Journal of Basic & Applied Mycology (Egypt) 4 (2013): 1-11

Otomycosis in Assiut, Egypt

A. M. Moharram[1,*], H. E. Ahmed[2] and Salma A-M. Nasr[1]

[1]Department of Botany and Microbiology, Faculty of Science
[2]Department of Otolaryngology, Faculty of Medicine, Assiut University, Assiut, Egypt

[*]Corresponding author: E-mail: ahmadmhrrm@yahoo.com
Received 20/8/2013, Accepted 7/9/2013

Abstract: During the period from October 2011 to August 2012 a total of 124 patients were clinically examined for mycotic otitis at the outpatient clinic of Otolaryngology Department, Assiut University Hospitals (AUH). The mycological analysis of ear swabs revealed the isolation of 18 fungal species and one variety belonging to 7 genera. Among the 92 mycologically-positive cases, 56 were males (60.9%) and 36 were females (39.1%). The disease was more prevalent among persons between 21-30 years where it was diagnosed in 29 patients matching 31.5% of total positive cases. *Aspergillus* infection was confirmed in 84.8 % of total cases. Fungi belonging to section *Nigri* were associated with infection of 39 cases (42.4%). Other species related to sections *Flavi* and *Terrei* were involved in 35 and 4 cases (38 % and 4.3% of the total cases respectively). *Candida* species (*C. albicans*, *C. glabrata*, *C. krusei* and *C. parapsilosis*) were involved in 16.3% of otomycotic cases. The remaining fungal species comprised *Chrysosporium keratinophilum*, *Cladosporium sphaerospermum*, *Mucor circinelloides*, *Phoma epicoccina* and *Stemphylium vesicarium* were rare etiologic agents of otomycosis. Extracellular proteolytic, lipolytic and ureolytic enzymes were produced by 95%, 76.2% and 36.6% of fungal isolates respectively. Aflatoxins were detected in cultures of 72.5% of *Aspergillus* species belonging to section *Flavi*. In-*vitro* sensitivity test showed that Terbinafine, Clotrimazole and Clove oil were the most effective antifungal agents.

Key words: Otomycosis, *Aspergillus*, *Candida*, protease, lipase, urease, aflatoxins, antifungal agents.

Introduction

Otomycosis is the fungal infection of the ear. Although it affects the external ear canal, the middle ear may be involved in case of a perforated tympanic membrane. The mastoid cavity may be affected if an open cavity mastoid surgery is performed previously (Ozcan 2011). Presence of itching in external ear canal must raise a high index of suspicion for otomycosis since it is the most frequent symptom. In some studies, itching was present in more than 90% of the patients (Ozcan *et al.* 2003a and Pradhan *et al.* 2003). Other common symptoms are otalgia, hearing loss, tinnitus and aural discharge (Ozcan *et al.* 2003a). Hearing loss and tinnitus are usually due to obstruction of the external ear canal by aural discharge or fungal hyphae. In some patients, fungal infection may supervene on bacterial external otitis and in that case itching may follow pain (Ozcan 2011).

Otomycosis shows a worldwide distribution and is particularly frequent in hot and humid regions (Kaur *et al.* 2000 and Ozcan *et al.* 2003a). The prevalence of otomycosis is as high as 54% in hot and humid regions (Than *et al.* 1980) whereas the prevalence decreases to 9% in temperate climates (Mugliston and O'Donoghue 1985). Wearing turban or other clothes on head has been reported as a risk factor for otomycosis in Bahrain, Turkey and India (Paulose *et al.* 1989, Ozcan *et al,* 2003a, Kumar 2005 and Ozcan 2011)). Swimming is also reported as a risk factor for otomycosis (Paulose *et al.* 1989, Ozcan *et al.* 2003a and Wang *et al.* 2005). Perforation of the tympanic membrane and previous ear surgery have also been reported as important risk factors for otomycosis (Falser 1984, Paulose *et al.* 1989, Ozcan *et al.* 2003a and Ho *et al.* 2006). The prevalence of dermatomycoses in patients with otomycosis was 36.5% in Turkey (Ozcan *et al.* 2003b) and 51% in India (Kumar 2005). In Turkey, 50% of patients who were refractory to otomycosis treatment had dermatomycoses such as tinea pedis and the same pathogenic fungi were isolated from dermatomycoses and otomycosis in nearly half of these patients (Ozcan *et al.* 2003b). Use of topical antibiotics has been reported as a predisposing factor for otomycosis by Jackman *et al.* (2005). Both absence (Paulose *et al.* 1989) and presence (Kaur *et al.* 2000) of earwax in the external ear canal have been accused as predisposing factors for otomycosis. Some authors suggested that cleaning external ear canal with matchsticks or cotton bud swabs was a predisposing factor (Kaur *et al.* 2000). Other proposed predisposing factors are immunosuppression, radiation therapy, cancer chemotherapy and prolonged treatment with systemic antibiotics (Falser 1984).

In Egypt, knowledge on otomycosis is still very limited. The present study aimed at identifying fungal species involved in otomycosis as well as their ability to produce proteolytic, lipolytic and ureolytic enzymes and their sensitivity to some antifungal therapeutic agents.

Materials and Methods

Patients

One hundred and twenty-four patients attending the outpatient clinic of Otolaryngology Department, Assiut University Hospitals (AUH)

were clinically examined for otomycosis during the period from October 2011 to August 2012.

Collection of samples

Sterile cotton swabs were used for collecting debris, fungal elements, and earwax from the external ear canal of patients showing symptoms of otomycosis. Samples were transferred immediately to the Assiut University Mycological Centre (AUMC) for further studies.

Mycological analysis

a) Direct microscopic examination: Direct smears from swabs were prepared and examined using Lactophenol Cotton Blue stain (LPCB) as recommended by Ellis *et al.* (2007).

b) Culturing of samples: Swabs were streaked on Sabouraud's dextrose agar medium (SDA) with the composition of (g/l): peptone, 15; dextrose, 40 and agar, 20 (Ellis *et al*. 2007). Cultures were incubated at 28° C for 7-15 days until fungal colonies appear. Cultures were also preserved on SDA slant agar at 4° C for further studies.

c) Identification of fungi

a) Phenotypic identification: Fungi were identified on the basis of macro-and microscopic features. The following references were consulted: Raper and Fennell (1965), Moubasher (1993), Barnett *et al*. (2000) and Domsch *et al.* (2007).

b) Genotypic identification: Some fungal isolates were selected and individually grown on yeast malt agar (YM) and incubated at 28° C for 3 days. A small amount of fungal growth was scrapped and suspended in 100µl autoclaved distilled water in 2ml sterile vials and boiled at 100°C for 15 minutes. Samples were sent to SolGent Company (Daejeon, South Korea) for rRNA gene sequencing. Fungal DNA was extracted and isolated using SolGent purification bead. Prior to sequencing, the ribosomal rRNA gene was amplified using the polymerase chain reaction (PCR) technique in which two universal fungal primers ITS1 (forward) and ITS4 (reverse) were incorporated in the reaction mixture. Primers used for gene amplification have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). PCR products were sequenced in the sense and antisense directions using ITS1 and ITS4 primers (White *et al.* 1990). Sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

Enzymatic activities of otomycotic fungi

a) Proteolytic activity: Test tubes containing modified **c**asein hydrolysis medium (Paterson and Bridge 1994) were used. The medium comprised (g/l): KH_2PO_4, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.2; $CaCI_2.2H_2O$, 0.1; 15% skimmed milk, 25 ml; glucose, 10; and agar, 20. After inoculation with the tested fungi, cultures were incubated at 25°C for 7 days. Degradation of milk protein was measured as depth of clear zone (in mm).

b) Lipolytic activity: The medium of Ullman and Blasins (1974) was used which has the following composition (g/l): peptone, 10; $MgSO_4$.$7H_2O$, 0.2; $CaCI_2.2H_2O$, 0.2; and agar, 20). The medium was sterilized by autoclaving at 121°C for 20 minutes. Tween 80 (10 ml) was autoclaved separately and added to the sterile and cooled basal medium. The medium was dispensed aseptically in test tubes (10 ml/tube) followed by inoculation by fungal isolates. After incubation at 25°C for 7 days, the lipolytic ability was observed as a visible precipitate due to the formation of crystals of calcium salt of the oleic acid liberated by the enzyme. The depth of precipitate (in mm) was measured.

c) Urease Activity: Christensen urea medium described by Ellis *et al* (2007) was employed with some modifications. The medium was prepared in 2 separate parts. The first part contained (g/l): peptone, 1; KH_2PO_4, 1; KCl, 0.05; yeast extract, 1; and phenol red, 0.012. These components were dissolved in 800 ml distilled water. The second part was composed of (g/l): glucose, 5; $MgSO_4.7H_2O$, 0.5; and distilled water, 200 ml. The two parts were mixed after autoclaving and cooled to 50°C. Aliquots of 5 ml of 40% solution of sterilized urea were added to each 100 ml of the medium which was then poured into sterile 5 ml test tubes (3 ml for each). After inoculation, cultures were incubated at 25°C for 3-5 days. Results were recorded as positive after appearance of a deep pink color in the broth medium.

Screening for aflatoxin production

a) Culturing of selected fungi: Thirty six fungal isolates belonging to *A. flavus* (26 isolates), *A. flavus* var. *columnaris* (9) and *A. parasiticus* (1) were cultivated in 250 ml conical flasks containing 50 ml of Potato Dextrose Broth (PDB) followed by sterilization, inoculation and incubation at 28C for 7 days (El-Kady and Moubasher 1982).

b) Extraction and detection of aflatoxins: Chloroform was the solvent for mycotoxin extraction. Pre-coated TLC silica plates were used for separation of mycotoxins followed by visualization under UV light (254 or 365 nm). Aflatoxins B and G when present fluoresce blue and greenish blue respectively. The intensity of the sample spots was compared with that of the standard aflatoxins (El-kady and Moubasher 1982 and Dorner 1998).

***In-vitro* sensitivity of otomycotic fungi to antifungal agents**

Disc diffusion method (CLSI 2010) was employed with some modifications as Mueller-Hinton II agar was replaced by Sabouraud's

dextrose agar medium. Inhibition zone around each disc was measured in mm and the fungal isolates were classified as sensitive, intermediate or resistant according to Ellis (2011) and Al-Hussaini *et al*. (2013).

Results and Discussion

Fungi recovered from cases of otomycosis

Eighteen fungal species and one variety belonging to seven genera were isolated and identified. The genus *Aspergillus* was represented by 9 species and one variety with the majority attributed to sections *Nigri* (39 isolates belonging to 6 species), *Flavi* (35 isolates of 2 species and one variety) and *Terrei* (one species). *Candida* was represented by 4 species whereas the remaining genera comprised only one species for each as shown in Table (1). Identification of some fungal isolates was confirmed by rRNA gene sequencing. Phylogenetic tree (Fig.1) showed three clades; one for *Aspergillus terreus* which showed close relationship with similar strains deposited in the GenBank. The second and third clades supported the identification of *A. flavus* and *Mucor circinelloides*. The majority of mycotic otitis cases were associated with *Aspergillus* (78 cases representing 84.8 % of total cases). Fungi belonging to section *Nigri* were associated with infection in 39 cases (42.4 %). Similarly fungal species related to sections *Flavi* and *Terrei* were involved in 35 and 4 cases (38 % and 4.3% of the total cases respectively). These findings are in great harmony with the previous reports from Brazil (Pontes *et al.* 2009), Iraq (Khammas *et al.* 2010), Iran (Barati *et al.* 2011 and Saki *et al.* 2013), Kingdom Saudi Arabia (Abou-Halawa *et al.* 2012), India (Sarvan *et al.* 2012, Gokale *et al*. 2013 and Panchal *et al.* 2013) and Spain (Garcia-Agudo *et al.* 2011).

Most reports indicate that *Aspergillus* species are involved in about 70% of fungal otitis cases, reinforcing the importance of *Aspergillus* otitis. Although *A. niger* is the globally most frequently recovered species, *A. flavus* occurs in equal frequency in Mexico (Bonifaz *et al*. 2010) as well as in Spain (Garcia-Agudo *et al*. 2011) and Iraq (Al-Abbasi *et al*. 2011). According to Marquez *et al*. (1999) and Araiza *et al* (2006) otitis caused by *A. fumigatus, A. terreus, A. clavatus, A. candidus,* and *A. nidulans* has been described. They also stated that several other hyalohyphomycetes (species of *Scopulariopsis, Penicillium* and *Fusarium*), phaeohyphomycetes (*Alternaria* spp., *Cladosporium* spp.) and *Candida albicans* have been isolated from patients with otomycosis. As mentioned by Araiza *et al.* (2006) growth of *Aspergillus* species is usually limited to the stratum corneum and produces a mild inflammatory process. In patients with excessive cerumen, the fungal filamentous masses intermingle to form a plug that may mechanically impair hearing. Actually this is a very superficial process which does not constitute a parasitic infection, but rather a saprotrophic condition to the auditory canal. Accordingly, all ornamentations and reproductive forms of *Aspergillus* spp. are seen in cases of *Aspergillus* otitis, in a similar proportion to what occurs in the environment and on culture media.

