MYCOLOGY

Prevalence and Biochemical Features of Melanized Fungi Isolated from Uli Community, Anambra State, Nigeria

F. O. Okereke[1], O. N. Enweonye[2], U. F. Onwuasoanya[3], V. N. Unaegbu[4], H. O. Ogwuegbu[4], G. C. Iheanacho[4], N. Opara-Nadi[1], C. C. Ekechukwu[5], U. C. Onwuasoanya[6]

[1]Department of Biological Sciences (Microbiology), Faculty of Natural and Applied Sciences, Spiritan University, Nneochi, Abia State, Nigeria
[2]Department of Science and Technology, Ministry of Education, Awka, Anambra State, Nigeria
[3]Department of Medical Microbiology and Public Health, Faculty of Medical Laboratory Science, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria
[4]Department of Microbiology, University of Agriculture and Environmental Sciences, Umuagwo, Imo State, Nigeria
[5]Department of Biology Education, Federal College of Education (Technical), Asaba, Delta State, Nigeria
[6]Department of Medical Laboratory Science, Faculty of Health Sciences, Chukwuemeka Odumegwu Ojukwu University, Igbariam Campus, Anambra State, Nigeria

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Abstract

Subcutaneous mycosis has been considered as mild infection previously but recent research has shown that several causative agents are highly pathogenic and could cause debilitating ailments if not properly checked. This study was conducted to evaluate the prevalence and biochemical features of melanized fungi isolated from Uli community, Anambra State, Nigeria. Two hundred and ten soil samples were collected from three communities (Umuoma, Umuaku, and Aluoha) at Uli community using soil auger. The soil samples were analyzed for the presence of melanized fungi using a standard microbiological technique followed by a prevalence enumeration of the fungal isolates using the same standard technique. The fungal isolates were characterized based on their cultural, microscopic, and biochemical features by measuring their ability to liquefy gelatin, hydrolyze skimmed milk, and utilize nitrate. Four black fungi were isolated from the three communities with variations in their occurrences (N=54). Umuoma community yielded the highest fungal isolate (22 (40.74%)) followed by Umuaku (20 (37.04%)) while the least was Aluoha (12 (22.22%)). The prevalence of the melanized fungi investigated was 31.48% and statistically, there was no significant difference (P=0.910) in the distribution of the fungal isolates in the three communities. Biochemically, *Exophiala jeanselmei* (88.2%) and *Scedosporium apiospermum* (85.7%) revealed high proteolytic activity and strong nitrate assimilation, *Ochroconis mirabilis* (58.3%) was moderate while *Cladosporium* (45.5%) showed weak proteolytic and moderate nitrate utilization. This study has shown that melanized fungi are moderately distributed in soils, and they exhibited high proteolytic activity, which indicates that they could be potent pathogens. However, further study is required to ascertain the *in vitro* and *in vivo* pathogenic potential of the melanized fungi in order to safeguard the health of individuals in the society, especially farmers in rural areas.

Keywords: Prevalence; Melanized fungi; Biochemical; Uli community

1 Introduction

Fungi are commonly found in the environment like bacteria and other microorganisms, and their functions are basically saprophytic and pathogenic, especially in individuals that have compromised immunity (Pakshir et al., 2013). Black or melanized fungi are group of fungi that have a colour

pigment in their cell wall otherwise known as melanin (Yew et al., 2014). The melanin enables them to appear dark coloured, especially on their reverse side on culture plate. The phylum of this group of fungi has been identified as ascomycota and medically important genera include *Exophiala*, *Cladosporium*, *Ochroconis*, *Scedosporium*, *Alternaria*, *Bipolaris* among others.

There is a wide distribution of melanized fungi in the environment such as in the soil, rotten wood, water, air, and hydrocarbon-contaminated soils (Yew et al., 2014). The ability of melanized fungi to survive in extreme environment has been ascribed to the presence of melanin, which provides resistance to ultraviolet light, dryness, breakdown by enzymes, high temperature, and heavy metals (Yew et al., 2014) This ability makes the fungi to be significant ecologically and of public health concern, especially in tropical and subtropical areas (Yew et al., 2014).

