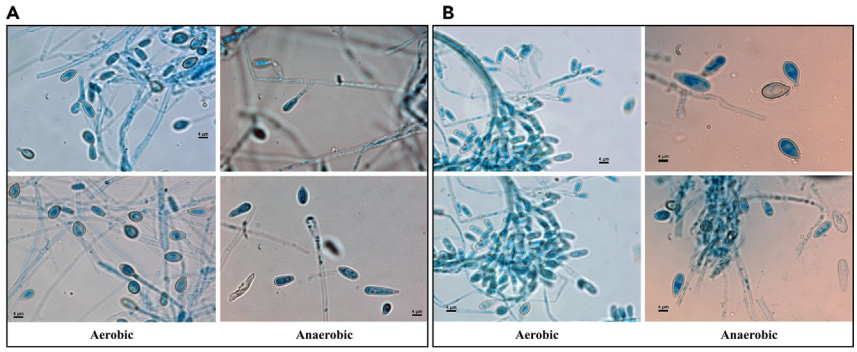

Figure 3. Representative microscopic images of *S. apiospermum* mycelia and spores grown aerobically and anaerobically

Staining was performed using lactophenol cotton blue. The number of spores was visually much lower under anaerobic conditions relative to aerobic conditions. Scale bars represent 4 μm.

(A) Mycelia and spores from *S. apiospermum* strain B4.

(B) Mycelia and spores from *S. apiospermum* strain B8.

sites become either hypoxic or anoxic during infection,[11,13] which is indicative of the anaerobic growth potential of *S. apiospermum*. de Hoog et al. (1994) demonstrated the facultative anaerobic nature of *Pseudallescheria boydii*, the teleomorphic state of *S. apiospermum*, and related species *S. prolificans*.[14] We confirm the findings of de Hoog et al. (1994) and demonstrate that *S. apiospermum* has equal or better growth abilities under hypoxic or anoxic conditions relative to aerobic conditions. Initial infections may occur under aerobic conditions. However, the eventual development of infection-induced hypoxia or anoxia does not arrest the proliferation due to the facultative nature of the pathogen and may lead to fatal infections. Prior studies have reported the intrinsic resistance of *S. apiospermum* against polyenes and variable susceptibility to other available antifungal drugs.[2,4] However, the characteristics of infections and responses to antifungal compounds under anaerobic conditions still needs to be reported. Based on the results of this study, we hypothesize that the robust growth of *S. apiospermum* under anaerobic conditions is the main culprit behind its widespread infections of hypoxic or anoxic pockets of the body. We recently demonstrated differential growth and sensitivity of bacterial pathogens to antimicrobial compounds under aerobic and anaerobic conditions.[15] Consequently, we investigated whether putative fungal pathogens show similar responses. We confirmed that all the previous drug sensitivity studies against fungal pathogens were performed only under aerobic conditions except for one study of *Saccharomyces cerevisiae*.[16] There is a general perception that fungi mainly use mitochondrial respiration and rarely grow under anaerobic conditions.[16] As a result, hypoxic or anoxic fungal AFST and MIC data are conspicuously lacking. Our results represent the first clear demonstration of the variable response of fungal pathogens to antifungals under aerobic and anaerobic conditions. We observed that antifungal azoles and polyenes were more effective against our *S. apiospermum* isolates under anaerobic conditions relative to aerobic conditions.

Primary antifungal therapy failure cases are increasing leading to fungal infections with high morbidity and mortality rate despite high sensitivity of fungal isolates to antifungal compounds tested in clinical laboratory set-up under aerobic condition.[9,17] Conditional morphological and physiological changes in pathogens induced by hypoxic or anoxic conditions of infection sites may alter drug susceptibility and tolerance patterns.[9,17–26] Compared to genetic mutations, the rate of development of phenotypic heterogeneity due to changes in growth conditions and environment is 10–90% higher in fungi.[17] In this study, we show that fungal growth under anaerobic conditions alters pigmentation and sporulation of the tested organisms. Altered growth conditions could also change the metabolic repertoire of the organisms leading to altered growth rate, cell wall composition, and lipid synthesis.[16] These modifications, driven by differential gene expression, may alter the sensitivity of fungal pathogens to antifungal agents.[11] For example, we observed dramatically lower (2- to 4-fold lower) MICs of azoles under anaerobic conditions relative to aerobic conditions. Similarly, strains were susceptible to high concentrations of amphotericin-B under anaerobic conditions but resistant under aerobic conditions.