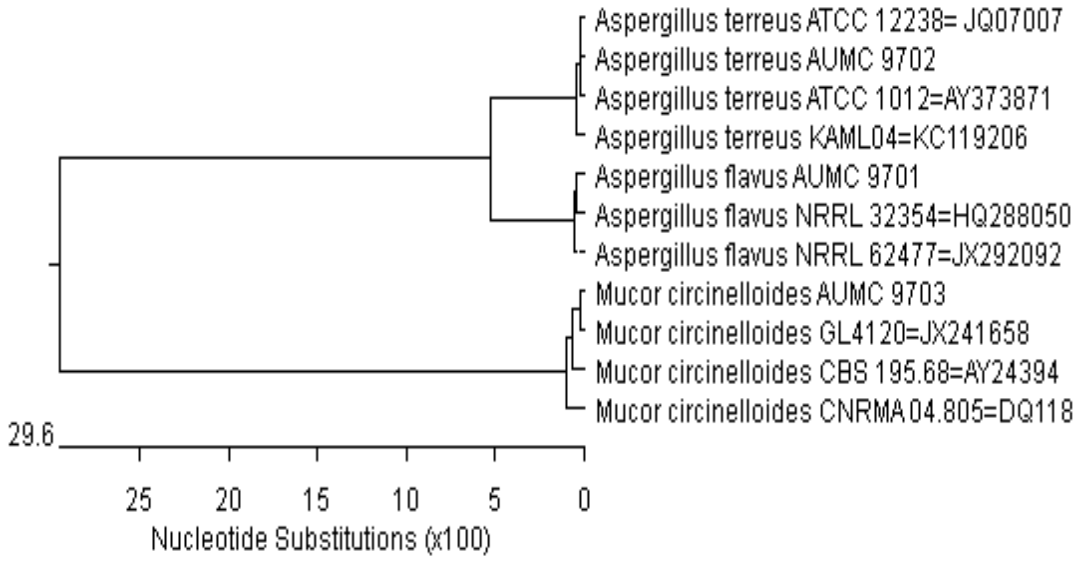


Fig.1 Phylogenetic tree for fungal species isolated from cases of otomycosis (given AUMC Numbers). Reference strains are included in the tree (given ATCC, KAML, NRRL, GL, CBS and CNRMA numbers).

Table (1): Incidence of fungi recovered from otomycotic infections.

Fungal species	Incidences out of 92 cases and (%)
***Aspergillus* species section *Flavi* (Total isolates)**	35 (38)
Aspergillus flavus Link	25 (27.1)
Aspergillus flavus var. c*olumnaris* Raper & Fennell	9 (9.7)
Aspergillus parasiticus Speare	1 (1.1)
***Aspergillus* species section *Nigri* (Total isolates)**	39 (42.3)
Aspergillus brasiliensis Raper & Fennell	28 (30.4)
Aspergillus carbonarius (Bainier) Thom	1 (1.1)
Aspergillus lacticoffeatus Frisvad & Samson	2 (2.2)
Aspergillus niger van Tieghem	4 (4.3)
Aspergillus piperis Samson & Frisvad	2 (2.2)
Aspergillus tubengensis Mosseray	2 (2.2)
Aspergillus terreus Thom	4 (4.3)
Cladosporium sphearospermum Penzig	2 (2.2)
Chrysosporium keratinophilum (Frey) Carmichael	1 (1.1)
Candida albicans (C.P Robin) Berkhout	2 (2.2)
Candida glabrata (Anderson) Meyer & Yarrow	1 (1.1)
Candida krusei (Castellani) Berkhout (Telemorph: *Pichia kudriavzevei* Boidin, Pignal & Besson)	10 (10.8)
Candida tropicalis (Castellani) Berkhout	2 (2.2)
Phoma epicoccina Punith., Tulloch & Leach (Synanamorph: *Epicoccum nigrum* Link)	1 (1.1)
Mucor circinelliodes van Tieghem	3 (3.2)
Stemphylium vesicarium (Wallr.) Simmons (Teleomorph: *Pleospora allii* (Rabenh.) Ces. & de Not.	1 (1.1)

Incidence of otomycosis in relation to sex, age and health status of patients

Among the 92 mycologically positive cases, 56 were males (60.9%) and 36 were females (39.1%). The disease was more prevalent among adults between 21-30 years where it was diagnosed in 29 patients (31.5% of total cases). The prevalence of otomycosis regularly decreased with the increase of age of patients as shown in Table (2). As mentioned by Bonifaz *et al*. (2010), *Aspergillus* otitis is a cosmopolitan condition that occurs more often in warm and humid climates. It affects both genders in the same proportion, with a slight predominance of females. The authors also added that *Aspergillus* species may be part of the habitual microbiota of the external auditory canal and *Aspergillus* otitis is almost always seen in people who practice water sports but rarely affects children. It is more frequent among adolescents and this has been related to growth spurts. However, the highest frequency is observed amongst young adults aging 20–30 years-old (Brook 1980, Muglestos and O'Donoghue 1985 and Zaror *et al*. 1991). In Nigeria, Fasunla *et al.* (2008) studied 5784 patients with ear diseases and found that 378 (6.54%) had otomycosis which consisted of 145 (38.36%) males and 233 (61.64%) females. Seventeen patients (4.50%) had recurrence within six months of treatment, 4 (1.06%) had poorly controlled plasma glucose. A significant number of the Nigerian patients, 52 (13.76%), had prior topical aural antibiotic treatment following misdiagnosis and the predominant etiological agents were *Aspergillus niger* (48.35%) and *Aspergillus fumigatus* (33.96%). In Australia, Ling and Sader (2008) reported a case of fungal malignant otitis externa caused by *A. flavus*. The case was a 77-year-old man with non-insulin dependent diabetes mellitus who presented with otalgia and otorrhea. In Iran Barati *et al.* (2011) reported that Otomycosis was confirmed after mycological diagnosis in 69% of clinically suspected patients. The highest incidence of otomycosis was in autumn and in patients aged 21-40 years old. Working in dry dusty environment was a major predisposing factor. *Aspergillus flavus* was the most common fungus in otomycosis followed by *A. niger*, *Candida albicans*, *A. fumigatus*, *A. nidulans* and *C. parapsilosis*. More recently, Saki *et al*. (2013) investigated 881 cases suspecting otomycosis in Iran. They reported that the 20–39 year age group had the highest prevalence of otomycosis. Positive cases (293 cases), comprised 162 (55.3%) women and 131 (44.7%) men. The fungal agents isolated were *A. niger* (67.2%), *A. flavus* (13%), *C. albicans* (11.6%), *A. fumigatus* (6.2%) and *Penicillium* species (2%).

In the present study (Tables 1& 2), *Candida* species were involved in 15 cases (16.3%) of

otomycotic cases. The remaining fungal species were rare etiologic agents of otomycosis. These included *Chrysosporium keratinophilum*, *Cladosporium sphaerospermum*, *Phoma epicoccina*, *Mucor circinelloides* and *Stemphylium vesicarium*. To the best of our knowledge *C. kertinophilum*, *P. epicoccina* and *S. vesicarium* are recorded for the first time in Egypt as causal agents of mycotic otitis externa. In India, Jadhav *et al*. (2003) investigated mycologically 79 patients (42 males and 37 females) clinically diagnosed as otomycotic cases. They found that *Candida albicans* was the sole pathogen in two patients (1 male and 1 female) aged 18 and 20 years respectively. Both patients had unilateral otomycosis and used antibiotic solution and removed wax with wooden sticks. Aneja *et al.* (2010) obtained *C. albicans* and *Penicillium* species from 10.2% and 2.7% of otomycotric samples respectively With reference to Table (3), only 16 out of 92 patients (17.4%) were diabetic and most of them were infected by *A. flavus.* The majority of otomycotic cases were unilateral (86 cases representing 93.4% of patients). Bilateral otomycosis was diagnosed in 6 patients, all of which were infected with *A. flavus.* Cases of disease recurrence were observed in 35 patients who were affected by *A. flavus* (48.6%) and other aspergilli attributed to Section *Nigri* (51.4%). There were 9 patients affected with a mixture of two fungi and all of them showed unilateral otomycosis. All patients infected with *Candida*, presented with unilateral non-recurrent otomycosis with the majority of them (13 out of 15, 86.7%) being non-diabetic. According to the P-value analysis, the relative frequency of infections with *Aspergillus* and *Mucor circinelloides* was significantly higher in diabetic than in non-diabitic patients. In United Arab Emirates (UAE), Hazarika *et al*. (2012) reported a case of mucormycosis of the middle ear in a 24-year-old male who was suffering from a left ear pain of 6-month-duration. Histopathology revealed non-septate, thin-walled fungal masses. Jia *et al.* (2011) reported recurrence of otomycosis in 8.89% of patients from Shanghai.

Enzymatic activities of otomycotic fungi

Lipolytic activity: Out of 101 isolates, 76 (75.2%) were able to produce lipase but with variable capabilities (Table 4). High lipase production was exhibited by 40 isolates which were mainly belonging to *Aspergillus* sections *Flavi* and *Nigri.* Although 50% of *A. terreus* isolates were moderately able to produce lipase, only 13.3% of *Candida* isolates were lipolytic showing low activity. Many investigators have emphasized the ability of several *Aspergillus* strains belonging to *A. niger*, *A. flavus*, *A. parasiticus* and *A. terreus* to produce extracellular lipases (Contesini, *et al*. 2009, Metwalli *et al.* 2013 and Srivastava and Gautamb 2013).

Proteolytic activity: Protease was produced by 96 out of 101 isolates which were almost of moderate proteolytic capabilities. All fungal isolates belonging to sections *Flavi* (35), *Nigri* (39) and *Terrei* (4) were proteolytic. On the other hand, 10 out of 15 *Candida* isolates were protease producers (Table 4). Reports from Serbia (Arsovic *et al.* 2004) showed that proteases were produced by 7 out of 8 *Candida* strains isolated from children with otomycosis and were belonging to *C. parapsilosis*, *C. famata*, *C. guilliermondii* and *C. albicans.* The authors suggested that protease enzymes enhance the ability of *Candida* spp. to colonize the skin and penetrate the host cells, which could be important in establishing the cause of the ear infection. It is known that the secreted proteases are important virulence factors in *Candida* species in cases of skin, mucosal and deep organ infections (Sohnle *et al.* 1976) and may help the yeast invasion through the keratin protective layer and facilitate the initiation of the infection of the ear canal (Negi *et al*. 1984). Fungal strains with higher proteolytic activity are considered more virulent (Hube 1996). As suggested by Kothary *et al*. (1984) variability among *A. flavus* isolates in proteinase production suggested strong correlation between isolate ability to express proteolytic activity and association with invasive aspergillosis in humans. The authors also added that *A. flavus* isolates causing invasive human lung infections are more likely to produce proteases than are environmental isolates.

Urease activity: Urease was only produced by 37.6 % fungal isolates which belong to *Aspergillus* sections *Flavi* (30 isolates) and *Terrei* (3) as well as the tested isolates of *Phoma*, *Mucor* and *Stemphylium.* Urease was not detected in fungal cultures related to *Aspergillus* sections *Nigri* and *Candida* (Table 3). The inability of *Candida* strains to produce urease was reported by Ellis *et al.* (2007). On the other hand, urease from *A. niger* was isolated and purified by Smith *et al* (1993) and Ghasemi *et al*. (2004). The enzyme was also detected in cultures of other filamentous and yeast fungi (Lubbers *et al*. 1996, Farley and Santosa 2002 and Mirbod *et al*. 2002). It is worthy to mention that fungal enzymes including lipase, protease and urease are considered as major virulence factors for pathogenecity for fungi causing human and animal diseases (Cox *et al* 2000, Karkowska-Kuleta 2009, Tomee and Kauffman 2000 and Watanabe *et al* 2008).