Previously, this group of fungi had been considered as ordinary microbes with limited knowledge on their pathogenic potentials but nowadays, the fungi have played vital roles in debilitating diseases such as mycetoma, chromoblastomycosis, phaeohyphomycosis, and allergic fungal sinusitis (Queiroz-Telles et al., 2017). Most common infection is subcutaneous though invasive and deadly cerebral phaeohyphomycosis had been widely reported (Revankar and Sutton, 2010). *Exophiala* species is a well-known cause of subcutaneous phaeohyphomycosis while *Scedosporium apiospermum* is responsible for lungs-related mycetoma and pneumonia. *Cladosporium* is the main cause of airborne allergens that activate asthma and allergic rhinitis while *Ochroconis* has been incriminated in keratitis and systemic infections in patients undergoing organ transplant (Revankar and Sutton, 2010).

In Nigeria, especially in South East geopolitical zone, rural communities, such as farmers, miners, fishermen are highly vulnerable to these infectious agents due to traumatic inoculation or by other mode of transmission. Moreover, prevalence and distribution of these agents have been limited though few studies have reported a prevalence range of 20-45% in soils (Vicente et al., 2014). Evaluating the prevalence and distribution of these fungal agents is essential for risk management and implementation of preventive measures.

It is imperative to note that melanized fungal identification based on morphology only could be cumbersome due to resemblance in colonial and microscopic features (Vicente et al., 2014) but biochemical features provide extra distinguishing features, especially on pathogenic potentials and adaptation to different ecosystem (Agrawal et al., 2021).

Despite the clinical relevance of melanized fungi, there is scarcity of data on their prevalence and biochemical features in rural communities in Anambra State, Nigeria. Most researchers in Nigeria had focused on dermatophytes and *Aspergillus* species. Moreover, there is poor correlation between biochemical virulence markers and environmental distribution patterns in soils found in Anambra State. Hence, the aim of this study was to determine the prevalence and biochemical features of melanized fungi isolated from Uli community, Anambra State. The findings from this study would provide baseline data on the environmental burden of melanized fungi in rural areas, highlight species with high virulence potential, and inform healthcare practitioners for optimum preventive measures.

2 Materials and methods

2.1 Study area

The study was conducted at Aluoha, Umuaku, Umuoma, Uli community, Ihiala Local Government Area, Anambra State.

2.2 Collection of soil samples

Two hundred and ten (210) soil samples were collected from three communities at Ihiala Local Government Area, Anambra State namely: Aluoha, Umuaku, and Umuoma. Seventy soil samples were randomly collected from different soil types (loamy, clay and sandy soil) in the three communities (Aluoha, Umuaku, and Umuoma) using a soil auger, which was sterilized using 70 % ethanol, prepared by mixing 30 mL of distilled water and 70 mL of ethanol. The soil samples were collected at the depth of 10 cm and transferred into a sterile polyethene bag. The samples were transported to the Department of Microbiology Laboratory, COOU, for immediate analysis. The samples were processed within 2 h of collection (Yew et al., 2014).

2.3 Isolation and purification of the isolates

A stock solution of the soil samples was prepared by weighing one gram using a weighing balance (DC-300), a little normal saline was added to the soil samples in a test tube, the mixture was shook and additional normal saline was added to make up 10 mL. The stock sample was serially diluted in tenfold, in which 9.0 mL of distilled water was dispensed into test tubes (pyrex) and 1.0 mL of the stock solution was dispensed into the first test tube, and was stirred after which 1.0 mL was taken from the tube using another pipette, and was transferred into the second test tube. The process continued until the last tube after which 1.0 mL was discarded. Inoculation was carried out by aseptically inoculating 0.1 mL of the diluted sample (10^{-5}) on Sabouraud Dextrose Agar (SDA) using spread plate method. The plates were incubated in an inverted position at 30±2°C for 7 days. After incubation, the grown colonies were sub-cultured by streaking a single colony aseptically on sterile poured plate (60 mm OD × 55 mm ID × 13mm high) of SDA. All the plates in triplicates were incubated inverted at 30±2°C for 7 days (Yew et al., 2014).

2.4 Identification of the isolates

The sub- cultured isolates were identified using their colonial and morphological descriptions. The colonial description was carried out to determine the appearances of the isolates on agar media plates, edges, texture, surface, and reverse pigmentation (Yew et al., 2014).

2.4.1 Slide culture technique

Slide culture technique was carried out so that the structural endowment of the fungi can be scrutinized as elucidated by Umedum and Iheukwumere (2013). Preparation of sabouraud dextrose agar (SDA) was carried out, followed by an agar block, which was cut at 4m2 area using a sterile glass slide. A Petri dish containing filter paper, glass slides (75 mm× 25 mm× 1 mm) and coverslip was put in an oven at 200 °C for 30 min. After heating, the filter papers in the Petri dishes were soaked with sterile water. The agar block was placed at the center of two slides that formed angle 90°. The colony of the fungi was inoculated at the margins of the agar, and a sterile coverslip was placed on the inoculated agar. The preparation was incubated at 30±2°C for 4 days as illustrated by Umedum and Iheukwumere (2013).

