Academic Science Journal (ASJ)
Vol. 4, No. 1, January 2026, pp. 59~67
DOI: 10.24237/04.01.612

Identification study of soil fungi in different areas of Diyala governorate

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Article Info

Article history:

Received 26, 12, 2024
Revised 13, 06, 2025
Accepted 15, 12, 2025
Published 30, 01, 2026

Keywords:

soil fungi,
Morphologies,
Aspergillus,
Czapek Dox Medium (CDA).

ABSTRACT

The present study dealt with isolation and characterization of fungi from different 33 soil samples from Diyala governorate including agricultural soil, residential area soil, sanitary landfill area soil and industrial area soil). The fungi have been isolated directly by soil dilution method using sabouraud's dextrose agar (SDA), The grown fungi colonies then have been sub cultured on Czapek Dox Medium (CDA) for identifying, also the fungi have been examined microscopically with Lactophenol cotton blue stain. The results showed that 87 fungal isolates of 25 species have been identified in 33 locations, categorised over four types of soil samples, where most of the samples were from agricultural areas, followed by residential areas, then sanitary landfill areas and industrial areas, and the results have shown that the highest percentage of fungi isolates was *Aspergillus flavus* with 14 (16.09 %)) at agricultural and residential areas with 14.54% and 16.66 % respectively(followed by *Aspergillus niger* with 12 (13.79%) The third one in the isolation percentage was *Trichoderma* sp with 8 (9.19 %). Other species were in lower percentages, with presence of pathogenic to humans and animals.

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1. INTRODUCTION

Fungi are a major component of biodiversity; they are essential for the survival of other organisms and are crucial in global ecological processes [1]. Soil fungi are important components of microbial communities, which inhabit dynamic soil environments and play critical functional roles as decomposers, mutualists, toxin producers and pathogens [2][3][4]. The biodiversity refers to the variability of life on the earth, including all of the living species of animals, plants and microorganisms. It has been estimated that there are approximately 12,000 species of fungi worldwide [5], and the actual number of fungi is still unknown [6]. Fungi provide us with very useful medication products, such as antibiotics and other valuable substances, including enzymes, organic acids, pigments and secondary metabolites used in the food industry and fermentation [7][8]. Soil fungi have important roles in various biological cycles, also they influence in plant nutrition, plant health, soil structure and soil fertility [9] Despite of the importance of microorganisms in soil processes, several soilborne microbial species are known to cause plant, animal and human diseases [10][11][12] and because of the importance of fungi, this study aimed to determine the species that could be isolated from soil, and which one was the most common.

2. METHOD

2.1 Collection of soil samples:

Thirty-three soil samples have been collected from 33 different places in Diyala governorate, including (21 of agricultural field soil, 7 of residential areas, 3 of sanitary landfill area soil, and 2 of industrial soil). The soil was taken at 10 cm depth, they were sieved then the samples were kept at 10 °C until used [13].

2.2: Media preparation:

1. Sabouraud's dextrose agar (SDA) medium was prepared by dissolving (65 gm) in (1 liter) of distilled water, autoclaved for 15 minutes at 121°C, after cooling it to (45°C), chloramphenicol (0.5%) was added, then the medium has been poured into petri dishes and let to solidify at room temperature, and used in the following steps (Younus and Hussain, 2015). This medium was used for primary isolation of fungi.

2. Czapek Dox Agar (CDA) medium was prepared by dissolving (49 gm) in (1 liter) of distilled water, autoclaved for 15 minutes at 121°C, then the medium has been poured into petri dishes and let to solidify at room temperature, and used in the following steps, this medium used for routine cultivation of fungi non-sporulating moulds [14].