Table (2): Incidence of otomycotic cases and causative fungi in relation to sex and age of patients

Fungi	Age group (years)														Total cases	
	≤ 10		11-20		21-30		31 – 40		41 – 50		51 - 60		≥ 61			
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Total cases	5	2	5	2	18	11	9	8	5	10	10	3	4	0	56	36
Aspergillus section *Flavi*	3	0	2	0	8	4	4	2	1	4	5	1	1	0	24	11
Aspergillus section *Nigri*	1	2	2	0	7	7	3	6	2	4	4	1	0	0	19	20
Aspergillus section *Terrei*	0	0	0	1	0	0	1	0	0	0	0	1	1	0	2	2
Candida species	2	1	1	0	3	0	0	3	1	1	0	0	3	0	10	5
Chrysosporium keratinophylum	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Cladosporium sphaerospermum	0	0	1	0	1	0	0	0	0	0	0	0	0	0	2	0
Phoma epicoccina	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Mucor circinelloides	0	0	0	0	0	0	0	1	1	0	1	0	0	0	2	1
Stemphylium vesicarium	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0

M= Males, F= Females

Table 3**:** Incidence of otomycotic fungi in relation to health status of patients

Fungal species	No. of cases	Diabetic	P -value	Non diabetic	Unilateral infection	Bilateral	Recurrence infection
Aspergillus section *Flavi*	35	12 (34.3%)	0.001*	23 (65.7%)	29	6	17
Aspergillus section *Nigri*	39	0 (0%)	0.000*	39 (100%)	39	0	18
Aspergillus section *Terrei*	4	0 (0 %)	0.792	4 (100%)	4	0	0
Candida species	15	2 (13.3%)	0.935	13 (86.6%)	15	0	0
Chrysosporium keratinophilum	1	1(100%)	0.387	0 (00%)	1	0	0
Cladosporium sphaerospermum	2	0 (0%)	0.512	2 (100%)	2	0	0
Phoma epicoccina	1	0 (0%)	0.645	1 (100%)	1	0	0
Mucor circinelloides	3	3 (100%)	0.002*	0 (0%)	3	0	0
Stemphylium vesicarium	1	0 (0%)	0.645	1 (100%)	1	0	0
Mixed fungal infection	9	2 (22.2%)	0.687	7 (77.8%)	9	0	0
Total number of cases	92	16	----	76	86	6	35

N. B.: mixed fungal infections were diagnosed in 9 cases

Table (4): Enzymatic activities of fungi recovered from otomycosis

Fungal species	Lipase					Protease					Urease				
	H	M	L	P	N	H	M	L	P	N	H	M	L	P	N
Total species (n=101)	40	28	8	76	25	1	87	8	96	5	16	9	13	38	63
Section *Flavi* (n= 35)	10	17	4	31	4	0	35	0	35	0	9	9	12	30	5
Section *Nigri* (n= 39)	30	4	1	35	4	0	37	2	39	0	0	0	0	0	39
Section *Terrei* (n=4)	0	2	0	2	2	0	4	0	4	0	2	0	1	3	1
Candida spp. (n=15)	0	0	2	2	13	0	5	5	10	5	0	0	0	0	15
Chrysosporium keratinophilum (n=1)	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1
Cladosporium sphaerospermum (n=2)	0	2	0	2	0	0	2	0	2	0	0	0	0	0	2
Phoma epicoccina (n=1)	0	0	1	1	0	0	0	1	1	0	1	0	0	1	0
Mucor circinellioides (n=3)	0	1	0	1	2	1	2	0	3	0	3	0	0	3	0
Stemphylium vesicarium (n=1)	0	1	0	1	0	0	1	0	1	0	1	0	0	1	0

Aflatoxins produced by otomycotic fungal isolates

Aflatoxins were produced by 26 out of 36 isolates representing 72.2% of tested fungi. Isolates of *A. flavus* 19 (73%) were able to produce aflatoxins B_1, B_2 and G_1 with variable levels. Relatively high levels of aflatoxin B1 was produced by 3 isolates of *A. flavus* as shown in Table (5). Six isolates of *A. flavus* var. *columnaris* produced low to intermediate levels of aflatoxins B1, B2 and G1. *A. parasiticus* produced low levels of aflatoxin B1. It is well established that the most potent of aflatoxins is B1 which is listed as a group I carcinogen by the International Agency for Research on Cancer (IARC 2002). Production of these toxins by fungi isolated from human corneal ulcers in Egypt has been demonstrated by Gharamah *et al.* (2013).

Sensitivity of fungi to antifungal agents

Data in Table 5 showed that Terbinafine (TER) followed by Clotrimazole (CC) and clove oil were the most effective antifungal agents where 55.1% – 68.3 % of total tested isolates were sensitive to these compounds. Results showed also that 90.8% of isolates were resistant to Fluconazole (FLC). Also, 50 - 54 % of isolates were resistant to Cetrimide (CET), Itraconazole (IT) and Amphoteicin B (AP). *Aspergillus* species belonging to sections *Flavi*, *Nigri* and *Terrei* were sensitive to Clotrimazole, Cetrimide, Terbinaine, Tioconazole and clove oil. On the other hand, most of these fungi showed resistance to Amphotericin B, Fluconazole, Itraconazole, Ketoconazole and Nystatin. *Candida* spp. showed variable degrees of sensitivity to Amphotericin B, Clotrimazole, Fluconazole, Ketoconazole and Nystatin. Fungal isolates related to *Chrysosporium*, *Phoma* and *Mucor* responded variably to the different antifungal agents as shown in Table (6). In this respect, Bonifaz *et al*. (2010) reported that topical antifungal agents are very helpful and should be started after proper cleaning of the auditory canal is obtained. The most widely used agents are Amphotericin B (1mg/ml which is active against most fungi but causes irritation) and Nystatin (100,000 U/ml). These agents are extremely effective in the treatment of *Aspergillus* otitis, while Imidazole derivatives such as Clotrimazole, Miconazole and Ketoconazole are less useful options. Jackman *et al*. (2005) used terbinafine for this purpose. Most patients with invasive *Aspergillus* otitis require long-term therapy with systemic antifungal drugs, which is usually associated with a complete recovery. The most widely recommended agent is Itraconazole at 100-200 mg/day for 15-30 days. In-*vitro* resistance to Itraconazole was described in isolates of *A. niger* causing otomycosis while susceptibility was retained to Voriconazole (Kaya and Kiraz 2007).

Conclusion: It is advised to establish a continuous collaboration between otolaryngologists and microbiologists for characterization of the pathogenic fungal species involved in otomycosis. In-*vitro* sensitivity test is of great importance to choose the most active antifungal agents. Patients are also advised to avoid removing of ear wax by metallic or stiff materials that may cause wounds subjecting ears to microbial infections. Keeping ear canal dry can also prevent or reduce the chance of otomycosis.

Table (5): Mycotoxins produced by otomycotic *Aspergillus* isolates

Fungal species	Aflatoxins	No. of producing isolates	Level of production (µg/L)			Non-producers
			L	M	H	
A. flavus (n=25)	B1	11	9	0	2	
	B2	1	0	1	0	
	B1 + B2	4	1	2	1	
	B1 + G1	2	2	0	0	
	G1	1	0	1	0	
	Total	19	12	4	3	6
A.flavus var. *columnaris* (n=9)	B1	2	2	0	0	
	B1 +B2	2	1	1	0	
	B1 + G1	1	1	0	0	
	G1	1	0	1	0	
	Total	6	4	2	0	3
A. parasiticus (n=1)	B1	1	1	0	0	0
Total isolates tested (n=35)	B1	14	11	0	3	
	B2	1	0	1	0	
	G1	2	3	0	0	
	B1 + B2	6	2	3	1	
	B1 + G1	3	1	0	0	
	Total	26	17	6	4	9

Levels of aflatoxin production (µg/L): L= Low (≤ 100), M= Moderate (>100- <500), H= High (≥ 500**).**

Table 6: Degree of sensitivity (DS): S = Susceptible, I = Intermediate, R= Resistant of common otomycotic fungal isolates to different antifungal agents.

Fungi tested (No. of isolates)	DS	AP	CC	CET	FLC	IT	KT	TER	NS	SER	TIO	CO
Aspergillus section *Flavi* (35)	S	3	29	14	1	1	8	34	8	19	31	22
	I	12	6	15	0	21	16	1	6	11	2	11
	R	20	0	6	34	13	11	0	21	5	2	2
Aspergillus section *Nigri* (39)	S	6	15	5	0	1	2	28	6	6	15	21
	I	13	16	8	1	7	8	6	13	17	11	9
	R	20	8	26	38	31	29	5	20	16	13	9
Aspergillus section *Terrei* (4)	S	0	2	1	0	1	1	3	0	0	3	3
	I	0	2	0	0	2	2	0	1	0	0	0
	R	4	0	3	4	1	1	1	3	4	1	1
Candida spp. (15)	S	6	12	2	7	3	15	2	10	0	3	5
	I	7	3	0	0	7	0	6	5	2	0	4
	R	2	0	13	8	5	0	7	0	13	12	6
Chrysosporium kertinophilum (1)	S	0	1	1	0	1	1	1	0	1	1	1
	I	0	0	0	0	1	1	1	0	0	0	0
	R	1	0	0	1	0	0	0	1	0	0	0
Mucor circinelloides (3)	S	2	0	0	0	0	0	0	0	1	0	2
	I	0	0	0	0	0	0	0	3	0	0	0
	R	1	3	3	3	3	3	3	0	2	3	1
Phoma epicoccina (1)	S	0	0	1	0	1	1	1	0	0	1	1
	I	0	0	0	0	0	0	0	1	0	0	0
	R	1	1	0	1	0	0	0	0	1	0	0
Total isolates (98)	S	17	59	22	8	7	27	67	24	27	54	55
	I	32	27	23	1	38	27	15	29	30	13	24
	R	49	12	53	89	53	44	16	45	41	31	19

AP:Amphotericine-B, CC: Clotrimazole, CET: Cetrimide, FLU: Fluconazole, IT: Itraconazole KT: Ketoconazole, TER: Terbinafine, NS: Nystatin, SER: Sertaconazole, TIO: Tioconazole, CO: Clove oil.

References

Al-Abbasi AM, Al-Sadoon A, and Sabbar BA (2011): Otomycosis in Basrah, Iraq. Journal of the Arab Board of Health Specializations 12: 28-33.

Al-Hussaini MS, Al-Tahtawy NF and Moharram AM (2013): Phenotypic and molecular characterization of Candida species in urine samples from renal failure patients. Science Journal of Clinical Medicine 2: 14-25.

Aneja KR, Sharma C and Joshi R. (2010): Fungal infection of the ear: a common problem in the north eastern part of Haryana. International Journal of Pediatric Otorhinolaryngology 74: 604-607.

Araiza J, Canseco P and Bonifaz A (2006): Otomycosis: Clinical and mycological study of 97 cases. Revue de Laryngologie Otologie Rhinologie 127: 251-254.

Arsovic NA, Banko AV, Dimitrijevi MV, Djordjevi V, Milovanovi JP and Arsenijevi VA (2004): Protease activities of *Candida* spp. isolated from otitis externa: Preliminary result. Acta Chirurgica Iugoslavica 151: 113-116.

Barati B, Okhovvat SAR, Goljanian A and Omrani MR (2011): Otomycosis in Central Iran: A clinical and mycological study. Iranian Red Crescent Medical Journal 13: 873-876.

Barnett JA, Payne RW and Yarrow D (2000): Yeasts: Characteristics and Identification, 3rd edition, Cambridge University Press, Cambridge, England, 1152 pp.

Bonifaz A, Chavolla-Magana R and Araiza J (2010): *Aspergillus* otitis. In: Pasqualotto AC (ed.). Aspergillosis: From Diagnosis to Prevention, pp. 999-1006.

Brook I (1980): Chronic otitis media in children. American Journal of Diseases of Children 134: 564–566.

CLSI (2010): Clinical and Laboratory Standards Institute; Method for antifungal disk diffusion susceptibility testing of non-dermatophyte filamentous fungi. Approved guidelines CLSI Document M51-A, Pennsylvania, USA, pp. 9.

Cox GM., Mukherjee J, Cole GT, Casadevall A and Perfect J (2000): Urease as a virulence factor in experimental Cryptococcosis. American Society for Microbiology 68: 443-448.

Contesini FJ, da Silva VCF, Maciel RF, de Lima RJ, Barros FFC and Carvalho PO (2009): Response surface analysis for the production of an enantioselective lipase from *Aspergillus niger* by solid-state fermentation The Journal of Microbiology 47:563-571.

Domsch KH, Gams W and Anderson TH (2007): Compendium of Soil Fungi. 2nd edition, Verlag, Eching, 672 pp.

Dorner JW (1998): Chromatographic analysis of mycotoxins. In: Chromatographic analysis of environmental and food toxicants. Shibamoto T (ed.), Marccel Dekker Inc. New York, 113 pp.

EL-Kady IA and Moubasher MH (1982): Toxigenicity and toxins of *Stachybotrys chartarum* isolates from wheat straw samples in Egypt. Experimental Mycology 6: 25-31.

Ellis D (2011): Antifungal susceptibility profile (Australian antifungal susceptibility data for *Candida* isolates from recurrent vulvovaginal candidiasis (2007- 2009) using the CLSI M44-A2 disc susceptibility standard for yeasts). Mycology Online, The University of Adelaide, Australia.

Ellis D, Davis S, Alexiou H, Handke R and Bartley R (2007): Descriptions of Medical Fungi. Mycology Unit Women's and Children's Hospital Adelaide, Australia 2nd edition, 198 pp.

Falser N (1984): Fungal infection of the ear: Etiology and therapy with bifonazole cream or Solution. Dermatologica 169:135-140.

Farley PC and Santosa S (2002): Reglulaiton of experssion of the *Rhizopus oryzae* uricase and urease enzymes. Canadian Journal of Microbiology 48: 1104-1108.

Fasunla J, Ibekwe T and Onakoya P (2008): Otomycosis in Western Nigeria. Mycoses 51: 67-70.

Gharamah, AA, Moharram AM, Ismail MA and Al-Hussaini AK (2013): Bacterial and fungal keratitis in Upper Egypt: In-*vitro* screening of enzymes, toxins and antifungal activity. Indian Journal of Ophthalmology (In press).

Ghasemi MF, Bakhtiari MR, Fallahpour M, Noohi A, Moazami N and Amidi Z (2004): Screening of Urease production by *Aspergillus niger* strains. Iranian Biomedical Journal 8: 47-50.

Garcia-Agudo L, Aznar-Marin P, Sanchez FG, Garcia-Martos P, Marin-Casanova P and Rodriguez-Iglesias M (2011): Otomycosis due to filamentous fungi. Mycopathologia 172: 307-310.

Gokale S K, Suligavi SS, Baragundi M, Anushka D and Manjula R (2013): Otomycosis: A clinico mycological study. International Journal of Medical and Health Sciences 2: 218-223.

Hazarika P, Zachariah J, Victor J, John M, Devi C and Abraham P (2012): Mucormycosis of the middle ear: A case report with review of literature. Indian Journal of Otolaryngology and Head and Neck Surgery 64: 90-94.