The mechanism(s) of antifungal resistance and altered response to antifungals under aerobic and anaerobic conditions for *S. apiospermum* has yet to be fully understood. Alterations in antifungal drug targets due to mutation, over- or under-expression of genes encoding efflux pumps or drug target proteins have been demonstrated for altered drug susceptibility, tolerance, and resistance in other fungal pathogens.[17] However, the effect of oxygen concentrations on drug susceptibility has not been well-studied.[13,27] However, in other fungal studies, oxygen has been shown to have a decisive role in these resistance mechanisms. For example, ergosterol is the primary target of azoles and polyenes. Therefore, over- and under-expression of the *Erg*11 genes and enzymes involved directly or indirectly in ergosterol biosynthesis could affect fungal ergosterol biosynthesis and, consequently, the sensitivity toward the drug. Using *S. cerevisiae*, Sud and Feingold (1981) demonstrated that anoxic conditions block ergosterol biosynthesis, leading to high sensitivity to Azole and Polyenes.[16] Due to this, the fungistatic effect of imidazoles against *S. cerevisiae* under aerobic conditions changes to a fungicidal effect under anaerobic conditions. However, the *S. apiospermum* strains tested in this study showed 3- to 4-fold higher sensitivity to azoles under anaerobic conditions relative to aerobic conditions. Similarly, the efficacy of amphotericin-B (polyenes) increased several fold under anaerobic conditions relative to aerobic conditions. The combination of anaerobic conditions and ergosterol-targeting antifungals is synergistic. Inhibition of ergosterol biosynthesis under anaerobic conditions likely results in its reduced accumulation in cell membranes of *S. apiospermum*, making them prone

Table 1. Results of antifungal sensitivity tests using disc-diffusion assays under aerobic and anaerobic conditions

	Mean diameter of zone of inhibition (mm)																							
	Azoles																							
	Imidazoles									Triazoles									Polyenes					
	Clotrimazole 10 μg/mL			Ketoconazole 50 μg/mL			Miconazole 50 μg/mL			Voriconazole 1 μg/mL			Itraconazole 30 μg/mL			Fluconazole 25 μg/mL			Nystatin 50 μg/mL			Amphotericin-B 50 μg/mL		
Fungal strains	A	An	P-value	A	An	P-value	A	An	P-value	A	An	P-value	A	An	P-value	A	An	P-value	A	An	P-value	A	An	P-value
B1	23	28	0.02	34	33	0.6	30	30	1	39	46	0.002	16	33	0	R	46	0	10	14	0.001	R	10	0
B2	23	31	0.001	37	36	0.3	29	29	1	45	49	0.01	18	36	0	21	44	0	13	20	0.002	R	14	0
B3	18	25	0.007	28	32	0.005	24	26	0.06	35	49	0	16	36	0.0001	17	37	0.0001	12	19	0.004	R	13	0
B3A	17	26	0.002	31	36	0.03	27	28	0.1	39	47	0.0002	15	38	0	20	34	0	10	19	0	R	13	0
B4	24	29	0.001	34	37	0.02	30	31	0.2	44	50	0.009	22	32	0.002	21	39	0.0002	11	17	0.001	R	11	0
B5	25	29	0.01	38	38	0.4	33	33	1	46	51	0.02	26	35	0.0002	29	41	0.0004	14	19	0	11	11	0.4
B6	22	29	0.001	33	34	0.4	29	29	1	47	52	0.02	19	37	0	29	42	0.009	9	14	0.0004	R	R	0
B6A	23	32	0.0001	31	33	1.4	26	29	0.02	42	51	0.0003	23	33	0.0002	27	41	0.0004	10	14	0.002	R	R	0
B7	25	28	0.008	35	38	0.05	32	32	0.4	42	50	0.0006	24	33	0.002	R	37	0	11	21	0.0001	R	13	0
B8	22	28	0.001	36	39	0.05	30	31	0.2	46	52	0.006	20	32	0.0001	29	37	0.02	12	17	0.0002	R	10	0
B9	17	25	0.0006	31	34	0.06	26	28	0.03	39	48	0.007	18	34	0	R	33	0	9	19	0	R	13	0
B10	23	28	0.05	35	37	0.2	26	30	0.005	41	47	0.0001	23	32	0.002	R	28	0	R	15	0	R	9	0
B11	21	27	0.009	35	37	0.07	27	32	0.04	41	48	0.001	23	35	0.0008	R	30	0	9	17	0	R	10	0
B11A	23	27	0.05	35	37	0.2	31	31	1	43	49	0.0005	24	33	0.0002	R	35	0	R	16	0	R	9	0
B12	17	26	0.004	31	35	0.03	26	30	0.005	40	49	0.002	20	37	0.0001	R	36	0	10	20	0.0001	R	13	0

Values are the mean diameter of zone of inhibition for strains grown in triplicate under aerobic and anaerobic conditions. Values are rounded a maximum of four significant digits. (**A** - aerobic condition, **An** - anaerobic condition, **R** - Resistant).