2.3: Isolation of fungi:

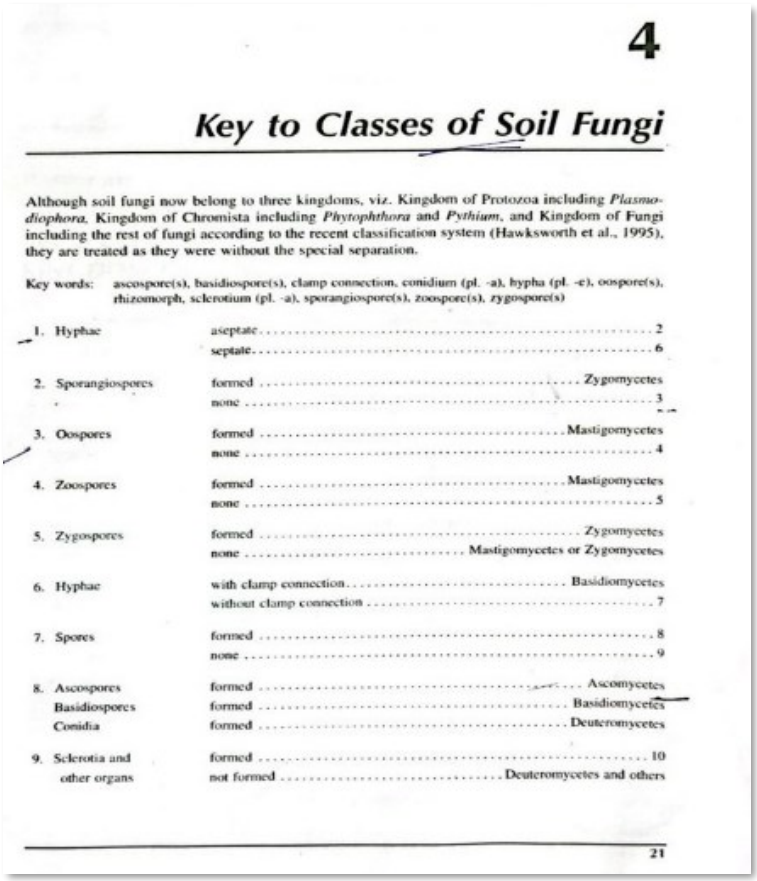
Soil dilution method have been used by using SDA medium. One gram from each soil samples was suspended in 10 ml sterile distilled water to prepare the microbial suspension. Three dilutions 10-1, 10- 2 and 10-3 have been prepared from the microbial suspension. One ml from the last dilution (10-3) was added to a sterile petri dish, then molten cooled (45°C) media of SDA were added and rotated gently to disperse the soil solution into the media, the petri dishes were incubated at 28± 2 0C, for 4-7 days (duplicate of each sample have been cultured), then the grown fungi colonies have been counted and the appearance percentage of each fungus have been recorded, according to the following equation [15].

$$Percentage\ of\ the\ fungal\ species = \frac{no.of\ fungal\ genus\ isolates * 100\ \%}{Total\ no.of\ fungal\ species\ isolates} \qquad (1)$$

The grown fungi colonies then have been sub cultured on CDA medium for identifying [14].

2.4: Identification of the soil fungi:

fungal species have been identified by observing the morphology of fungi, colony features, size, width, thickness, color and texture, other cultural characteristics such as tint in colony surface and reverse, smell or fragrance, quantity of aerial hyphae, pigment exuded and fruiting structures [16]. Also, they were examined microscopically by staining with lacto phenol cotton blue stain, observed under an optical microscope for the hyphae (septate or aseptate), conidia, conidiophores and arrangement of spores, yeasts were also diagnosed by observing the size and shape of blastopores and the sprouting cells and the way they aggregated [14]. The identification of fungi was also done with the help of literature Watanabe, (2002), the classification key presented in the above reference was used to identify some species as shown in figure 1.



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Key to Classes of Soil Fungi

Although soil fungi now belong to three kingdoms, viz. Kingdom of Protozoa including *Plasmodiophora*, Kingdom of Chromista including *Phytophthora* and *Pythium*, and Kingdom of Fungi including the rest of fungi according to the recent classification system (Hawksworth et al., 1995), they are treated as they were without the special separation.