Ho T, Vrabec JT, Yoo D and Coker NJ (2006): Otomycosis: Clinical features and treatment implications, Otolaryngology and Head and Neck Surgery 135: 787-791.

Hube B. (1996): *Candida albicans* secreted aspartyl proteinases. Current Topics in Medical Mycology 1: 55-69.

International Agency for Research on Cancer (2002): Some traditional herbal medicines,

some mycotoxins, naphthalene and styrene. IARC monographs on the evaluation of carcinogenic risks to humans (vol. 82), Lyon, France: World Health Organization, 556 pp.

Jackman A, Ward R, April M and Bent I (2005): Topical antibiotic induced otomycosis, International Journal of Pediatric Otorhinolaryngology I69: 857-860.

Jadhav J, Pal M and Mishra S (2003). Etiological significance of *Candida albicans* in otitis externa, Mycopathologia 156: 313-315.

Jia X, Liang Q, Chi F and Cao W (2011): Otomycosis in Shanghai: Aetiology, clinical features and therapy. Mycoses 55: 404-409.

Karkowska-Kuleta J□, Rapala-Kozik M and Kozik A (2009): Fungi pathogenic to humans: Molecular bases of virulence of *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Acta Biochimica Polonica 56: 211-224.

Kaur R, Mittal N, Kakkar M, Agarwal AK and Mathur MD (2000): Otomycosis: A clinicomycologic study. Ear, Nose and Throat Journal 79: 606-609.

Kaya AD and Kiraz N (2007): In vitro susceptibilities of *Aspergillus* spp. causing otomycosis to Amphotericin B, Voriconazole and Itraconazole. Mycoses 50: 447-450.

Khammas AH, Abbas AK, Ilabi S and Al-Najjar AR (2010): Isolation and identification of fungi associated with otomycosis. Iraqi Journal of Common Medicine 3:186-189.

Kothary MH, Chase TJ and MacMillan JD (1984): Correlation of elastase production by some strains of *Aspergillus fumigatus* with ability to cause invasive aspergillosis in mice. Infection and Immunity 43(1): 320-325.

Kumar A (2005): Fungal spectrum in otomycosis paitents. JK Science (India) 7: 152-155.

Ling SS and Sader C (2008): Fungal malignant otitis externa treated with hyperbaric oxygen. International Journal of Infectious Diseases 12: 550-552

Lubbers MW, Rodriguez SB, Honey NK and Thornton RJ (1996): Purification and characterization of urease from *Schizosaccharomyces pombe*. Canadian Journal of Microbiology 42: 132-140.

Márquez A, García-Martos P, Marín P, Delgado D, García-Santos MD and Mira J (1999): Otomycosis of uncommon etiology. Enfermedades Infecciosas y Microbiología Clínica 17: 243-245.

Metwally MA, Mubarak HM, Gamea AM and El Zawawy NA (2013): Characterization and inhibition effect of conyza leaf extract (*Pluchea dioscoridis*) on lipase enzyme produced by *Aspergillus niger*, isolated from otomycosis disease. Research Journal of Microbiology 8: 92-100.

Mirbod F, Schaller R.A and Cole T (2002): Purification and characterization of urease isolated from the pathogenic fungus *Coccidioides immitis*. Medical Mycoogy 40: 35-44.

Moubasher A H (1993): Soil Fungi in Qatar and other Arab Countries, the Centre for Scientific and Applied Research, University of Qatar, Doha, Qatar, 566 pp.

Mugliston T and O'Donoghue G (1985): Otomycosis: A continuing problem. The Journal of Laryngology and Otology 99: 327-333.

Negi M, Tsubai R, Matsui T and Ogawa H (1984): Isolation and characterization of proteinase from *Candida albicans*: Substrate specificity. Journal of Investigative Dermatology 83:32-36.

Ozcan M (2011): Otomycosis in Turkey: A review In: Rai M. and Kovics G (eds.), Progress in Mycology, Springer, Netherlands, pp.182-192.

Ozcan K M, Ozcan M, Karaarslan A and Karaarslan F (2003a): Otomycosis in Turkey: Predisposing factors, aetiology and therapy. Journal of Laryngology and Otology I17: 39-42.

Ozcan M, Ozcan KM, Karaarslan A and Karaarslan F (2003b): Concomitant otomycosis and dermatomycoses: A clinical and microbiological study. European Archives of Otorhinolaryngology 260: 24-27.

Panchal P, Pethani J, Patel D, Rathod S and Shah P (2013): Analysis of various fungal agents in clinically suspected cases of otomycosis. Indian Journal of Basic and Applied Medical Research 2: 865-869.

Paterson RRM and Bridge PD (1994): Biochemical Techniques for Filamentous Fungi. International Mycological Institute, CAB International, Survey, UK, 125 pp.

Paulose KO, Khalifa SA, Shenoy P and Sharma RK (1989): Mycotic infection of the ear (otomycosis): A prospective study. The Journal of Laryngology and Otology l03: 30-35.

Pontes ZB, Silva AD, Lima E, Guerra M, Oliviera N, Carvalho M and Guerra FS (2009): Otomycosis: a retrospective study. Brazilian Journal of Otorhinolaryngology 75:367-370.

Pradhan B, Tuladhar NR and Amatya RM (2003): Prevalence of otomycosis in outpatient Department of Otolaryngology in Tribhuvan University Teaching Hospital, Kathmandu, Nepal. The Annals of Otology, Rhinology and Laryngology I12: 384-387.

Raper KB and Fennell DI (1965): The Genus *Aspergillus*, the Williams and Wilkins Company, Baltimore, USA, 686 pp.

Saki N, Rafiei A, Nikakhlagh S, Amirrajab N and Saki S (2013): Prevalence of otomycosis in Khouzestan Province, South-West Iran. The

Journal of Laryngology and Otology 127: 25-27.

Sarvan RR, Kikani KM, Mehta SJ and Joshi PJ (2012): Clinico-mycological study of otomycosis. International Journal of Biological and Medical Research 3: 2469-2470.

Smith PT, Douglas AKJ and Goodman N (1993): Isolation and characterization of urease from *Aspergillus niger*. Journal of General Microbiology 139: 957-962.

Sohnle PG, Frank MM and Kirkpatrick CH (1976): Mechanisms involved in elimination of organisms from experimental cutaneous *Candida albicans* infections in guinea pigs. Journal of Immunology 117: 523-530.

Srivastava AK and Gautamb N (2013): Lipase activity of some fungi isolated from otitis Media Advances in Bioresearch 2: 63- 66.

Tiwari S, Singh SM and Jain S (1995): Chronic bilateral suppurative otitis media caused by *Aspergillus terreus*. Mycoses 38: 297-300.

Than KM, Naing KS and Min M (1980): Otomycosis in Burma, and its treatment. The American Journal of Tropical Medicine and Hygiene 29: 620-623.

Tomee JFC and Kauffman HF (2000): Putative virulence factors of *Aspergillus fumigatus*. Clinical and Experimental Allergy 30: 476-484.

Ullman U and Blasins C (1974): A simple medium for the detection of different lipolytic activity of microorganisms. Zentralblatt fur Bakteriologie und Hygiene 229: 264-267.

Wang ME, Liu EY, Shiao A S and Wang T (2005): Ear problems in swimmers. Journal of the Chinese Medical Association 68: 347-352.

Watanabe A, Hashimoto Y, Higurashi H, Ochiai E, Toyotome T, Nagayoshi M and Kamei K (2008): Candidates for virulence factors of *Aspergillus fumigatus*. Nihon Ishinkin Gakkai Zasshi 49: 263-267.

White TJ, Bruns T, Lee S and Taylor J (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenitics. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds.) PCR Protocols: a Guide to Methods and Applications, Academic Press, New York, pp. 315-322.

Zaror L, Fischman O, Suzuki F A and Felipe RG (1991): Otomycosis in Sao Paulo. Revista Do Instituto De Medicina Tropical De Sao Paulo 33: 169-173.

Zohri AA (1990): Mycoflora and mycotoxins of some meat products. Ph. D. Thesis, Botany Department, Faculty of Science, Assiut University, Assiut, Egypt.

Journal of Basic & Applied Mycology (Egypt) 4 (2013): 13-19

In-*vitro* response of microbiota isolated from human eye infections to some antibiotics

M. A. Abdel-Sater[1,2,*], A. H. Al- Zubeiry[2] and K. A. Badah[2]

[1]Department of Botany & Microbiology, Faculty of Science, Assiut University, Egypt
[2]Department of Microbiology, Faculty of Science, Taiz University, Taiz, Yemen

*Corresponding author: e-mail: masater59@yahoo.com
Received 13/4/2013, Accepted 3/6/2013

Abstract: Five antifungal drugs commonly used in the treatment of fungal eye diseases were tested against the common fungi associated with eye infections using standard agar disk diffusion method. All drugs examined except Floconazole (2%) showed high to moderate antifungal activity. All the 74 bacterial isolates tested against ten antibacterial drugs were affected by Cephotaxime, Ceftriaxone, Ciprofloxacin, Norfloxacin and Tetracycline. Most of drugs tested showed high to moderate effect on Gram-positive bacteria, and weak effect on Gram-negative bacteria, except Ciprofloxacin and Norfloxacin showed high or moderate effect against *Pseudomonas aeruginosa*. Treatment with proper antifungal and/or antibacterial agents will be of great advantage and ophthalmologists should be aware of the drug of choice to be used.

Key words: Eye infection, drugs, Antibacterial, antifungal, fungi, bacteria.

Introduction

The concentration of the drug applied to the eye may be increased by the preparation of fortified eye drops. Frequent topical application of drops is a useful means of achieving therapeutic levels in the eye. Ointments and subconjunctival injections may prolong the contact time between the antifungal and the corneal and conjunctival tissue (Aminov 2009, Dash *et al.* 2011 and Kavitha *et al*. 2012). Only Amphotericin B and Miconazole are available as ophthalmic ointments (Friedberg *et al*. 1991 and Mauger and Craig 1994). Miconazole was reported to be useful for the therapy of mycotic keratitis (Foster 1981 and Fitzsimons and Peters 1986). Intravenous and topical Miconazole and Ketoconazole administrations have also been reported to be useful in therapy of keratitis due to *Scedosporium apiospermum* (Nunery *et al*. 1985 and Ruben 1991).

Oral Fluconazole therapy has been used with success in some cases, treating mycotic keratitis (Thakar 1994), multifocal choroiditis due to coccidioidomycosis (Cunningham *et al*. 1998), chorioretinitis and iridocyclitis complicating disseminated coccidioidomycosis and endogenous *Candida* endophthalmitis (Luttrull *et al*. 1995). Ketoconazole is currently available as an oral preparation worldwide. Ishibashi (1983) reported that oral ketoconazole therapy was effective with mycotic keratitis. Corneal ulcer due to *Fusarium solani* was treated with Ketoconazole and Miconazole and responded well (Mirza 2005).

Topical antibiotics are used to treat bacterial blepharitis, conjunctivitis, keratitis and external hordeola. The most likely pathogens are *Staphylococcus aureus*, coagulase negative staphylococci, streptococci, *Haemophilus influenza*, coliforms and *P. aeruginosa* (Seal *et al.* 1982). Chlortetracycline is a broad spectrum bacteriostatic antibiotic. Tetracyclines are actively concentrated within phagocytes so that it is useful against intracellular pathogens such as *Chlamydia* (Neu 1978). Chloramphenicol's spectrum of activity covers the majority of ocular pathogens (Seal *et al*. 1982).

Aminoglycosides such as Gentamycin has the broadest antibacterial spectrum, with activity against *P. aeruginosa* (Gwon 1992, Chattopadhyay and Grossart 2010 and Palaniappan and Holley 2010). Grag *et al*. (1999) carried out a survey of 37 ophthalmic centers in the United Kingdom and showed that the majority of used antibiotics are not commercially available. Fortified Gentamycin is frequently combined with Cefuroxime in the treatment of bacterial keratitis (Timewell *et al*. 1983). Vancomycin and Teicoplanin are also useful agents against Gram-positive bacteria while Ceftazidime is used mainly as an anti-pseudomonad. Erythromycin, used in the form of an eye ointment, is an alternative to Tetracycline in the treatment of infections caused by Gram-positive organisms. Also it is used in combination with systemic Tetracycline therapy for *Chlamydia*. Amikacin has been shown to be a useful antibiotic against *Pseudomonas* with multiple drug resistance (Shanmuganathan *et al*. 2005).

The aim of the work is to study the sensitivity of microorganisms, isolated from eye infections in Taiz City, Yemen, towards some pharmaceutical drugs.

Materials and Methods

Organisms and culture maintenance

Eighty-five isolates (11 of fungi and 74 of bacteria) recovered from the anterior lid margins and

ulcerated area (blepharitis), conjunctival sac (dacryocystitis) and cornea (keratitis) in Taiz City, Yemen (refer to Abdel-Sater *et al.* 2012), were used in the present study. Fungal isolates were routinely maintained on Sabouraud's dextrose agar and incubated at 28ºC, whereas bacterial isolates were maintained on nutrient agar and incubated at 37ºC and *Streptococcus* spp. were maintained on chocolate agar medium at 37ºC.