Table 2. Results of antifungal sensitivity tests using MIC strip tests under aerobic and anaerobic conditions

	Azoles																														
	Imidazoles									Triazoles												Echinocandins								Polyenes		
	Clotrimazole			Ketoconazole			Miconazole			Voriconazole			Posaconazole			Itraconazole			Fluconazole			Caspofungin			Anidulafungin			Micafungin			Amphotericin-B		
Fungal strains	A	An	p value	A	An	p value	A	An	p value	A	An	p value	A	An	p value	A	An	p value	A	An	p value	A	An	p value	A	An	p value	A	An	p value	A	An	p value
B1	0.5	0.2	0	0.4	0.3	0	0.5	0.4	0.11	0.07	0.06	0.37	0.5	0.3	0.019	1.5	0.8	0.005	2.7	1.2	0.016	0.9	0.5	0.007	0.8	0.3	0	R	R	0	R	16	0
B2	0.5	0.3	0	0.4	0.4	0	0.5	0.4	0	0.05	0.04	0.17	0.4	0.4	0	3.7	1.0	0.001	0.5	0.3	0.019	0.8	0.8	0	0.2	0.15	0.11	R	1.0	0	R	8	0
B3	0.4	0.3	0	0.5	0.3	0	1.0	0.3	0.0001	0.10	0.05	0.08	0.8	0.3	0	3.0	1.5	0	1.7	0.8	0.005	1.3	0.4	0.006	0.5	0.2	0	R	1.0	0	R	16	0
B3A	0.8	0.3	0	0.8	0.3	0	1.0	0.4	0	0.11	0.06	0.008	0.6	0.2	0.009	2.7	1.0	0.007	3.3	1.7	0.011	R	1.3	0	0.2	0.13	0.013	R	1.0	0	R	16	0
B4	0.4	0.2	0	0.3	0.2	0	0.25	0.3	0.37	0.06	0.05	0.23	0.5	0.3	0.047	0.8	1.0	0	4	1.3	0.00009	R	0.2	0	R	0.13	0	R	1.5	0	R	16	0
B5	0.5	0.3	0	0.4	0.3	0	0.5	0.4	0	0.05	0.04	1	0.4	0.2	0	0.5	0.5	0	1.7	0.3	0.002	R	0.5	0	0.002	0.01	0.003	R	1.0	0	R	16	0
B6	0.8	0.4	0	0.5	0.4	0	1.0	0.4	0	0.06	0.05	1	1.0	0.3	0	R	2.0	0	R	6.0	0	R	0.2	0	0.11	0.007	0.0005	1	1.5	0	R	R	0
B6A	0.8	0.3	0	0.5	0.3	0	0.6	0.3	0.02	0.06	0.06	0	0.7	0.3	0.007	R	1.3	0	R	3.7	0	R	0.4	0	0.05	0.002	0	2	3.0	0	R	R	0
B7	0.4	0.1	0	0.5	0.2	0	0.5	0.5	0	0.05	0.04	1	0.4	0.2	0.0006	1.5	0.8	0	4.7	1.5	0.009	0.7	0.3	0.024	0.3	0.02	0.002	0.4	0.8	0	R	12	0
B8	0.5	0.2	0	0.5	0.1	0	0.5	0.4	0	0.06	0.03	0.03	0.5	0.2	0.003	1.5	1.5	0	12	5.3	0.0006	R	0.3	0	0.005	0.002	0.017	0.5	2.0	0	R	R	0
B9	0.8	0.3	0	0.5	0.3	0	0.5	0.3	0.009	0.05	0.05	1	0.4	0.2	0	1.0	0.4	0	3.3	0.8	0.002	0.5	0.2	0.0001	R	0.004	0	1.5	0.8	0	R	8	0
B10	0.5	0.3	0	0.5	0.3	0	0.5	0.4	0.12	0.06	0.05	1	0.4	0.2	0	1.0	0.9	0.37	4.0	1.3	0.00009	0.9	0.5	0.008	0.5	0.15	0.002	0.8	1.0	0	R	8	0
B11	0.5	0.2	0	0.5	0.2	0	0.5	0.4	0	0.04	0.03	0.23	0.4	0.2	0	0.8	0.5	0	4.0	1.2	0.00007	0.6	0.5	0.37	0.4	0.2	0.005	0.8	1.0	0	R	24	0
B11A	0.5	0.2	0	0.5	0.2	0	0.4	0.3	0.37	0.06	0.03	0	0.7	0.2	0.005	0.8	0.4	0.001	4.0	1.83	0.0002	0.9	0.9	1	0.08	0.2	0.005	0.8	0.8	0	R	24	0
B12	0.8	0.3	0	0.9	0.2	0	0.5	0.3	0	0.08	0.06	0.09	0.5	0.2	0	1.5	0.8	0.027	4.7	1.2	0.007	1.2	0.5	0.016	0.2	0.05	0.002	1.0	1.0	0	R	8	0
Mean	0.6	0.3	NA	0.5	0.3	NA	0.6	0.4	NA	0.06	0.05	NA	0.5	0.2	NA	1.6	1.0	NA	3.9	1.9	NA	0.9	0.5	NA	0.25	0.1	NA	1.0	1.2	NA	R	14	NA
SD	0.2	0.07	NA	0.2	0.08	NA	0.2	0.06	NA	0.02	0.01	NA	0.2	0.06	NA	1	0.5	NA	2.7	1.7	NA	0.3	0.3	NA	0.2	0.1	NA	0.47	0.6	NA	0	5.8	NA
Std. Error	0.04	0.02	NA	0.04	0.02	NA	0.06	0.02	NA	0.005	0.003	NA	0.05	0.02	NA	0.3	0.1	NA	0.8	0.4	NA	0.09	0.08	NA	0.07	0.02	NA	0.1	0.2	NA	0	1.7	NA
F-stat	46.8			31.1			12.6			9.1			36.7			4.6			5.5			9			5.4			1.5			0		
p value	0			0			0.001			0.005			0			0.04			0.03			0.006			0.03			0.2			0		

Values are mean MIC (μg/mL) for fungal strains grown in triplicate under aerobic and anaerobic conditions. Each MIC strip contained drug concentrations ranging from 0.002 μg/mL to 32 μg/mL. Values are rounded to a maximum of four significant digits. (**A** – Aerobic, **An** – Anaerobic, **R** – Resistant, **NA** – Not applicable).