Key words: ascospore(s), basidiospore(s), clamp connection, conidium (pl. -a), hypha (pl. -e), oospore(s), rhizomorph, sclerotium (pl. -a), sporangiospore(s), zoospore(s), zygospore(s)

1. Hyphae	aseptate	2
	septate	6
2. Sporangiospores	formed	Zygomycetes
	none	3
3. Oospores	formed	Mastigomycetes
	none	4
4. Zoospores	formed	Mastigomycetes
	none	5
5. Zygospores	formed	Zygomycetes
	none	Mastigomycetes or Zygomycetes
6. Hyphae	with clamp connection	Basidiomycetes
	without clamp connection	7
7. Spores	formed	8
	none	9
8. Ascospores	formed	Ascomycetes
Basidiospores	formed	Basidiomycetes
Conidia	formed	Deuteromycetes
9. Sclerotia and other organs	formed	10
	not formed	Deuteromycetes and others

21

Figure 1. Key to classification of soil fungi adapted from the book "Pictorial Atlas of Soil and Seed Fungi: Morphologies of Cultured Fungi and Key to Species" [16].

3. RESULTS AND DISCUSSION

The results showed that 87 fungal isolates of 25 species have been identified in 33 locations, categorised over four types of soil samples (tables 1,2,3 and 4 show the distribution of fungal species according to the soil types), where most of the samples were from agricultural areas, followed by residential areas, then sanitary landfill areas and industrial areas. Figures: 2, 3, 4 and 5 explain the distribution of isolated fungi in different locations of Diyala governorate and show the need for a broader future study for all regions in our governorate to cover the full fungal microbial content. However, the results have shown that the highest percentage of fungi isolates was *Aspergillus flavus* with 14 (16.09 %) at aagricultural and residential areas with 14.54% and 16.66 % respectively followed by *Aspergillus niger* with 12 (13.79%) The third one in the isolation percentage was *Trichoderma sp* with 8 (9.19 %) (Figure 6). The results also showed the presence of other species in lower percentages, some of which are pathogenic to humans and animals. Tables 5, 6, 7 and 8 show the percentages of fungal isolates with different areas.

The fungal diversity of any soil depends on many soil factors such as pH, organic content, and moisture [16], and there was much of a variance in the four types of soil (agricultural, residential, sanitary landfill and industrial areas of soil), the diversity was found to be higher in the agricultural as compared to other four soils [1].

Table 1: Distribution of fungi in agricultural soils

NO. OF SAMPLE (LOCATION)	DUPLICATE 1	DUPLICATE 2
1	*Rhizopus oryzae* Yeast	*Rhizopus oryzae* *Aspergillus flavus* Yeast
2	*Sakscenea* *Aspergillus flavus* *Trichoderma* sp *Penicillium* sp *Curvularia* sp	*Sakscenea* *Aspergillus flavus* *Trichoderma* sp *Penicillium* sp *Curvularia* sp
3	*Sakscenea* *Aspergillus niger* *Basidiobolus ranarum* *Gongronella butleri*	*Aspergillus niger* *Basidiobolus ranarum*
4	*Cunninghamella* *Trichoderma* sp *Fusarium* sp	*Cunninghamella* *Trichoderma* sp *Candida* sp
5	*Aspergillus flavus* *Aspergillus niger* *Rhizopus* sp	*Aspergillus flavus* *Aspergillus niger* *Rhizopus* sp
9	*Rhizopus* sp *Aspergillus niger* *Aspergillus fumigatus*	*Rhizopus* sp *Aspergillus niger* *Aspergillus fumigatus*
10	Actinomycetes *Aspergillus flavus* *Trichderma* sp	Actinomycetes *Aspergillus flavus* *Trichderma* sp
11	*Cryptococcus neoformans* *Penicillium* sp	*Cryptococcus neoformans* *Penicillium* sp
12	*Aspergillus niger*	*Aspergillus niger*
14	*Trichoderma* sp *Rhizopus stolonifer*	*Trichoderma* sp *Rhizopus stolonifer*
15	*Aspergillus flavus* *Bipolaris* sp *Trichderma* sp	*Aspergillus flavus* *Bipolaris* sp *Trichderma* sp
17	*Rhizopus stolonifer* *Aspergillus terreus*	*Rhizopus stolonifer* *Aspergillus terreus*
18	*Rhizopus stolonifer*	*Rhizopus stolonifer*
21	*Aspergillus niger* *Cryptococcus neoformans*	*Aspergillus niger* *Cryptococcus neoformans*
22	*Aspergillus flavus* *Aspergillus niger* Actinomycetes	*Aspergillus flavus* *Aspergillus niger* Actinomycetes
25	*Aspergillus niger* *Aspergillus terreus* *Aspergillus flavus*	*Aspergillus niger* *Aspergillus flavus*