2.4.2 Lactophenol cotton blue staining

After incubation, the coverslip was removed from the agar block using a sterile forceps and was placed on a clean slide containing a drop of lactophenol cotton blue. The margin of the coverslip was sealed using candle wax to conserve the stain. The slide was then viewed using a digital microscope of X10 and X40 objective lenses (Umedum and Iheukwumere, 2013).

2.5 Biochemical characterization of the isolates

2.5.1 Gelatin liquefaction

Proteolytic activity of the isolates was evaluated using gelatin agar containing 1% gelatin, 1% of sodium chloride, 1% of peptone, 2% of agar, pH= 7.0. The medium was then autoclaved at 121 °C (15 psi) for 15 minutes and dispensed in sterile test tubes. The tubes were inoculated using an inoculum which had been standardized using 0.5 McFarland standard. An uninoculated tube was included as a negative control. The tubes were incubated at room temperature (30±2°C) for 3 weeks and read three times a week for proteolytic activity after being placed in a refrigerator at 4°C for periods of 15, 30, and 60 min (until the uninoculated gelatin solidified). Each tube was observed for liquefaction of the gelatin, determined by the failure of the substrate to solidify at 4°C as described by Agrawal et al. (2021).

2.5.2 Skimmed milk hydrolysis

Proteolytic activity of the isolates was also determined using skimmed milk broth medium containing 5% NaCl, 3% peptone, 10% skimmed milk (v/v), pH= 7.0 and 100 mL of distilled water. The medium was then autoclaved at 121 °C (15 psi) for 15 minutes while the skimmed milk was added after sterilization. The medium (5mL) was dispensed in tubes, which were later inoculated with a small amount of inoculum which had been standardized using 0.5 McFarland standard. A negative control tube was not inoculated. The inoculated tubes and the control tube were incubated at room temperature (30±2°C) for 3 weeks. The tubes were checked daily for the presence of clot formation (coagulation) or precipitation of casein found in the skimmed milk (6).

2.5.3 Nitrate utilization

The ability of the isolates to utilize nitrate as the sole source of nitrogen was determined using Czapek Dox agar (sucrose: 30 g/L; $NaNO_3$: 2g/L; K_2HPO_4: 1g/L; $MgSO_4$: 0.5g/L; KCl: 0.5g/L; $FeSO_4$: 0.010g/L; Agar: 15g/L; pH=7.00). The medium was constituted and sterilized at 121 °C (15 psi) for 15 minutes. The medium was then cooled to 45°C and dispensed (5mL) into sterile test tubes, which were slightly bent to provide large surface area for fungal growth. The test fungal isolates which had been standardized using 0.5 McFarland standard were inoculated and incubated at room temperature (35°C) for 7 days. Tubes were checked daily for the presence of fungal growth (de Hoog et al., 2011).

3 Results

3.1 Prevalence and features of the fungal isolates

The prevalence of the fungal isolates in the sampled communities is presented in Table 1. The result revealed that four melanized fungal species were isolated from soil samples, which had different occurrences in the sampled communities as elucidated below. A total of 54 fungi were isolated; Umuaku community (n=20, 37%), Umuoma community (n=22, 40.7%), and Aluoha community (n=12, 22.2%). The overall prevalence (Figure 1) showed isolate X as the most prevalent (31.48%), followed by isolate Y (25.93%), isolate M (22.22%), and the least was isolate Z (20.37%). The chi-square test of independence across communities was non-significant (P>0.05). The high P-values showed uniform distribution, which means that difference in counts is due to chance and sampling differences instead of ecological differences among communities.

The cultural and microscopic features of the fungal isolates are presented in Table 2. The result revealed that all the fungal isolates exhibited variations in colour on the selective agar media and their surface textures were mostly rough, downy and raised. The hyphae were septate and the conidia were dark and sizeable compared to other filamentous fungi that often possess tiny conidia. The reverse colour of all the fungal isolates was dark, which showed that they are black, melanized, or dematiaceous fungi.

Table 1. Prevalence of the fungal isolates in the sampled communities.