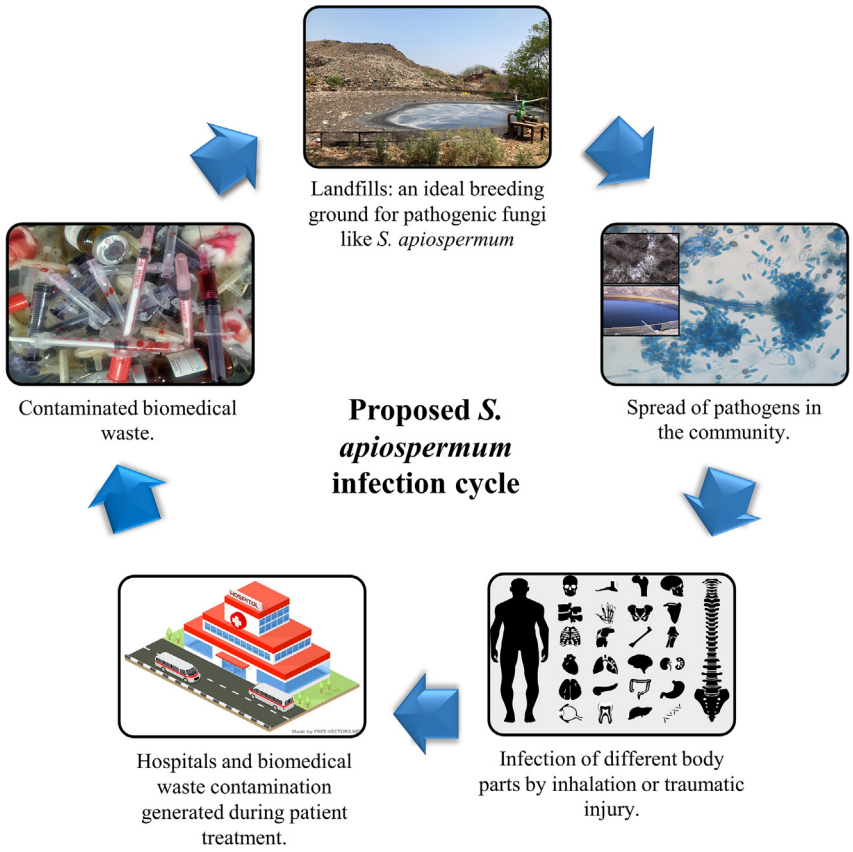

Figure 4. Proposed infection cycle of *S. apiospermum*

to lysis and more susceptible to antifungal drugs. Consequently, the killing efficiency can increase several fold in the absence of oxygen. We also noticed that the killing efficiency of the antifungal compounds was arrested after 3–4 days of incubation in aerobic conditions. In contrast, this potential was not affected under anaerobic conditions and remains unchanged. We hypothesize that under aerobic conditions, the generation of reactive oxygen species (ROS) and positive redox potential of the medium induces oxidative damage leading to degradation of drugs. In contrast, the absence of ROS and negative redox potential of the medium prevent oxidative damage under anaerobic conditions, leading to prolonged efficacy of the antifungal compounds. This is an exciting observation but needs additional verification.

Since fungal cells are eukaryotic, prolonged therapy of invasive fungal infections with antifungal drugs exerts adverse toxic effects on the host organs, especially the liver and kidneys.[28] Thus, we anticipate a need to re-evaluate clinical breakpoints for antifungal drugs leading to lower doses for prolonged therapy of invasive fungal infections in hypoxic or anoxic environments (e.g., abscesses of lungs, brains, and abdomens) leading to successful treatment with lower toxicity. In addition, our data unequivocally demonstrate that amphotericin-B is effective against *S. apiospermum* strains under anaerobic conditions while ineffective under aerobic conditions. We recommend that this aspect be considered in treating infections of hypoxic or anoxic body parts caused by *Scedosporium*, particularly in combination with azoles.

Since the *Scedosporium* strains characterized in this study were isolated from landfill materials, we hypothesize that poorly managed landfills can serve as breeding grounds for *Scedosporium* and may serve a focal spreading point to landfill workers and consequently nearby communities. In addition, landfill leachates can reach freshwater resources (e.g., groundwater, streams, etc.) and agricultural irrigation drawing from these waters could further disseminate these pathogens. We depict possible routes of contamination and spread in Figure 4. More positively, landfill materials with facultative fungal taxa, including *Scedosporium*, could serve as biotechnological resources containing organisms with a broad repertoire of enzymes functioning under anaerobic conditions. These organisms also likely contribute to the degradation of complex organic *in situ* in deeper anoxic layers of landfills.