26	*Aspergillus fumigatus* *Penicillium* sp *Trichderma* sp	*Penicillium* sp *Trichderma* sp
27	*Aspergillus nidulans* *Cunninghamella* sp *Rhizopus stolonifer*	*Aspergillus nidulans* *Cunninghamella* sp *Rhizopus stolonifer*
29	*Aspergillus flavus* *Cunninghamella* sp *Rhizopus microsporus*	*Cunninghamella* sp *Rhizopus microsporus*
31	*Cunninghamella* sp	*Cunninghamella* sp
33	*Cladosporium* sp	*Cladosporium* sp

Table 2: Distribution of fungi in agricultural soils

NO. OF SAMPLE (LOCATION)	DUPLICATE 1	DUPLICATE 2
6	*Cunninghamella* sp *Aspergillus niger* *Aspergillus flavus*	*Cunninghamella* sp *Aspergillus niger* *Aspergillus flavus*
7	*Rhizopus* sp *Mucor* sp *Aspergillus flavus* *Penicillium* sp *Bipolaris* sp	*Rhizopus* sp *Aspergillus flavus* *Penicillium* sp *Bipolaris* sp
16	*Mucor* sp *Trichderma* sp *Aspergillus flavus*	*Mucor* sp *Trichderma* sp *Aspergillus flavus*
19	*Cryptococcus neoformans*	*Cryptococcus neoformans*
20	*Penicillium* sp *Microsporum canis* *Cladosporium* sp	*Penicillium* sp *Microsporum canis* *Cladosporium* sp
23	Actinomycetes	Actinomycetes
30	*Cunninghamella* sp *Rhodotorulla mucillaginosa*	*Cunninghamella* sp *Rhodotorulla mucillaginosa*

Table 3: Distribution of fungi in sanitary landfill area soils

NO. OF SAMPLE (LOCATION)	DUPLICATE 1	DUPLICATE 2
24	*Aspergillus niger* *Cryptococcus neoformans* *Rhodotorulla mucillaginosa*	*Aspergillus niger* *Cryptococcus neoformans* *Rhodotorulla mucillaginosa*
28	*Cryptococcus neoformans* *Aspergillus niger* *Aspergillus flavus*	*Cryptococcus neoformans* *Aspergillus niger* *Aspergillus flavus*
32	*Trichoderma* sp *Mucor* sp *Penicillium* sp	*Trichoderma* *Mucor* sp *Penicillium* sp

Table 4: Distribution of fungi in industrial area soils

NO. OF SAMPLE (LOCATION)	DUPLICATE 1	DUPLICATE 2
8	*Aspergillus niger* *Aspergillus flavus*	*Aspergillus niger* *Aspergillus flavus*
13	*Aspergillus niger* *Aspergillus flavus* *Sakscenea* sp	*Aspergillus niger* *Aspergillus flavus* *Sakscenea* sp

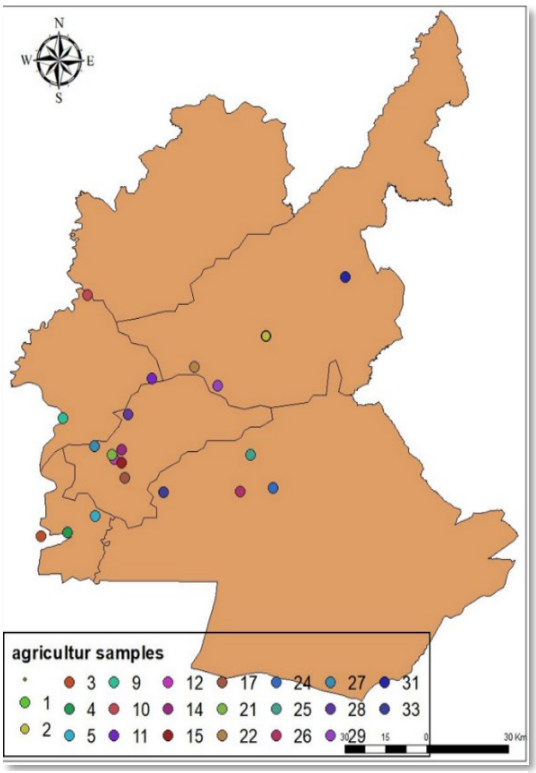

Figure 2: Distribution of fungi in some agricultural areas of soil Diyala governorate