Pharmaceutical agents

Five types of antifungal drugs, namely Ketoconazole (1%), Clotrimazole (1%), Floconazole (2%), Nystatin (1%) and Miconazole (1%), obtained from the Yemen Commercial Center, were used in this study. Also, ten antibacterial drugs [Cephotaxime (30 µg), Chloramphenicol (30 µg), Gentamycin (10 µg), Amoxicillin (10 µg), Ciprofloxacin (5 µg), Ceftraxone (30 µg), Tetracycline (30 µg), Erythromycin (15 µg), Clindamycin (2 µg), and Norofloxacin (10 µg)] were used.

Determination of antimicrobial activity

The standard agar disk diffusion method (Cheesbrough 1992) was adopted and performed as follows: sterile 6 mm filter paper disks (Whatman No.1) were impregnated in newly synthesized pharmaceutical drugs. The disks were then air dried for 1 hour. By using a sterile loop, the fungal or bacterial suspensions were spreaded on the surface of Sabouraud's dextrose agar or nutrient agar and blood agar plates, respectively. The discs saturated with the tested compounds were then placed on the agar surface (5 disks for each plate). Each plate had one control disk impregnated with the solvent. The plates were incubated at 28ºC for one week in case of fungi and at 37ºC for 24-48 hours in case of bacteria. The inhibition zones (in mm) around disks were then measured (Cheesbrough 1992).

Results and Discussion

Five antifungal drugs commonly used in the treatment fungal eye infection were tested against 11 isolates of the common fungi associated with eye infections (Table 1). All isolates were sensitive to Miconazole (1 %), Clotrimazole (1%), Ketoconazole (1%) and Nystatin (1%), while all isolates tested were resistant to Floconazole (2%). Miconazole (1%) exhibited high activity against all fungal isolates tested. Clotrimazole (1%) had high or moderate effect against isolates of *Aspergillus flavus*, *A. oryzae* and *Candida albicans*, whereas it showed moderate activity towards *A. awamori*, *A. niger* and *Penicillium griseofulvum*. Ketoconazole (1 %) showed moderate activity against most fungal isolates (9 out of 11) whereas it showed high activity against *A. oryzae* isolates and weak activity against *A. niger*. Nystatin (1%) was less effective against the tested isolates, showing moderate effect towards *A. flavus* (2 out of 4) and isolates of *A. niger, C. albicans* and *P. griseofulvum* (one isolate each) and weak effect towards isolates of *A. awamori* and *A. oryzae* (Table 1).

There are relatively few therapeutically useful antifungal agents compared to the large number of antibacterial agents that are available. This is due mainly to the fact that fungi and man are both eukaryotes and most substances that kill or inhibit fungal pathogens are also toxic to the host. Most antifungal agents exploit differences in the sterol composition of the fungal cell membrane. Antifungal agents vary considerably in their spectrum of activity (Kwon-Chung and Bennett 1992 and Midgley and Moore 1998).

In the 1980s, Miconazole was reported to be useful for therapy of mycotic keratitis in two series of patients. In the first series (Foster 1981), Miconazole resulted in resolution of all lesions in seven patients with mycotic keratitis (four cases due to *C. albicans*, two due to *A. fumigatus*, and one due to *A. flavus*). In the other series (Fitzsimons and Peters 1986), Miconazole was used in combination with Ketoconazole to treat 17 patients with mycotic keratitis (eight cases due to *Fusarium* sp., and 4 to *Aspergillus* sp., 3 to *Drechslera* sp. and one of each of *Curvularia* sp. and *Candida* sp.). Topical Miconazole administration has been reported to be useful in therapy of superficial keratitis due to *Scedosporium apiospermum* (*Pseudallescheria boydii*) (Ruben 1991) or as primary therapy (Panda *et al*. 1996). Intravenous administration of Miconazole was reported to result in successful outcomes in patients with *S. apiospermum* orbital infection (Nunery *et al*. 1985).

Ishibashi (1983) reported that oral Ketoconazole therapy was effective in two patients with mycotic keratitis, one case due to *F. solani*, and the other due to an unidentified fungus. Keratitis due to dematiaceous fungi other than *Curvularia* spp. appears to respond to primary therapy with oral and/or topical Ketoconazole and oral Ketoconazole with topical Miconazole (Rosa *et al*. 1994 and Garg *et al*. 2000).

Two doses of Ketoconazole (100 mg per day) with topical Miconazole ointment for 6 weeks have been recommended for treatment of mycotic blepharitis due to *Candida* spp. (Huber-Spitzy *et al*. 1991).

Dacryocystitis due to *C. albicans* resolved with surgery alone in one patient and with surgery and topical Miconazole therapy in another (Purgason *et al*. 1992). Miconazole was totally ineffective in patients with *Fusarium* keratitis (Thomas 2003).

A steady accumulation in both normal corneas and those infected with *C. albicans* was noted when Fluconazole was given in a twice-daily divided dose. The presence of inflammation induced by fungal infection did not influence corneal uptake (O'Day *et al*. 1990). Cryptococcal chorioretinitis can be treated with oral Fluconazole (Agarwal *et al*. 1991).

In the study of Algalibi (2000), Clotrimazole (1%) and Ketoconazole (2%) were effective *in vitro* against all tested fungi but with variable capabilities. Miconazole nitrate (2%), Isoconazole nitrate (1%) and Tioconazole (1%) were also effective against all tested fungi except *Rhizopus stolonifer*. Also, Tioconazole (1%) and Miconazole (2%) exhibited moderate to low activities against *A. niger, A. ochraceus, A. terreus, A.versicolor* and *Trichothecium roseum* as compared with Clotrimazole (1%), Isoconazole nitrate (1%) and Ketoconazole (2%) which showed high activities against tested *Aspergillus* species and *Trichothecium roseum.*

In another *in vitro* study of Abdul-Rahman (2004), Miconazole nitrate (1%) and Ketoconazole (2%) were the most effective antifungal drugs in vitro against all tested fungi but with variable capabilities. Of the tested species, *Penicillium funiculosum*, *A. flavus*, *A. ochraceus*, *P. chrysogenum* and *P. citrinum* were the most resistant fungal species. They showed no inhibition with Griseofulvin and Nystatin, while *F. solani* and *A. niger* showed the most sensitive fungal isolates against tested antifungal drugs.

Also, the different antifungal therapeutic agents containing Terbinafine HCL, Itraconazole, Sertaconazole, Tioconazole, and Clotrimazole were highly effective *in vitro* on *A. flavus*, *A. fumigatus*, *C. albicans*, *Chrysosporium tropicum* and *Scopulariopsis brevicaulis* and less effective on *Geotrichum candidum*, *Trichophyton interdigitale*, *T. mentagrophytes* and *T. rubrum* (Sallam 2006).

Table (1): Effect of some antifungal drugs on fungal isolates commonly recovered from human eye infections (measured as diameter of inhibition zone in mm).

Species	NIT	Clotrimazole (1%)				Ketoconazole (1%)				Miconazole (1%)				Nystatin (1%)			
		NIP	H	M	W	NIP	H	M	W	NIP	H	M	W	NIP	H	M	W
Aspergillus awamori	1	1	-	1	-	1	-	1	-	1	1	-	-	1	-	-	1
A. flavus	4	4	2	2	-	4	-	4	-	4	4	-	-	4	2	2	-
A. niger	1	1	-	1	-	1	-	-	1	1	1	-	-	1	-	1	-
A. oryzae	3	3	1	2	-	3	1	2	-	3	3	-	-	3	-	-	3
Candida albicans	1	1	1	-	-	1	-	1	-	1	1	-	-	1	-	1	-
Penicillium griseofulvum	1	1	-	1	-	1	-	1	-	1	1	-	-	1	-	1	-
Total isolates	11	11	4	7	0	11	1	9	1	11	11	0	0	11	2	5	4
% of positive isolates		100				100				100				100			

NIT= number of isolates tested; NIP= number of positive isolates.
H= high effect (more than 6.0 mm); M= moderate effect (3.0-6.0 mm); W= weak effect (less than 3.0 mm).
*Floconazole at 2 % concentration showed no effect against all fungi tested.

The antibacterial effect of the 10 drugs commonly used in treatment of eye diseases against 74 bacterial isolates were tested (Table 2). Most drugs tested had antibacterial effect against numerous bacterial isolates. All bacterial isolates tested were affected by Cephotaxime, Ceftriaxone, Ciprofloxacin, Norfloxacin and Tetracycline, while 98.6% of the isolates were inhibited by Gentamycin, 90.5% by Chloramphenicol and 89.2% by Clindamycin and Erythromycin. On the other side, only 44 isolates (59.5% of total isolates) were inhibited by Amoxycillin. On the other hand, Amoxycillin have no effect on isolates of *Staphylococcus epidermidis, E. coli, Pseudomonas aeruginosa* and *Alcaligenes* sp. Similarly, Chloramphenicol showed no effect on *S. pyogenes* and *P. aeruginosa*, Clindamycin and Erythromycin on *E. coli* and *P. aeruginosa* and Gentamycin on *Streptococcus pyogenes*.

Also, the current data presented in table (2) showed that most of drugs tested had high or moderate effect against Gram-positive bacteria and weak effect against Gram-negative bacteria, except Ciprofloxacin and Norfloxacin which showed high or moderate effect against *P. aeruginosa*. Among the Gram-positive bacteria, it was noticed that *B. cereus* and *S. aureus* were the most sensitive towards most drugs while *S.*

epidermidis and *S. pyogenes* were less sensitive. Among the Gram-negative bacteria tested, *Alcaligenes* sp. was the most resistant towards the examined drugs, followed by *E. coli* and *P. aeruginosa*.

McClellan *et al*. (1989) found 95% of culture-proven cases were caused by bacteria and most bacterial isolates were sensitive to Gentamicin.

Insler *et al*. (1991) recommended the use of topical Ciprofloxacin for methicillin-resistant *S. aureus* keratitis. Also, Ciprofloxacin ophthalmic ointment was effective and safe topical antimicrobial agent for the treatment of bacterial keratitis caused by susceptible microorganisms (Wilhelmus *et al*. 1993).

Of 1558 corneal isolates, 478 (30.7%) were not sensitive to Ciprofloxacin. Among the isolates, 355 (32.5%) of the 1091 Gram-positive cocci were not sensitive to Ciprofloxacin, and 2 of the 20 Gram-positive bacilli, 22 of the 165 Gram-negative organisms, and 99 of the 282 actinomycetes and related organisms were not sensitive to Ciprofloxacin (Kunimoto *et al*. 1999).

In the study of Algalibi (2000) most of the tested antibiotics were active *in vitro* against Gram-positive bacteria. Only four drugs showed high inhibitory action towards all tested bacteria (percentage of inhibition between 75%-100% for all bacteria). These antibiotics were Ofloxacin, Ciprofloxacin, Gentamicin and Cephradine. Seven antibiotics exhibited low or no activity against Gram-negative bacteria. These include; Clindamycin, Erythromycin, Neomycin, Penicillin G, Tobramycin, Tetracyclin and Chloramphenicol. *P. aeruginosa* followed by *Serratia marcescens* were the most resistant bacteria against most of the tested antibiotics. On the other hand, *Bacillus cereus* was the most susceptible organism to antibiotics. Also, Abdul-Rahman (2004) found that Ciprofloxacin, Chloramphenicol, Gentamycin and Tetracyclin were the most active drugs *in vitro* against Gram-negative bacteria, while Penicillin, Colistin sulphate and Tobramycin were the lowest active drugs against bacteria. *E. coli* and *Neisseria gonorrhoeae* showed high resistance to all drugs, followed by *Proteus mirabilis* and *P. aeruginosa*, which showed resistance to 12 types of drugs (Abdul-Rahman 2004). Bacterial isolates that were resistant to Ciprofloxacin and Ofloxacin were also resistant to Levofloxacin (Kowalski *et al*. 2001).

Chloramphenicol eye drops were useful for the treatment of Methicillin-resistant *S. aureus* (MRSA) ocular surface infections (Fukuda *et al*. 2002). Lomholt and Kilian (2003) found that the vast majority of eye isolates of *P. aeruginosa* from European countries are fully susceptible to Ciprofloxacin.

Sharma *et al*. (2004) showed that, a significant resistance of *S. aureus* to many antibiotics including Ciprofloxacin and referred to the need for an alternative to Ciprofloxacin monotherapy for the treatment of staphylococcal keratitis.

All *Pseudomonas* isolates (100%) while only 86 % of Gram-negative isolates were sensitive to Ciprofloxacin. *Alcaligenes* sp. was resistant to all antibiotics tested with the exception of Ceftazidime (Briscoe *et al*. 2005). However, no Ciprofloxacin resistance was identified in all *Pseudomonas* isolates tested by Leibovitch *et al*. (2005).

The efficacy of topical Moxifloxacin (0.5%) in the treatment of *P. aeruginosa* keratitis in rabbits suggests a potential value for topical Moxifloxacin as a broad-spectrum agent in the treatment of bacterial keratitis (Aliprandis *et al*. 2005).

Most microorganisms isolated from patients with bacterial keratitis (Coagulase-negative staphylococci, *P. aeruginosa*, *Corynebacterium* spp., *S. aureus* and *Streptococcus* spp.) showed susceptibility to Ciprofloxacin and aminoglycosides (Ly *et al*. 2006).

Conclusion: Treatment with a proper antibiotic based on culturing and sensitivity testing will be of great advantage. Also, ophthalmologists should be aware of the drug of choice to be used for eye infection.

Table (2): Effect of some antibacterial drugs on bacterial isolates commonly recovered isolated from human eye infections (measured as diameter of inhibition zone in mm).