In conclusion, we isolated 15 strains of *S. apiospermum* from landfill samples. AFST showed that azoles and amphotericin-B were more effective against fungal growth under anaerobic conditions with very low MICs relative to growth under aerobic conditions. This study demonstrates the need for setting new clinical breakpoints for antifungal compounds with a lower dose for prolonged therapy when infections are located in anoxic or hypoxic body sites. We hypothesize that the facultative anaerobic growth of *S. apiospermum* substantially contributes to its pathogenesis and allows the transition of these fungi from host to the environment and back to host through intermediaries such as landfills. This is the first study demonstrating the differential sensitivity to antifungal compounds by fungal pathogens under aerobic and anaerobic

conditions and emphasizing the urgent need to re-evaluate fungal response to antimicrobials under anaerobic conditions across a broader range of taxa.

Limitations of the study

This study examined *S. apiospermum* isolates from a single landfill site, and further study of other more distantly related and clinically relevant strains is necessary to evaluate response to antifungal compounds under anaerobic conditions. Analyses were performed in pure culture under optimal growth conditions, and the interaction of antifungal compounds with fungal pathogens will differ *in situ* relative to culture conditions. Thus, a future extension of this study should be conducted using simulated physiological conditions of infection sites in terms of buffering redox potential and oxygen conditions for clinically better AFST data.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108304.

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AUTHOR CONTRIBUTIONS

Conceptualization, O.P.; Methodology, O.P. and K.K.Y.; Sampling and data curation, K.K.Y. and Y.N.; Investigation, K.K.Y.; Writing – Original Draft, O.P. and K.K.Y.; Writing – Review & Editing, S.J.G., S.D., R.S., M.N.D., and V.R.R.; Funding Acquisition, O.P.; Resources, O.P.; Supervision, O.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

1. McCarthy, M.W., Katragkou, A., Iosifidis, E., Roilides, E., and Walsh, T.J. (2018). Recent Advances in the Treatment of Scedosporiosis and Fusariosis. JoF *4*, 73. https://doi.org/10.3390/jof4020073.
2. Goldman, C., Akiyama, M.J., Torres, J., Louie, E., and Meehan, S.A. (2016). Scedosporium apiospermum infections and the role of combination antifungal therapy and GM-CSF: A case report and review of the literature. Med. Mycol. Case Rep. *11*, 40–43. https://doi.org/10.1016/j.mmcr.2016.04.005.
3. Cortez, K.J., Roilides, E., Quiroz-Telles, F., Meletiadis, J., Antachopoulos, C., Knudsen, T., Buchanan, W., Milanovich, J., Sutton, D.A., Fothergill, A., et al. (2008). Infections Caused by *Scedosporium* spp. Clin. Microbiol. Rev. *21*, 157–197. https://doi.org/10.1128/CMR.00039-07.
4. Perlin, D.S., Rautemaa-Richardson, R., and Alastruey-Izquierdo, A. (2017). The global problem of antifungal resistance: prevalence, mechanisms, and management. Lancet

Infect. Dis. *17*, e383–e392. https://doi.org/10.1016/S1473-3099(17)30316-X.