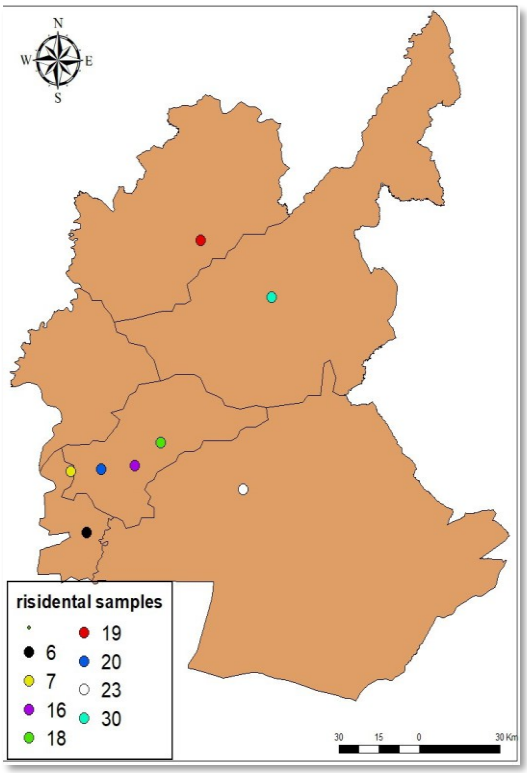

Figure 3: Distribution of fungi in some of residential areas of soil of Diyala governorate

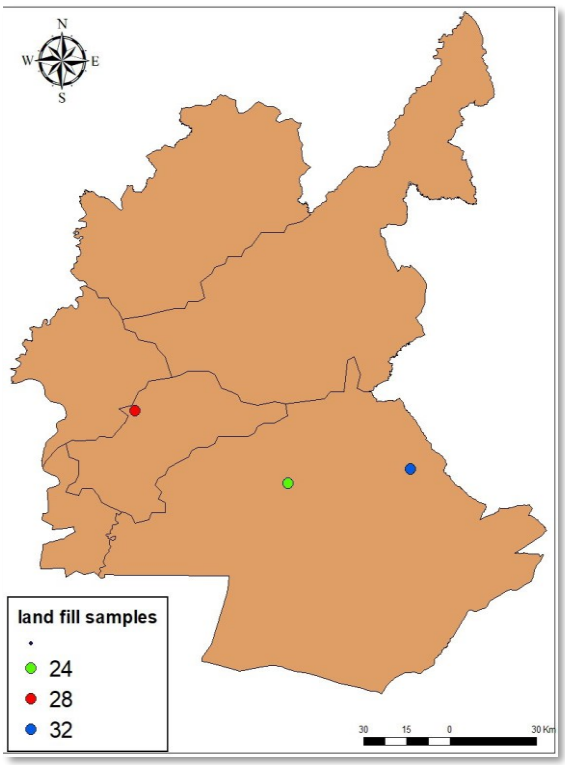

Figure 4:Distribution of fungi in some sanitary landfill areas of soil of Diyala governorate