Species	NIT	Amoxycillin (10 µg)				Cephotaxime (30 µg)				Ceftriaxone (30 µg)				Chloramphenicol (30 µg)				Ciprofloxacin (5 µg)			
		NIP	H	M	W	NIP	H	M	W	NIP	H	M	W	NIP	H	M	W	NIP	H	M	W
Staphylococcus aureus	40	40	15	25	-	40	-	40	-	40	29	11	-	40	30	10	-	40	35	5	-
S. epidermidis	20	-	-	-	-	20	16	4	-	20	13	5	2	20	-	17	3	20	-	20	-
Streptococcus pyogenes	1	1	-	1	-	1	-	1	-	1	-	1	-	-	-	-	-	1	1	-	-
Bacillus cereus	3	3	-	-	3	3	3	-	-	3	3	-	-	3	2	1	-	3	3	-	-
Escherichia coli	2	-	-	-	-	2	-	-	2	2	-	-	2	2	-	-	2	2	-	1	1
Pseudomonas aeruginosa	6	-	-	-	-	6	-	-	6	6	-	-	6	-	-	-	-	6	4	2	-
Alcaligenes sp.	2	-	-	-	-	2	-	-	2	2	-	-	2	2	-	-	2	2	-	1	1
Total isolates	74	44	15	26	3	74	19	45	10	74	45	17	12	67	3	60	4	74	43	29	2
% of positive isolates		59.5				100				100				90.5				100			

Table (2): Cont.

Species	NIT	Clindamycin (2 µg)				Erythromycin (15 µg)				Gentamycin (10 µg)				Norfloxacin (10 µg)				Tetracycline (30 µg)			
		NIP	H	M	W	NIP	H	M	W	NIP	H	M	W	NIP	H	M	W	NIP	H	M	W
Staphylococcus aureus	40	40	-	37	3	40	-	40	-	40	-	34	6	40	-	10	30	40	32	8	-
S. epidermidis	20	20	-	17	3	20	-	-	20	20	-	16	4	20	-	13	7	20	5	15	-
Streptococcus pyogenes	1	1	-	-	1	1	-	1	-	-	-	-	-	1	-	-	1	1	-	1	-
Bacillus cereus	3	3	1	2	-	3	-	-	3	3	2	1	-	3	-	2	1	3	3	-	-
Escherichia coli	2	-	-	-	-	-	-	-	-	2	-	-	2	2	-	1	1	2	-	-	2
Pseudomonas aeruginosa	6	-	-	-	-	-	-	-	-	6	-	6	-	6	5	1	-	6	-	-	6
Alcaligenes sp.	2	2	-	-	2	2	-	-	2	2	-	-	2	2	-	-	2	2	-	-	2
Total isolates	74	66	1	56	9	66	-	41	25	73	3	60	10	74	6	23	45	74	43	21	10
% of positive isolates		89.2				89.2				98.6				100				100			

NIT= number of isolates tested; NIP= number of positive isolates.
H= high effect (22.0-27.5 mm); M= moderate effect (16.0-21.0 mm); W= weak effect (8.0-15.0mm).

References:

Abdul-Rahman MA (2004): Bacteria and fungi responsible of human eye infections in Sana'a, Yemen. Ph. D. Thesis, Botany Department, Faculty of Science, Sana'a University, Yemen.

Abdel-Sater MA, Al-Zubeiry AH and Badah KA (2012): Microbiota associated with human eye infections in Taiz city, Yemen. Journal of Basic and Applied Mycology 3(1): 1-11.

Agarwal A, Gupta A, Sakhuja V, Talwar P, Joshi K and Chugh KS (1991): Retinitis following disseminated cryptococcosis in a renal allograft recipient: Efficacy of oral Fluconazole. Acta Ophthalmologica (Copenhagen) 69: 402-405.

Alghalibi SMS (2000): Studies on fungi and bacteria responsible for human corneal ulcers in Upper Egypt. Ph. D. Thesis, Botany Department, Faculty of Science, Assiut University, Assiut, Egypt.

Aliprandis E, Ciralsky J, Lai H, Herling I and Katz HR (2005): Comparative efficacy of topical Moxifloxacin versus Ciprofloxacin and Vancomycin in the treatment of *P. aeruginosa* and Ciprofloxacin-resistant MRSA keratitis in rabbits. Cornea 24(2): 201-205.

Aminov RT (2009): The role of antibiotics and antibiotic resistance in nature. Environmental Microbiology 11(12): 2970-2988.

Briscoe D, Rubowitz A and Assia EI (2005): Changing bacterial isolates and antibiotic sensitivities of purulent dacryocystitis. Orbit 24(2): 95-98

Chattopadhyay MK and Grossart HP (2010): Antibiotic resistance, intractable and here's why. British Medical Journal 341: 6848.

Cheesbrough M (1992): Medical laboratory manual for tropical countries, Vol. II. Butterworth- Heinemann, Cambridge.

Cunningham ET, Seiff SR, Berger TG, Lizotte PE, Howes EL and Horton JC (1998): Intraocular coccidioidomycosis diagnosed by skin biopsy. Archives of Ophthalmology 116: 674-677.

Dash BK, Sultana S and Sultana N (2011): Antibacterial activities of methanol and acetone extracts of fenugreek (*Trigonella foenum*) and coriander (*Coriandrum sativum*). Life Sciences and Medicine Research 27: 1-8.

Fitzsimons R and Peters AL (1986): Miconazole and Ketoconazole as a satisfactory first-line treatment for keratomycosis. American Journal of Ophthalmology 101: 605-608.

Foster CS (1981): Miconazole therapy for keratomycosis. American Journal of Ophthalmology 91: 622-629.

Friedberg ML, Pleyer U and Mondino BJ (1991): Device drug delivery to the eye. Collagen shields, iontophoresis and pumps. Ophthalmology 98: 725-732

Fukuda M, Ohashi H, Matsumoto C, Mishima S and Shimomura Y (2002): Methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative *Staphylococcus* ocular surface infection efficacy of Chloramphenicol eye drops. Cornea 21(7): 586-589.

Garg P, Gopinathan U, Choudhary K and Rao GN (2000): Keratomycosis: clinical and microbiologic experience with dematiaceous fungi. Ophthalmology 107: 574-580.

Grag P, Sharma S and Rao GN (1999): Ciprofloxacin-resistant *Pseudomonas* keratitis. Ophthalmology 106: 1319-1323.

Gwon A (1992): Topical Ofloxacin compared with Gentamicin to the treatment of external ocular infection. British Journal of Ophthalmology 76: 714-718.

Huber-Spitzy V, Baumgartner I, Bohler-Sommeregger K and Grabner G (1991): Blepharitis- a diagnostic and therapeutic challenge. Graefe's Archive for Clinical and Experimental Ophthalmology 229: 224-227.

Insler MS, Fish LA, Silbernagel J, Hobden JA, O'Callaghan RJ and Hill JM (1991): Successful treatment of methicillin-resistant *Staphylococcus aureus* keratitis with topical Ciprofloxacin. Ophthalmology 98(11): 1690-1692.

Ishibashi Y (1983): Oral Ketoconazole therapy for keratomycosis. American Journal of Ophthalmology 95: 342-345.

Kavitha T, Nelson R, Thenmozhi R and Priya E (2012): Antimicrobial activity and phytochemical analysis of *Anisomeles malabarica* (L.) R. British Journal of Microbiology and Biotechnology Research 2 (1): 1-5.

Kowalski RP, Pandya AN, Karenchak LM, Romanowski EG, Husted RC, Ritterband DC, Shah MK and Gordon YJ (2001): An *in-vitro* resistance study of Levofloxacin, Ciprofloxacin, and Ofloxacin using keratitis isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Ophthalmology 108(10): 1826-1829.

Kunimoto DY, Sharma S, Garg P and Rao GN (1999): *In-vitro* susceptibility of bacterial keratitis pathogens to Ciprofloxacin:

Emerging resistance. Ophthalmology 106(1): 80-85.

Kwon-Chung KJ and Bennett JE (1992): Medical mycology. Lea and Febiger, Philadelphia, London, pp. 162-169.

Leibovitch I, Lai TF, Senarath L, Hsuan J and Selva D (2005): Infectious keratitis in South Australia: emerging resistance to Cephazolin. European Journal of Ophthalmology 15(1): 23-26.

Lomholt JA and Kilian M (2003): Ciprofloxacin susceptibility of *Pseudomonas aeruginosa* isolates from keratitis. British Journal of Ophthalmology 87(10): 1238-1240.

Luttrull JK, Wan WL, Kubak BM, Smith MD and Oster HA (1995): Treatment of ocular fungal infections with oral Fluconazole. American Journal of Ophthalmology 119: 477-481.

Ly CN, Pham JN, Badenoch PR, Bell SM, Hawkins G, Rafferty DL and McClellan KA (2006): Bacteria commonly isolated from keratitis specimens retain antibiotic susceptibility to Fluoroquinolones and Gentamicin plus Cephalothin. Clinical and Experimental Ophthalmology 34(1): 3-5.

Mauger TF and Craig EL (1994): Havener's ocular pharmacology, 9th ed. Mosby CV, Louis St., Mo.

McClellan KA, Bernard PJ and Billson FA (1989): Microbial investigations in keratitis at the Sydney eye hospital. Australian and New Zealand Journal of Ophthalmology 17(4): 413-416.

Midgley G and Moore MK (1998): Onychomycosis. Revista Iberoamericana de Micología 15: 113-117.

Mirza SH (2005): Fungal keratitis due to *Fusarium solani*. Journal of College of Physicians and Surgeons, Pakistan 15(9): 576-577.

Neu HC (1978): Symposim on the Tetracyclines: a major appraisal. Bulletin of the New York Academy of Medicine 54: 141-155.

Nunery WR, Welsh MG and Saylor RL (1985): *Pseudallescheria boydii* (*Petriellidium boydii*) infection of the orbit. Ophthalmic Surgery 16: 296-300.

O'Day DM, Foulds G, Williams TE, Robinson RD, Allen RH and Head WS (1990): Ocular uptake of Fluconazole after oral administration. Archives of Ophthalmology 108: 1006-1008.

Palaniappan K and Holley RA (2010): Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. International Journal of Food Microbiology 140: 164-168.

Panda A, Sharma N and Angra SK (1996): Topical Fluconazole therapy of *Candida* keratitis. Cornea 15: 373-375.

Purgason PA, Hornblass A and Loeffler M (1992): A typical presentation of fungal dacryocystitis: A report of two cases. Ophthalmology 99: 1430-1432.

Rosa RH, Miller JD and Alfonso EC (1994): The changing spectrum of fungal keratitis in South Florida. Ophthalmology 101: 1005-1013.

Ruben S (1991): *Pseudallescheria boydii* keratitis. Acta Ophthalmologica (Copenhagen) 69: 684-686.

Sallam AM (2006): Mycological studies on skin diseases in Assiut, Egypt. Ph. D. Thesis, Botany Department, Faculty of Science, Assiut University, Assiut, Egypt.

Seal DV, Barrett SP and McGill JI (1982): Aetiology and treatment of acute bacterial infection of the external eye. British Journal of Ophthalmology 66: 357-360.

Shanmuganathan VA, Armstrong M, Buller A and Tullo AB (2005): External ocular infections due to methicillin-resistant *Staphylococcus aureus* (MRSA). Eye 19(3): 284-291.

Sharma V, Sharma S, Garg P and Rao GN (2004): Clinical resistance of *Staphylococcus* keratitis to Ciprofloxacin monotherapy. Indian Journal of Ophthalmology 52(4): 287-292.

Thakar M (1994): Oral Fluconazole therapy for keratomycosis. Acta Ophthalmologica (Copenhagen) 72: 765-767.

Thomas PA (2003): Current perspectives on ophthalmic mycoses. Clinical Microbiology Reviews 16: 730-797.

Timewell RM, Rosenthal AL, Smith JP and Cagle GD (1983): Safety and efficacy of Tobramycin and Gentamicin sulfate in the treatment of external ocular infections of children. Journal of Pediatric Ophthalmology and Strabismus 20: 22-26.

Wilhelmus KR, Hyndiuk RA, Caldwell DR, Abshire RL, Folkens AT and Godio LB (1993): 0.3% Ciprofloxacin ophthalmic ointment in the treatment of bacterial keratitis. The ciprofloxacin ointment/bacterial keratitis study group. Archives of Ophthalmology 111(9): 1210-1218.