5. Limper, A.H., Knox, K.S., Sarosi, G.A., Ampel, N.M., Bennett, J.E., Catanzaro, A., Davies, S.F., Dismukes, W.E., Hage, C.A., Marr, K.A., et al. (2011). An Official American Thoracic Society Statement: Treatment of Fungal Infections in Adult Pulmonary and Critical Care Patients. Am. J. Respir. Crit. Care Med. *183*, 96–128. https://doi.org/10.1164/rccm.2008-740ST.
6. Pappas, P.G., Lionakis, M.S., Arendrup, M.C., Ostrosky-Zeichner, L., and Kullberg, B.J. (2018). Invasive candidiasis. Nat. Rev. Dis. Primers *4*, 18026. https://doi.org/10.1038/nrdp.2018.26.
7. Taj-Aldeen, S.J., Gamaletsou, M.N., Rammaert, B., Sipsas, N.V., Zeller, V., Roilides, E., Kontoyiannis, D.P., Henry, M., Petraitis, V., Moriyama, B., et al. (2017). Bone and joint infections caused by mucormycetes: A challenging osteoarticular mycosis of the twenty-first century. Med. Mycol. *55*, 691–704. https://doi.org/10.1093/mmy/myw136.
8. Nam, S.H., Chung, Y.-S., Choi, Y.J., Lee, J.H., and Kim, J.H. (2020). Treatment outcomes in acute invasive fungal rhinosinusitis extending to the extrasinonasal area. Sci. Rep. *10*, 3688. https://doi.org/10.1038/s41598-020-60719-7.
9. Fisher, M.C., Alastruey-Izquierdo, A., Berman, J., Bicanic, T., Bignell, E.M., Bowyer, P., Bromley, M., Brüggemann, R., Garber, G., Cornely, O.A., et al. (2022). Tackling the emerging threat of antifungal resistance to human health. Nat. Rev. Microbiol. *20*, 557–571. https://doi.org/10.1038/s41579-022-00720-1.
10. McKeown, S.R. (2014). Defining normoxia, physoxia and hypoxia in tumours—implications for treatment response. BJR *87*, 20130676. https://doi.org/10.1259/bjr.20130676.
11. Chung, H., and Lee, Y.-H. (2020). Hypoxia: A Double-Edged Sword During Fungal Pathogenesis? Front. Microbiol. *11*, 1920. https://doi.org/10.3389/fmicb.2020.01920.
12. Stone, N., Gupta, N., and Schwartz, I. (2021). Mucormycosis: time to address this deadly fungal infection. Lancet. Microbe *2*, e343–e344. https://doi.org/10.1016/S2666-5247(21)00148-8.
13. André, A.C., Laborde, M., and Marteyn, B.S. (2022). The battle for oxygen during bacterial and fungal infections. Trends Microbiol. *30*, 643–653. https://doi.org/10.1016/j.tim.2022.01.002.
14. de Hoog, G.S., Marvin-Sikkema, F.D., Lahpoor, G.A., Gottschall, J.C., Prins, R.A., and Guého, E. (1994). Ecology and physiology of the emerging opportunistic fungi Pseudallescheria boydii and Scedosporium prolificans: Ökologie und Physiologie der opportunistischen Pilze Pseudallescheria boydii und Scedosporium prolificans. Mycoses *37*, 71–78. https://doi.org/10.1111/j.1439-0507.1994.tb00780.x.
15. Kovale, L., Nimonkar, Y.S., Green, S.J., Shouche, Y.S., and Prakash, O. (2021). Antibiotic susceptibility of human gut-derived facultative anaerobic bacteria is different under aerobic versus anaerobic test conditions. Microbes Infect. *23*, 104847. https://doi.org/10.1016/j.micinf.2021.104847.
16. Sud, I.J., and Feingold, D.S. (1981). Mechanisms of Action of the Antimycotic Imidazoles. J. Invest. Dermatol. *76*, 438–441. https://doi.org/10.1111/1523-1747.ep12521036.
17. Berman, J., and Krysan, D.J. (2020). Drug resistance and tolerance in fungi. Nat. Rev. Microbiol. *18*, 319–331. https://doi.org/10.1038/s41579-019-0322-2.
18. Ernst, J.F., and Tielker, D. (2009). Responses to hypoxia in fungal pathogens. Cell Microbiol. *11*, 183–190. https://doi.org/10.1111/j.1462-5822.2008.01259.x.
19. Grahl, N., and Cramer, R.A. (2010). Regulation of hypoxia adaptation: an overlooked virulence attribute of pathogenic fungi? Med. Mycol. *48*, 1–15. https://doi.org/10.3109/13693780902947342.
20. Grahl, N., Puttikamonkul, S., Macdonald, J.M., Gamcsik, M.P., Ngo, L.Y., Hohl, T.M., and Cramer, R.A. (2011). In vivo Hypoxia and a Fungal Alcohol Dehydrogenase Influence the Pathogenesis of Invasive Pulmonary Aspergillosis. PLoS Pathog. *7*, e1002145. https://doi.org/10.1371/journal.ppat.1002145.
21. Grahl, N., Shepardson, K.M., Chung, D., and Cramer, R.A. (2012). Hypoxia and Fungal Pathogenesis: To Air or Not To Air? Eukaryot. Cell *11*, 560–570. https://doi.org/10.1128/EC.00031-12.
22. Schaffer, K., and Taylor, C.T. (2015). The impact of hypoxia on bacterial infection. FEBS J. *282*, 2260–2266. https://doi.org/10.1111/febs.13270.
23. Gresnigt, M.S., Rekiki, A., Rasid, O., Savers, A., Jouvion, G., Dannaoui, E., Parlato, M., Fitting, C., Brock, M., Cavaillon, J.-M., et al. (2016). Reducing hypoxia and inflammation during invasive pulmonary aspergillosis by targeting the Interleukin-1 receptor. Sci. Rep. *6*, 26490. https://doi.org/10.1038/srep26490.