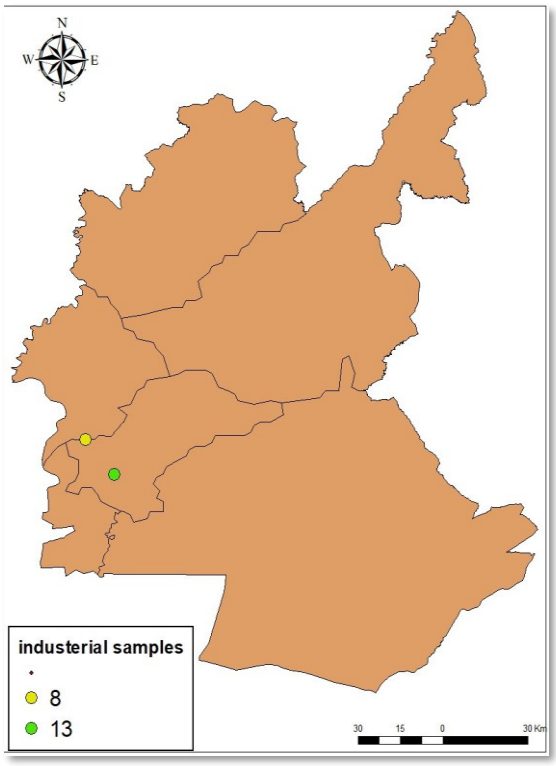

Figure 5: Distribution of fungi in industrial areas of Diyala governorate

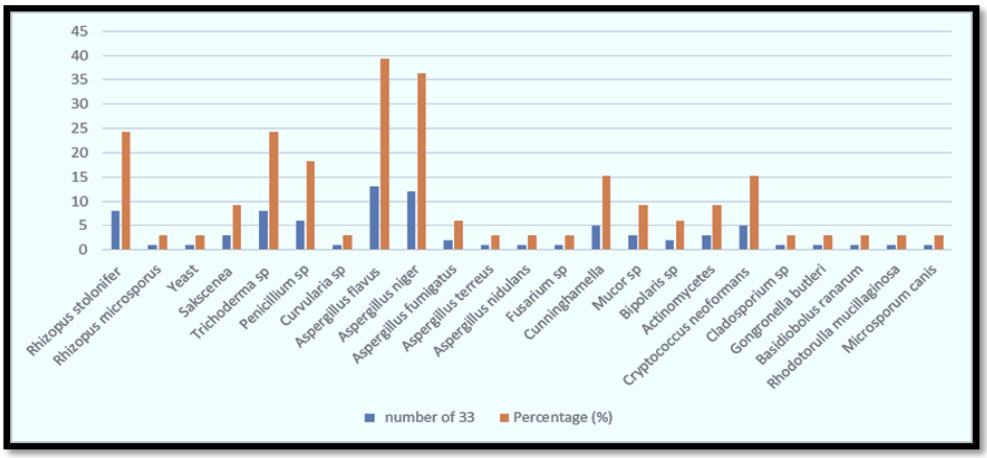

Figure 6: Distribution of fungi in different areas of Diyala soils

The results of isolating fungal species in agricultural and residential areas show the dominance of *Aspergillus flavus* (figure 7) over the rest of the species (Figure 8) with 14.54 % and 16.66 % respectively. *Aspergillus* has high enzymatic activity for various substrate nutrients [17]. *Aspergillus* species have developed a remarkable tolerance to highly stressful circumstances and the ability of *Aspergillus* species to adapt to different environments is partly due to their structural constituents like the rodlet layer in their surfaces which binds covalently to the cell wall, functions in spore dispersion and fixation to the soil [18]. Since the soil in most of Diyala areas is agricultural, even residential [19], we notice the spread of this genus (as obvious in tables 5 and 6). When studying the sanitary landfill area, two pathogenic fungi were observed to dominate the soil of sanitary landfill areas (Table 7), represented by the species *Aspergillus niger* and *Cryptococcus neoformans*, which are characterized as opportunistic pathogens [14], which explains why they are prevalent in this type of soil where medical waste is located. However, previous research have confirmed the prevalence of pathogenic fungi in the environment of sanitary landfills; a study by [20] showed the prevalence of *A. fumigatus* and other pathogenic species in soils containing medical waste.

As for the soil in the industrial sites of the governorate, it was found that there were two species of *Aspergillus* and a species of zygomycota fungi (table 8), and these results were consistent with the study conducted by Dalas *et al.,* [21], they showed the spread of fungal species in soils contaminated with crude oil (industrial soil). This indicates that *Aspergillus* species have a high ability to survive in soils contaminated with industrial hazards, as it is known that they possess the lipolytic enzyme) (lipase) [17]. Ren *et al*., (2009) explained that *A. niger* can result in a high leaching efficiency of heavy metals from a contaminated soil; they revealed to that the highest metal removal efficiencies were 97.5%, 88.2%, 26% and 14.5% for Cu, Cd, Pb and Zn, respectively [22]. Many of pathogenic species of zygomycota (figure 9) were appeared in soil sample in all locations with different percentages, and these results were identical to the findings of the study presented by Younus and Dalas *et al.,* [21] [23].