Journal of Basic & Applied Mycology (Egypt) 4 (2013): 21-32

Vaginal yeast infection in patients admitted to Al-Azhar University Hospital, Assiut, Egypt

A. M. Moharram[1]*, Manal G. Abdel-Ati[2] and Eman O. M. Othman[1]

[1]Department of Botany and Microbiology, Faculty of Science, Assiut University
[2]Department of Obstetrics and Gynecology, Faculty of Medicine, Al-Azhar University, Assiut, Egypt

***Corresponding author: e-mail: ahmadmhrrm@yahoo.com**
Received 24/9/2013, Accepted 30/10/2013

Abstract: In the present study, 145 women were clinically examined during the period from December 2011 to July 2012 for vaginal yeast infection. Direct microscopy and culturing of vaginal swabs revealed that only 93 cases (64.1 %) were confirmed to be affected by yeasts. The majority of patients were 21-40 years old representing 70% of the positive cases. Yeast infection was more encountered in women receiving oral contraceptives (40%) than in those complaining of diabetes mellitus (25%) or treated with corticosteroids (17%). Phenotypic and genotypic characterization of yeast isolates showed that *Candida albicans* was the most prevalent species affecting 45.2% of patients, followed by *C. krusei* and *C. tropicalis* (20.4 % and 10.8% respectively). *C. glabrata* and *C. parapsilosis* were rare (3.3% and 1.1% respectively). *Rhodotorula mucilaginosa* and *Geotrichum candidum* occurred in 18.3% and 1.1% of vaginal samples respectively. Protease was produced by 83 out of 93 isolates tested (89.2%) with active isolates belonging to *C. albicans* and *C. krusei*. Lipase was produced by 51.6% of isolates with active producers related to *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei*. All isolates of *Candida* and *Geotrichum* were unable to produce urease enzyme, but those of *R. mucilaginosa* produced this enzyme. In-*vitro* sensitivity test showed that *C. albicans* isolates were sensitive to all antifungal agents (12 types) with the most effective drugs being Nystatin and Terbinafine affecting 100% of strains. Cetrimide, Fluconazole, Clotrimazole, Miconazole and Amphotericin-B affected 59.5.8% - 85.7% of the tested isolates.

Key words: vaginal candidiasis, antifungal agents, enzymes.

Introduction

Candida species are commensals of diseased skin and mucosal surfaces of the gastrointestinal, genitourinary, and respiratory tracts. Vulvovaginal candidiasis (VVC) occurs most commonly in post-pubertal women who have diabetes mellitus, have been taking systemic antibacterial agents, or in the third trimester of pregnancy, or those who are sexually active (Odds 1994). Although the evidence is contradictory, estrogen contraceptive therapy probably predisposes women to the infection. Although an intestinal reservoir or a sexual-partner with *Candida* balanitis has been said to be the source of recurrent vulvovaginitis, the best current evidence indicates that relapse comes from organisms persisting in the vagina (O'Connor *et al.* 1986 and Reed 1992). Sobel (1985) mentioned that *Candida* vaginitis is the second most common vaginal infection. During the childbearing years, 75% of women experience at least one episode of VVC, and 40%-50% of these women experience a second attack. *Candida* is isolated from the genital tract of approximately 10%-20% of asymptomatic, healthy women of childbearing age.

The present work was designed to study the prevalence of vaginal yeast infection in women admitted to Al-Azhar University Hospital in Assiut City. Possible risk factors for infection as well as the ability of isolated fungi to produce some enzymes were studied. Sensitivity or resistance of yeasts to common antifungal agents was also tested.

Materials and methods

1. Patients and sample collection: Eligible participants were women admitted to the outpatient clinic of Gynecology of Al-Azhar University Hospital, Assiut city, during the period from December 2011 to July 2012. Vaginal swabs were taken from women with proven clinical diagnosis of vaginal yeast infection. A questionnaire was also prepared to get information on age of patients as well as on possible risk factors.

2. Mycological analyses

a- Direct microscopic examination (DME): Wet and dry smears stained with lactophenol cotton blue (LPCB) were prepared. Appearance of budding cells with or without pseudohyphae under microscope indicates positive results (Ellis *et al.* 2007).

b- Culturing of specimens: Samples were streaked on the surface of Sabouraud glucose agar (SGA) supplemented with chloramphenicol (0.5 gm/l) to suppress bacterial growth. Cultures were incubated at 37°C for 24-96 h or until the

appearance of colonies. The growing fungi were kept in SGA slants for further investigation (Ellis *et al*. 2007).

c- Phenotypic identification of yeasts: CHROMagar *Candida* medium kindly supplied by the CHROMagar Company, Paris, France, has been recommended for rapid identification of many common *Candida* species (Pfaller *et al*. 1996). Yeast cultures were streaked on the medium surface and incubated at 37ºC for 48 hours. Chemical colorimetric reaction on agar allows distinction between *C. albicans* (green colonies), *C. tropicalis* (metallic blue colonies), *C. krusei* (pink-fuzzy colonies), *C. glabrata* (mauve-dark pink colonies), and *C. parapsilosis* (white-pale pink colonies) (Odds and Bemaerts 1994). Yeasts were also cultured on corn meal agar (CMA) amended with Tween 80 for 4-7 days to ensure production of chlamydospores (Ellis *et al*. 2007). The ability of *Candida* species to grow on SGA at 45°C was studied in an attempt to differentiate the *C. albicans* isolates from the *C. dubliniensis* isolates (Pinjon *et al.* 1998). Germ tube (GT) production was also tested by inoculating yeasts in small tubes containing 0.5 ml of human serum (male AB plasma, Sigma-Aldrich, Steinem, Germany) containing 0.5 % glucose and incubated at 37ºC for 2-3 hours (Ellis *et al.* 2007). API *Candida* strips (Biomuriex) which allow the performance of 12 identification tests were used to confirm identification of *Candida* species (Bernal *et al.* 1998).

d- Genotypic identification of yeast isolates: Nucleotide sequencing of rRNA genes of some yeast species was done with the help of Solgent Company, Daejeon, South Korea. Primers used for gene amplification have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC - 3'). Then the amplification was carried out in a thermal cycler under the following conditions: one round of denaturation at 95°C for 15 sec followed by 30 cycles of denaturation at 95°C for 20 sec, annealing at 50°C for 40 sec and extension at 72°C for 1 min, with a final extension step at 72 °C for 5 min. The PCR products were then purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) prior to sequencing. Purified PCR products were reconfirmed (using size marker) by electrophoreses on 1% agarose gel. Then the bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture. Each sample was sequenced in the sense and antisense directions using ITS1 and ITS4 primers (White *et al.* 1990). Sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

3. Extracellular enzyme production

a- Protease production was tested on the medium of Paterson & Bridge (1994) containing skimmed milk. The medium was dispensed aseptically in 15 cm test tubes (10 ml/tube) which were inoculated on the surface of agar by 25 µl of yeast cell suspension and incubated at 37°C for 7 days. Formation of clear zone around yeast colonies indicates hydrolysis of skimmed milk by proteolytic enzymes.

b- Lipase production was tested using the medium of Ullman & Blasins (1974) which incorporated Tween 80 as a substrate. The medium was dispensed aseptically in 15 cm test tubes (10 ml/tube), inoculated on the surface of agar by 25 µl of yeast cell suspension and incubated at 37ºC for 10 days. The lipolytic ability was observed as a visible precipitate due to the formation of crystals of calcium salt of the oleic acid liberated by the enzyme. The depth of each visible precipitate (in mm) was measured.

c- Urease production was tested in Difco urea broth suspended into tubes,in aliquots of 0.5 ml. A loopful of cells from 1-2-old-day culture was suspended in the broth and incubated at 37^0C. A change of the color to bright pink or red indicates urease activity (Barnett *et al*. 2000).

4. Antifungal susceptibility test

The disk diffusion method adopted by the Clinical and Laboratory Standards Institute (CLSI 2004) was used to evaluate the in-*vitro* sensitivity of yeasts to 12 antifungal agents and to clove oil. The antifungal agents, producing companies and the interpretative breakpoints of antifungal agents given by Ellis (2011) and Al- Hussaini *et al*. (2013) are shown in Table (1).

Table 1: Interpretative breakpoints of antifungal agents (Ellis 2011 and Al-Hussaini *et al* 2013).

Antifungal agents	Abbr.	Producing Company	Dose/ disc	Zone diameter in mm		
				Susceptible	Intermediate	Resistan
Amphotericin B	AP	Hi-Media	100 units	≥ 15	10 – 14	≤ 10
Cetrimide	CET	Pharco Pharm	5.6 µg	≥ 15	14 – 9	≤ 8
Clotrimazole	CC	Hi-Media	10 µg	≥ 20	12 – 19	≤ 11
Clove oil	C. Oil	Philo Pharm	15 µl	≥ 15	14 – 9	≤ 8
Fluconazole	FLU	Hi-Media	10 µg	≥ 19	15 – 18	≤ 14
Itaconazole	IT	Hi-Media	10 µg	≥ 23	14 – 22	≤ 13
Ketoconazole	KT	Hi-Media	10 µg	≥ 28	27 – 21	≤ 20
Miconazole	MIC	Hi-Media	30 µg	≥ 20	19 - 12	≤ 11
Nystain	NS	Hi-Media	100 units	≥ 15	10 – 14	< 10
Sertaconazole	SER	October Pharm	450 µg	≥ 23	14 – 22	≤ 13
Terbifene HCL	TER	Novartis Pharm	300 µg	≥ 23	14 – 22	≤ 13
Tioconazole	TIO	Pfizer Egypt Pharm	300 µg	≥ 23	14 – 22	≤ 13
Voriconazole	VRC	Hi-Media	1 µg	≥17	16 – 14	≤ 13

Results and discussion

Prevalence of yeast infection in relation to age of patients

During the present work, 145 women were clinically examined for vaginal yeast infection. The direct microscopic examination (DME) revealed that only 64 out of 145 cases (44.1%) were positive showing budding yeast cells either alone or in combination with pseudohyphae. The number of positive culture on SGA cases was higher (93 patients representing 64.1% of total samples) with the majority of cases being affected by a single yeast species. Mixed fungal infections were only observed in few numbers of patients. Results of the present work showed that yeast vaginitis was more common in the age group 21 - 40 years (65 patients representing 69.8% of total positive cases) as shown in Figure (1). These results are nearly similar to those reported from the United States (Sobel 1985) where 75% of women experience at least one episode of vulvovaginal candidiasis (VVC), and 40%-50% of these women experience a second attack. Sobel (1985) suggested that *Candida* organisms gain access to the vagina from the adjacent perianal area and then adhere to vaginal epithelial cells, but *Candida albicans* adheres to vaginal epithelial cells in significantly greater numbers than non-*albicans Candida* species. In Nigeria, Jombo *et al*. (2010) found that the prevalence of *Candida* infection in women was 29.1% (n=2458); no isolate was recovered from persons less than 10 years of age, while the peak age group of infection was 30 - 39 years (11.8%, n=997); the age-group 20 - 49 years accounted for over 25% of the entire infections. In the same country, Nwadioha *et al*. (2010) mentioned that VC was a leading cause of abnormal vaginal discharge in the study constituting 60% (n=420) of the 700 female genital discharge of microbial causes. The result was almost similar to some earlier studies (Sobel *et al*. 1998 and Nwokedi and Anyiam 2003) which recorded 52.5% and 60% respectively.

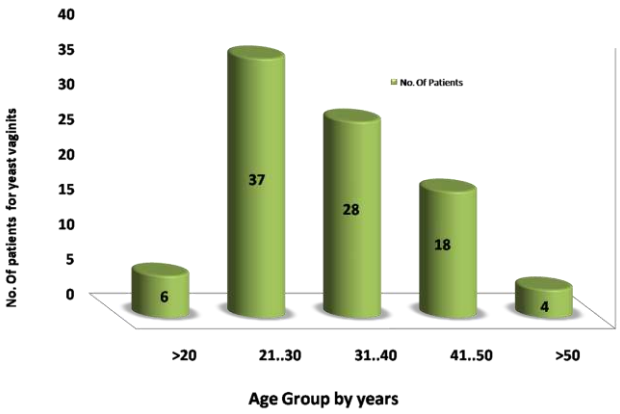


Fig 1: Incidence of vaginal yeast infection in relation to age.

Predisposing factors for vaginal yeast infection

Major risk factors included treatment with oral contraceptives, corticosteroids or diabetes mellitus (Fig 2). As mentioned by Reed (1992), several factors were associated with increased rates of asymptomatic vaginal colonization with *Candida* as well as *Candida* vaginitis including pregnancy (30%-40%), oral contraceptives with a high estrogen content, and uncontrolled diabetes mellitus. The hormonal dependence of VVC is illustrated by the fact that *Candida* is seldom isolated from premenarchial girls, and the

prevalence of *Candida* vaginitis is lower after the menopause, except in women taking hormone replacement therapy. Other predisposing factors include corticosteroids, antimicrobial therapy, intrauterine devices, and high frequency of coitus. As mentioned by Vazquez and Sobel (2003), vaginal colonization with *Candida* is more common in diabetic patients, and poorly controlled diabetes predisposes to symptomatic vaginitis. Glucose tolerance tests have been recommended for women with recurrent vulvovaginal candidiasis (RVVC); however, the yield is low and testing is not justified in otherwise healthy premenopausal women. Diets high in refined sugar may precipitate symptomatic *Candida* vaginitis.

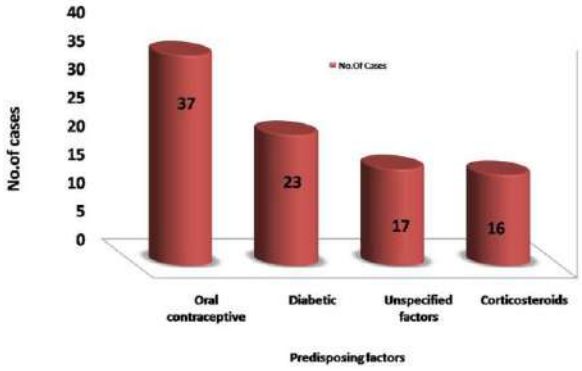


Fig 2: Incidence of vaginal yeast infection in relation to predisposing factors.