24. Li, Z., and Nielsen, K. (2017). Morphology Changes in Human Fungal Pathogens upon Interaction with the Host. JoF *3*, 66. https://doi.org/10.3390/jof3040066.
25. Kowalski, C.H., Morelli, K.A., Schultz, D., Nadell, C.D., and Cramer, R.A. (2020). Fungal biofilm architecture produces hypoxic microenvironments that drive antifungal resistance. Proc. Natl. Acad. Sci. USA *117*, 22473–22483. https://doi.org/10.1073/pnas.2003700117.
26. Patra, S., Raney, M., Pareek, A., and Kaur, R. (2022). Epigenetic Regulation of Antifungal Drug Resistance. JoF *8*, 875. https://doi.org/10.3390/jof8080875.
27. Gupta, S., Laskar, N., and Kadouri, D.E. (2016). Evaluating the Effect of Oxygen Concentrations on Antibiotic Sensitivity, Growth, and Biofilm Formation of Human Pathogens. Microbiol. Insights *9*, 37–46. https://doi.org/10.4137/MBI.S40767.
28. Bellmann, R., and Smuszkiewicz, P. (2017). Pharmacokinetics of antifungal drugs: practical implications for optimized treatment of patients. Infection *45*, 737–779. https://doi.org/10.1007/s15010-017-1042-z.
29. Burland, T.G. (1999). DNASTAR's lasergene sequence analysis software. In Bioinformatics Methods and Protocols, S. Misener and S.A. Krawetz, eds. (Humana Press), pp. 71–91. https://doi.org/10.1385/1-59259-192-2:71.
30. Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389–3402. https://doi.org/10.1093/nar/25.17.3389.
31. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. *28*, 2731–2739. https://doi.org/10.1093/molbev/msr121.
32. Sharma, R., Prakash, O., Sonawane, M.S., Nimonkar, Y., Golellu, P.B., and Sharma, R. (2016). Diversity and Distribution of Phenol Oxidase Producing Fungi from Soda Lake and Description of Curvularia lonarensis sp. nov. Front. Microbiol. *7*, 1847. https://doi.org/10.3389/fmicb.2016.01847.
33. Prakash, O., and Ranade, D.R. (2021). Technical consideration for cultivation and purification of anaerobic microorganisms. In Anaerobes and Anaerobic Processes, O. Prakash and D.R. Ranade, eds. (CRC Press, Taylor & Francis Group).
34. Floch, C., Alarcon-Gutiérrez, E., and Criquet, S. (2007). ABTS assay of phenol oxidase activity in soil. J. Microbiol. Methods *71*, 319–324. https://doi.org/10.1016/j.mimet.2007.09.020.
35. Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (2020). Recommandations Version 1.2. https://www.sfm-microbiologie.org/wp-content/uploads/2020/10/CASFM2020_Octobre2020_V1.2.pdf.
36. Berkow, E.L., Lockhart, S.R., and Ostrosky-Zeichner, L. (2020). Antifungal Susceptibility Testing: Current Approaches. Clin. Microbiol. Rev. *33*, e00069-19. https://doi.org/10.1128/CMR.00069-19.
37. Szekely, A., Johnson, E.M., and Warnock, D.W. (1999). Comparison of E-Test and Broth Microdilution Methods for Antifungal Drug Susceptibility Testing of Molds. J. Clin. Microbiol. *37*, 1480–1483. https://doi.org/10.1128/JCM.37.5.1480-1483.1999.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
HM Itraconazole	HiMedia	Cat#EM073
HM Ketoconazole	HiMedia	Cat#EM074
HM Fluconazole	HiMedia	Cat#EM072
HM Clotrimazole	HiMedia	Cat#EM144
HM Miconazole	HiMedia	Cat#EM146
HM Voriconazole	HiMedia	Cat#EM086
HM Posaconazole	HiMedia	Cat#EM120
HM Nystatin	HiMedia	Cat#EM145
HM Amphotericin-B	HiMedia	Cat#EM071
HM Micafungin	HiMedia	Cat#EM121
HM Anidulafungin	HiMedia	Cat#EM122
HM Caspofungin	HiMedia	Cat#EM119
HM Flucytosine	HiMedia	Cat#EM118
HM Griseofulvin	HiMedia	Cat#EM143
HM Terbinafine	HiMedia	Cat#EM142
HM Fluconazole	HiMedia	Cat#SD232
HM Voriconazole	HiMedia	Cat#SD277
HM Amphotericin-B	HiMedia	Cat#SD270
HM Clotrimazole	HiMedia	Cat#SD115
HM Itraconazole	HiMedia	Cat#SD276
HM Ketoconazole	HiMedia	Cat#SD274
HM Miconazole	HiMedia	Cat#SD272
HM Nystatin	HiMedia	Cat#SD271
Mueller Hinton agar with 2% glucose with methylene Blue	HiMedia	Cat#M1825
2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt	HiMedia	Cat#RM9270
Deposited data		
ITS region sequence data	This paper	GenBank: OP520726-OP520740
Software and algorithms		
SeqMan	Burland[29]	https://www.dnastar.com/software/lasergene/seqman-ngen/
NCBI-BLAST	Altschul et al.[30]	https://blast.ncbi.nlm.nih.gov/Blast.cgi
MEGA7	Tamura et al.[31]	https://www.megasoftware.net/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Om Prakash (prakas1974@gmail.com, dyhead_scccs@siu.edu.in).