Table 5: Distribution of fungi in agricultural soils

SPECIES	ISOLATE NUMBER OF 55 ISOLATES	PERCENTAGE (%)
RHIZOPUS ORYZAE	1	1.818
ASPERGILLUS FLAVUS	8	14.54
YEASTS	1	1.818
SAKSCENEA SP	2	3.636
TRICHODERMA SP	6	10.909
PENICILLIUM SP	3	5.454
CURVULARIA SP	1	1.818
ASPERGILLUS NIGER	7	12.727
BASIDIOBOLUS RANARUM	1	1.818
GONGRONELLA BUTLERI	1	1.818
CUNNINGHAMELLA SP	4	7.272
FUSARIUM SP	1	1.818
RHIZOPUS SP	2	3.636
ASPERGILLUS FUMIGATUS	2	3.636
ACTINOMYCETES	2	3.636
CRYPTOCOCCUS NEOFORMANS	2	3.636
RHIZOPUS STOLONIFER	4	7.272
BIPOLARIS SP	1	1.818
ASPERGILLUS TERREUS	2	3.636
ASPERGILLUS NIDULANS	1	1.818
RHIZOPUS MICROSPORUS	1	1.818
CLADOSPORIUM SP	1	1.818
CANDIDA SP	1	1.818

Table 6: Distribution of fungi in residential area soils

SPECIES	ISOLATE NUMBER OF 18 ISOLATES	PERCENTAGE (%)
CUNNINGHAMELLA SP	2	11.11
ASPERGILLUS NIGER	1	5.55
ASPERGILLUS FLAVUS	3	16.66
RHIZOPUS SP	1	5.55
MUCOR SP	2	11.11
PENICILLIUM SP	2	11.11
BIPOLARIS SP	1	5.55
TRICHDERMA SP	1	5.55
CRYPTOCOCCUS NEOFORMANS	1	5.55
MICROSPORUM CANIS	1	5.55
CLADOSPORIUM SP	1	5.55
RHODOTORULLA MUCILLAGINOSA	1	5.55
ACTINOMYCETES	1	5.55

Table 7: Distribution of fungi in sanitary landfill area soils

SPECIES	ISOLATE NUMBER OF 9 ISOLATES	PERCENTAGE (%)
ASPERGILLUS NIGER	2	22.22
RHODOTORULLA MUCILLAGINOSA	1	11.11
CRYPTOCOCCUS NEOFORMANS	2	22.22
ASPERGILLUS FLAVUS	1	11.11
TRICHODERMA	1	11.11
MUCOR SP	1	11.11
PENICILLIUM SP	1	11.11

Table 8: Distribution of fungi in industrial area soils

SPECIES	ISOLATE NUMBER OF 5 ISOLATES	PERCENTAGE (%)
ASPERGILLUS NIGER	2	40
ASPERGILLUS FLAVUS	2	40
SAKSCENEA	1	20

The fungal diversity of any soil depends on a large number of factors of the soil such as pH, organic contents, and moisture [21] and there was no much of a variance in the four types of soil samples (agricultural, residential, landfill and industrial), the diversity was found to be higher in the agriculture land soils as compared to the residential, sanitary landfill and industrial sites of soils in addition to dominance of *Aspergillus* flavus in the governorate

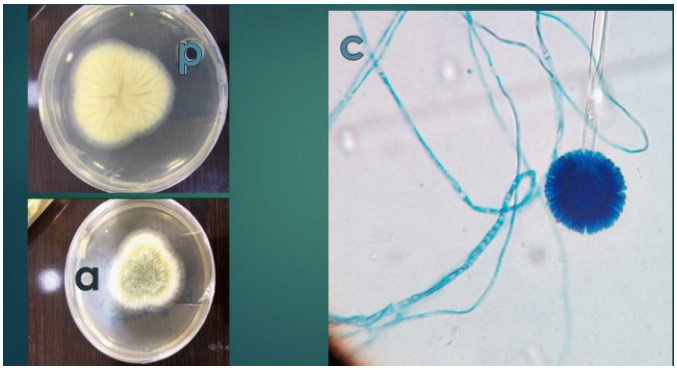

Figure 7: *Aspergillus flavus*, a- macroscopic top view grown on SDA at 28±2ºC and pH 5.6 for 7 days of incubation, b-reverse view, c –microscopic image stained with lacto phenol cotton blue at 40X.