Isolate code	Umuaku n (%)	Umuoma n (%)	Aluoha n (%)	Total (N = 54)	Prevalence (%)	χ^2	p-value
X	6 (30.00)	7 (31.80)	4 (33.3)	17	31.48	0.180	0.910
Y	5 (25.00)	6 (27.30)	3 (25.00)	14	25.93	0.120	0.940
Z	4 (20.00)	5 (22.70)	2 (16.70)	11	20.37	0.220	0.890
M	5 (25.00)	4 (18.20)	3 (25.00)	12	22.22	0.150	0.920
Total	20 (37.04)	22 (40.74)	12 (22.22)	54 (100)	100		

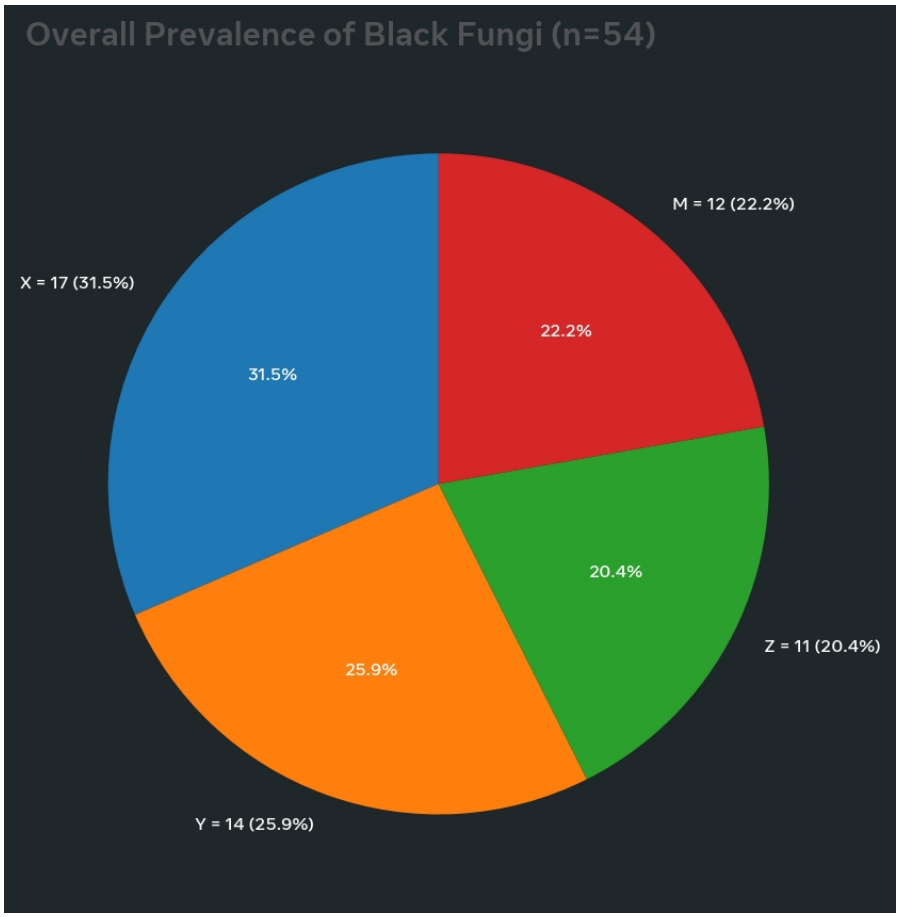


Figure 1. Overall prevalence of the fungal isolates (n = 54).

Table 2. Cultural and microscopic features of the fungal isolates.

Isolate code	Appearance on SDA	Texture and elevation	Microscopic appearance	Conidia	Reverse colour	Possible fungus
X	Dark brown	Velvety, flat, rough surface and smooth edge	Brown septate hyphae	Brownish annello-conidia	Dark brown	*Exophiala jeanselmei*
Y	Olive grey	Raised, rough surface and smooth edge	Dark septate hyphae	Dark blastoconidia	Black	*Scedosporium apiospermum*
Z	Olive brown	Velvety, downy, rough surface and smooth edge	Dark septate hyphae	Dark blastoconidia with branching chains	Black	*Cladosporium carrionii*
M	Greyish brown	Downy, rough edge and flat surface	Brown septate hyphae	Dark poroconidia	Black	*Ochroconis mirabilis*

SDA = Sabouraud dextrose agar.