Materials availability

This study did not generate any new unique material or reagents.

Data and code availability

- The ITS region sequences of fungal isolates were submitted to GenBank and are publicly available as of the date of publication. Accession numbers listed in the key resources table and Table S1.
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

This study does not include any model organisms or cell lines. All the fifteen strains of *S. apiospermum* used in this study were isolated from anaerobic enrichment initiated using Okhla landfill samples. All the strains are submitted to National Centre for Microbial Resource, Pune, India and their accession numbers are provided in Table S1.

METHOD DETAILS

Enrichment, isolation, and purification of fungi

Fungal strains were isolated from the Okhla landfill (Latitude: 28° 38′ 41.2800" N, Longitude: 77° 13′ 0.1956" E) in Delhi, India. Anaerobic enrichment was initiated using nitrate mineral salt (NMS) medium and samples collected from Okhla landfill in 125 ml serum vials with mix (N_2:CO_2:H_2 - 85:10:5) gas in head space. For isolation, enriched media (six-month-old enrichment) was spot inoculated and spread on fresh anaerobic potato dextrose agar (PDA) and NMS agar medium. Plates were incubated at 35°C in an anaerobic chamber (ThermoFisher) filled with mixed gas (N_2:CO_2:H_2 - 85:10:5) for ten days. Fungal colonies were purified by successive inoculation on fresh PDA plates using the hyphal tip method.[32] All anaerobic media used in this study was prepared using strict anoxic and aseptic techniques.[33]

Molecular identification and phylogenetic analysis of isolated fungi

DNA was isolated from pure cultures and the internal transcribed spacer (ITS) region was PCR amplified as described previously.[32] Amplicons were sequenced using capillary electrophoresis (3730 DNA Analyser), and contigs were generated using the software package SeqMan.[29] The most similar sequences in the NCBI GenBank database were identified using BLAST,[30] and phylogenetic tree construction was performed using the software package *MEGA7*.[31]

Optimization of media and growth conditions

The growth of fungal strains on five different media was tested, including Oatmeal Agar (OA), Malt Extract Agar (MEA), Czapek Dox Agar (CDA), Sabouraud Dextrose Agar (SDA) and PDA. Freshly grown fungal discs (8 mm) were inoculated in the center of plates and incubated at 30°C under aerobic conditions, as described above. After fifteen days, the diameter of the fungal growth was measured. All tested strains grew optimally on SDA plates. Therefore, subsequent physiological tests were conducted using SDA. For determination of optimal conditions of pH, temperature, and salinity, fungal discs were inoculated on SDA plates in replicate and incubated at a range of temperatures (5 to 40°C), pH (5, 7, 9, and 11) and NaCl concentrations (0 to 4%). Appropriate pH buffers such as citrate, phosphate (pH-5 and pH-7), and bicarbonate (pH-9 and pH-11) were used to maintain pH. Growth response was measured after fifteen days of incubation, as described above.

Comparison of aerobic and anaerobic growth

To study aerobic and anaerobic growth response, discs of fungal strains were inoculated in the center of SDA plates and incubated at 35°C under aerobic (BOD incubator) and anaerobic conditions (anaerobic chamber) for 15 days; growth was measured periodically. Morphological changes like pigmentation, mycelial morphology, and variations in the sporulation patterns were also assessed. Sporulation patterns and structures were studied by staining mycelia with lactophenol cotton blue and observing stained specimens using a phase contrast light microscope (Olympus BX53 Digital Microscope). Enzyme production under aerobic and anaerobic conditions was also assessed. Aerobic and anaerobic media containing starch, carboxymethylcellulose, and pectin were prepared for amylase, cellulase, and pectinase tests. Plates were inoculated and incubated under aerobic and anaerobic conditions for four days. Amylase, cellulase and pectinase activities were tested using iodine solution. Polyphenol oxidase (PPO) production was tested using the ABTS (2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay.[34] Water agar plates containing ABTS (2 mmol) were prepared. Plates were inoculated and incubated under both (aerobic and anaerobic) conditions and production of a green colour zone surrounding growth as a result of PPO activity was measured. All tests and observations were conducted in triplicate.

Determination of antifungal drug susceptibility and minimum inhibitory concentration (MIC) values under aerobic and anaerobic conditions

Aerobic and anaerobic antifungal drug susceptibility testing was conducted as described previously(Kovale et al., 2021),[15] following standards and guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). A disc diffusion assay was conducted to test drug susceptibility. Fungal strains were grown on SDA plates for 15 days to ensure good sporulation. Spore suspensions were prepared in normal saline and turbidity was adjusted to yield 1×10^6 to 5×10^6 spores per ml (*i.e.*, 0.5 McFarland standards). Subsequently, 200 µl of spore inoculum was plated onto modified Mueller Hinton Agar plates with 2% glucose + 0.5 µg ml-1 methylene blue.[35–37] Plates were dried for

10 minutes, antifungal discs were emplaced, and plates were subsequently incubated at 35°C under aerobic and anaerobic conditions as described above. Plates were examined after 36 hours of incubation and zones of inhibition measured. Strains showing sensitivity to antifungal agents were selected for Minimum Inhibitory Concentration (MIC) analysis; resistant strains were not characterized further. For MIC measurements, Ezy MIC™ (HiMedia, Mumbai, India) strips were used according to the manufacturer's instructions (experimental setup and conditions same as AFST). Plates were incubated at 35°C for 36 hours under aerobic and anaerobic conditions, as described above. Analysis of each condition and strain was performed in triplicate.

QUANTIFICATION AND STATISTICAL ANALYSIS

All experiments in this study were carried out in triplicate. Average and standard deviation values were calculated in the software package Excel. p values and F statistics were calculated using one-way analysis of variance (ANOVA) tests.