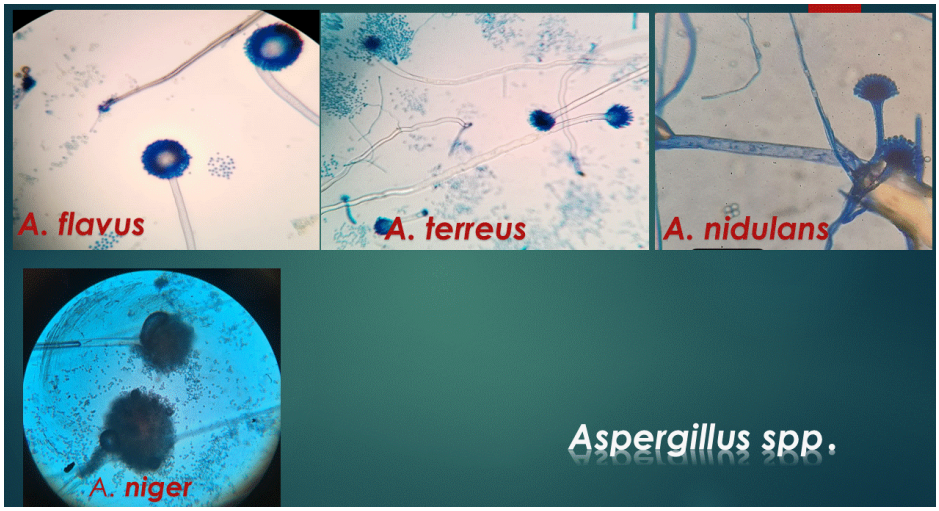

Figure 8: Microscopic image of some *Aspergillus spp* stained with lacto phenol cotton blue at 40X.

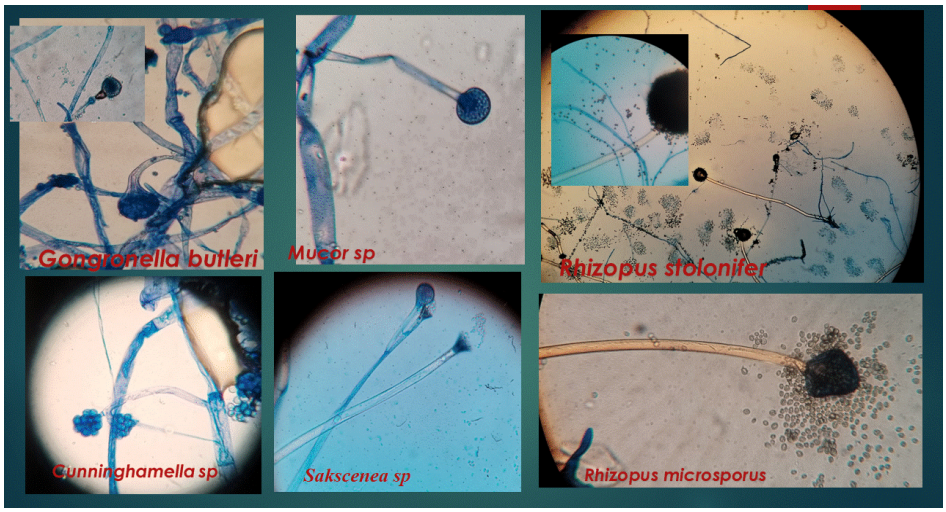

Figure 9: Microscopic image of some species of zygomycetes fungi stained with lacto phenol cotton blue at 40X.

4. CONCLUSION

Due to the large number of the fungi category spread in the soil the study will need in the future molecular techniques to identify fungal species that are difficult to distinguish phenotypically using cultural and microscopic methods and because of their important, it should receive much more attention, and more researches should be done to find any promising species that could be used in the industry, medicine and agriculture .also to preserve any important species.

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