3.2 Biochemical characterization of the isolates

The biochemical characterization of the fungal isolates is presented in Table 3. The result revealed that *Exophiala jeanselmei* showed the highest gelatin liquefaction (88.2%), followed by *S. apiospermum* (85.7%) and *O. mirabilis* (58.3%) while the least was *Cladosporium* (45.5%). Similar progression was followed in skimmed milk hydrolysis and nitrate assimilation although weak proteolytic activity was observed in *Cladosporium* while moderate proteolytic activity and nitrate assimilation was observed in *O. mirabilis*. Statistically, there was a significant difference ($P<0.05$) in gelatin liquefaction and skimmed milk hydrolysis among the fungal isolates while nitrate assimilation was not significant ($P>0.05$).

Table 3. Biochemical characterization of the isolates.

Fungal isolate	GL n (%)	SH n (%)	NA n (%)	Interpretation
Exophiala jeanselmei (n = 17)	15 (88.2)	14 (82.4)	16 (94.1)	Highly proteolytic and nitrate positive
Scedosporium apiospermum (n = 14)	12 (85.7)	11 (78.6)	13 (92.9)	Highly proteolytic and nitrate positive
Cladosporium (n = 11)	5 (45.5)	4 (36.4)	9 (81.8)	Weakly proteolytic, strongly nitrate positive
Ochroconis mirabilis (n = 12)	7 (58.3)	6 (50.0)	8 (66.7)	Moderately proteolytic, moderate nitrate positive
χ^2	8.34	7.89	4.12	
p-value	0.039	0.048	0.249	

GL = gelatin liquefaction; SH = skimmed milk hydrolysis; NA = nitrate assimilation.

4 Discussion

The occurrence of melanized or black fungi in soil samples is of a public health concern due to their pathogenic potentials and frequent exposure of some individuals to soil. The threat posed by subcutaneous infection associated with melanized fungi has attracted attention of several researchers resulting in their isolation and characterization, which are highly essential in disease prevention and controls. The overall prevalence (31.48%) observed in the present study revealed a substantial distribution of the infectious agents in the sampled communities, especially at Umuoma community. The variation in the occurrences of the fungal isolates in the sampled communities could be attributed to different soil types used for the study (Loamy, clay, and sandy soil). Research had revealed that loamy soil has high concentration of humus, high biodegradation, and high water-holding capacities, which are essential factors that create conducive atmosphere for the proliferation of the fungal species. Melanized fungi are generally known as saprophytes, and their high accumulation in soil sample indicates high biodegradation. This observation conforms to the finding of several researchers (Yew et al., 2014) who studied the distribution and pathogenicity of melanized fungi in soil samples. The dark reverse pigmentation of the fungal isolates could be attributed to the presence of a dark pigment known as melanin in their cell wall, which provides protection in adverse environmental condition, and also serve as a virulent factor as documented by several researchers (Pakshir et al., 2013; Yew et al., 2014; Revankar and Sutton, 2010). The four fungal species isolated from this study corroborates the isolation carried out by (1) and (2), who also isolated similar fungi using environmental samples. However, the fungal isolates observed in the present study did not conform to the finding of (4) who isolated mostly melanized fungi of pleosporales such as *Bipolaris*, *Curvularia*, and *Alternaria* species. The variation could be attributed to geographical location.

The high proteolytic activity observed in *Exophiala* could be attributed to the presence of subtilisin-like serine protease that degrades human collagen and keratin. This is a principal virulent factor for phaeohyphomycosis, which farmers and rural labourers contract via traumatic inoculation during farming activities. This observation is consistent with the findings of de Hoog et al. (2011). Similarly, high caseinase production by the fungal isolates is a clear indication of potential for keratitis and subcutaneous infection. Low milk hydrolysis observed in *Cladosporium* despite high nitrate assimilation suggests metabolic shift to nitrogen assimilation rather than protein degradation, and this ability was facilitated by an enzyme known as nitrate reductase, which enabled the fungus to optimize nitrate as sole source of nitrogen as supported by Revankar and Sutton (2010).

5 Conclusion

The present study has shown that melanized or black fungi are found in soil samples with variations in their distribution in the sampled communities. The highest number of the fungal isolate was observed at Umuoma community (22), followed by Umuaku (20) while the lowest was observed at Aluoha community (12). Fungal distribution in the three communities revealed that *Exophiala* had the highest prevalence (most common) of 31.48%. The biochemical features of the fungal isolates revealed that they produced enzymes such as caseinase, gelatinase, and nitrate reductase, which enabled them to break down skimmed milk and gelatin, and assimilate nitrate as the sole source of nitrogen, respectively. The production of these enzymes has shown that the fungal isolates are capable of posing threat to public health as far as subcutaneous infection is concerned, especially towards rural farmers and individuals that have weakened immune cells.

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