Yeasts identified in the present study

Candida was the most prevalent genus being recovered from 75 out of 93 cases representing 80.6% of positive vaginal yeast infections. Five species of *Candida* were identified of which *C. albicans* was the most prevalent (isolated from 45.2% of cases) (Table 2). Colonies of *C. albicans* appeared green on CHROMagar with positive germ tube test and production of chlamydospores (Fig 3). Biochemical reactions using API *Candida* strips as well as molecular characterization based on rRNA gene sequencing and establishment of the phylogenetic tree (Fig 4) confirmed the phenotypic identification of *Candida* species. *C. krusei* and *C. tropicalis* were obtained from 20.4 % and 10.8% of positive cases respectively. *C. glabrata* and *C. parapsilosis* were rarely recovered from vaginal dischange (3.3% and 1.1% of cases respectively). *Rhodotorula mucilaginosa* was found in 18.3% of vaginal samples whereas *Geotrichum candidum* was only obtained from one case (1.1% of the total cases). As mentioned by Ferrer (2000), *Candida albicans* is by far the most common species in gynecology (80-90% of cases) and *Candida glabrata* is the second most common species, causing approximately 5-15% of cases of vaginal candidiasis. In Brazil, Ribeiro *et al.* (2001) found that the prevalence of vaginal yeast isolates was 60% among patients with symptoms of vulvovaginitis and *C. albicans* was the most frequently isolated species followed by *C. glabrata*. According to Nyirjesy *et al.* (1995) an increase of non-*albicans* species has been observed, particularly in recurrent cases. These species are found in approximately 20-30% of cases of recurrent vaginal candidiasis. Among them *C. glabrata* is the most common. Reports from Iran showed that the commonest species was *C. albicans* (78.75%). While other species such as *C. glabrata* (8.75%), *C. krusei* (6.25%) and *C. tropicalis* (2.5%) were less frequent (Pakshir *et al.* 2007). Our results are also in agreement with those of Van Dyck *et al*. (1999) who reported that vulvovaginal candidiasis is created by the fungus *C. albicans* in approximately 85% of cases, with *C. glabrata* being responsible for the remaining 15%. An explanation for the predominance of *C. albicans* in the previous study may be ascribed to its predominance over other *Candida* species in the environment and more importantly, is its favourable survival ability in a depressed human immune system. More recently, Monjaraz-Rodriguez *et al*. (2012) identifying *Candida* species by conventional culturing on chromogenic media as well as by biochemical tests and PCR analysis, found that the most frequent agent of vaginal infection was *C. glabrata* whereas other yeasts as *C. albicans*, *C. krusei* and *C. parapsilosis* were less common.

Table 2: Incidence of yeast species isolated from 93 different vaginal swabs.

Fungal species	Number of cases
Candida (total species)	75 (80.6%)
Candida albicans (Robin) Berkhout	42 (45.2%)
Candida glabrata (Anderson) Meyer and Yarrow	3 (3.3%)
Candida krusei (Castell.) Berkhout	19 (20.4%)
Candida parapsilosis (Ashford) Langeron and Talice	1 (1.1%)
Candida tropicalis (Castell.) Berkhout	10 (10.8%)
Geotrichum candidum (Butler and Peterson) Redhead and Malloch	1 (1.1%)
Rhodotorula mucilaginosa (Jorgensen) Harrison	17 (18.3%)

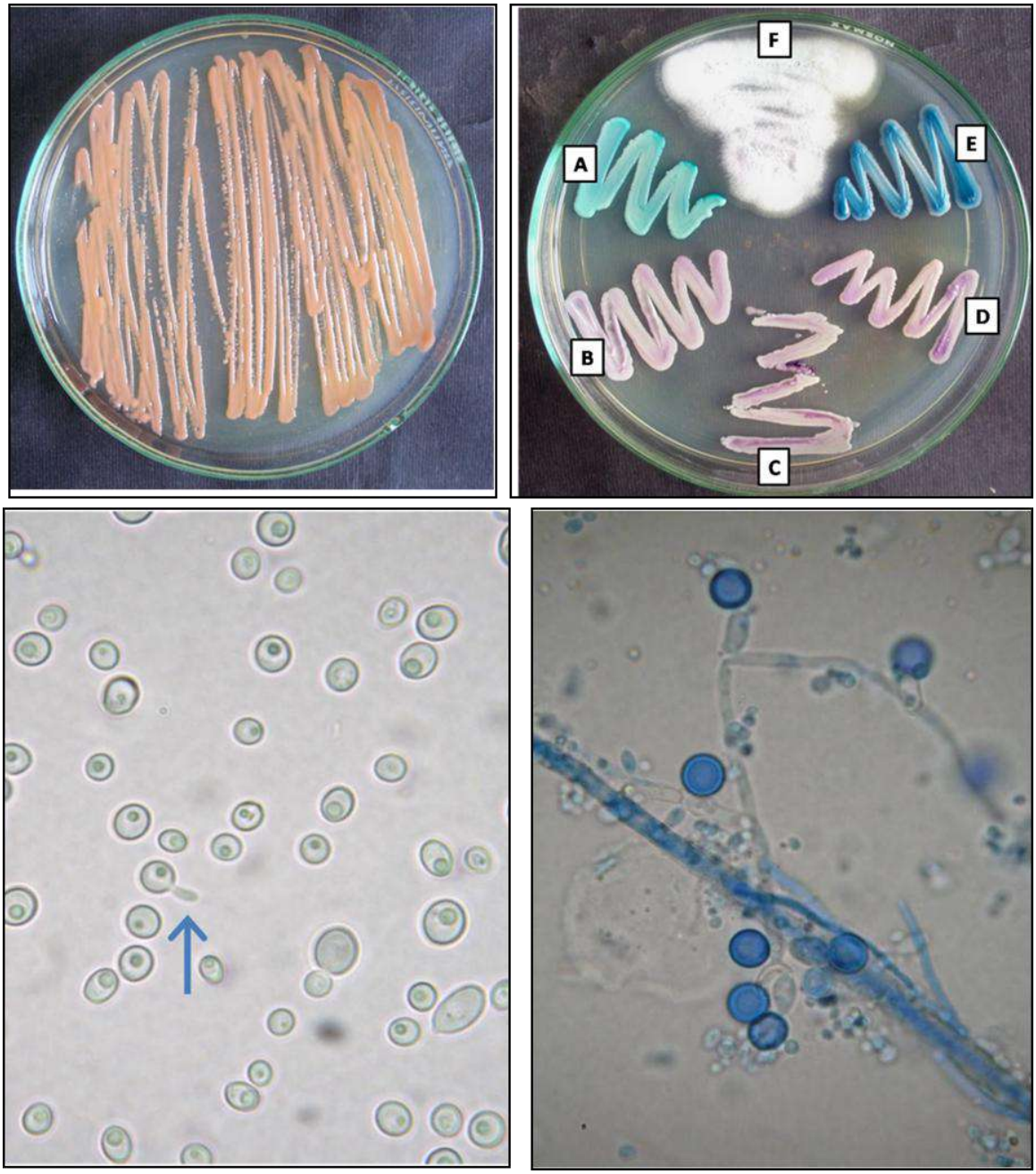


Figure 3: Colony colour of *Rhodotorula mucilaginosa* (top left), *Candida* and *Geotrichum* (top right) as shown on CHROMagar *Candida:* A) Green *Candida albicans*, B) Dark pink *Candida glabrata*, C) Pale pink fuzzy *Candida krusei*, D) Pale pink *Candida parapsilosis*, E) Blue *Candida tropicalis*, F) Chalky white *Geotrichum candidum;* Germ tube (bottom left) and Chlamydospores (bottom right) of *Candida albicans.*

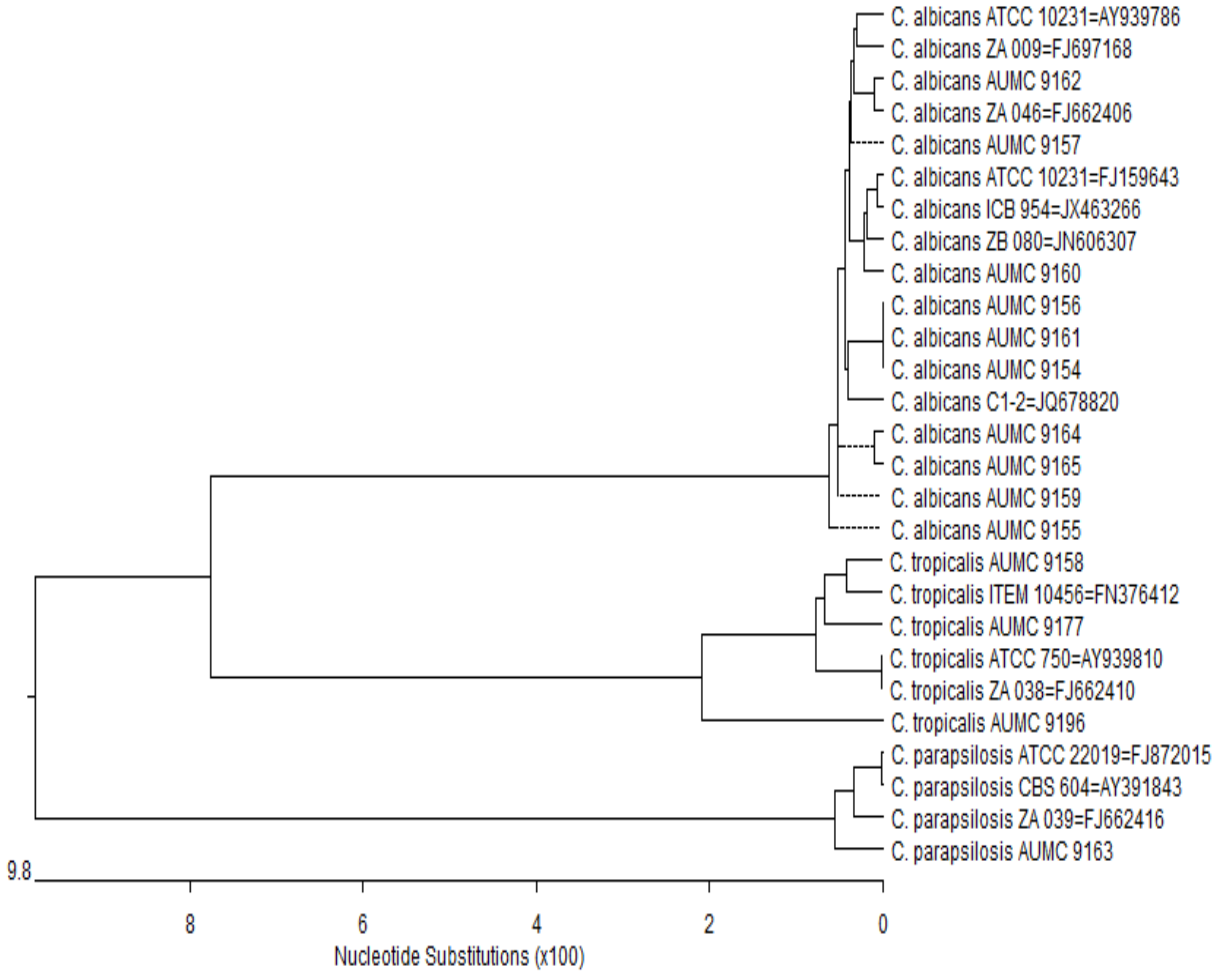


Figure 4: Phylogenetic tree for some *Candida* species isolated (C = *Candida*, given AUMC No.). The scale indicates the number of nucleotide substitutions per site. Reference strains of corresponding fungi are involved in the tree

Extracellular enzymes produced by the isolated yeasts

Since phospholipids and proteins represent the major chemical constituents of the host cell membranes (outer and inner membranes) and the cell contents are mainly of proteins, it was necessary to shed light on the ability of the isolated fungi to produce lipolytic and proteolytic enzymes. Results of the present study showed that protease was produced by 83 (89.2%) of the 93 isolates tested and most isolates of *C. albicans* and *C. krusei* produced moderate to high levels of this enzyme as shown in Table (3). *C. tropicalis* produced variable levels of the proteolytic and lipolytic enzymes. On the other hand, the tested isolate of *C. parapsilosis* yielded low level of protease but the lipolytic activity was not detectable. According to Chakrabarti *et al.* (1992) isolates of *C. glabrata* have shown to be capable of proteinase production. High rates of proteinase in *C. albicans* have also been reported by other workers (Wu *et al.*, 1996 and Koelsch *et al.*, 2000). Kantarcioglu and Yucel (2002) reported that the positivity rate for protease activity from *Candida* was 78.9%. Oksuz *et al.* (2007) reported protease production in 64 (52.4%) isolates. Protease-positive isolates consisted of 46 (56.7%) *C. albicans* and 18 (43.9%) non-*albicans Candida* isolates, namely *C. parapsilosis* and *C. tropicalis* isolates. More recently, Sachin *et al* (2012) detected proteinase in 65 (59.1%) of *Candida* isolates and maximum production was seen in *C. albicans* and *C. tropicalis*. The same authors ascribed the pathogenicity of *Candida* to several putative virulence factors, including germination, adherence to host cells, phenotypic switching and production of extracellular hydrolytic enzymes.

Table 3: Number of yeast isolates showing positive enzymatic activities.

Fungal species	No of isolates tested	Proteolytic			Lipolytic		
		Low	Moderate	High	Low	Moderate	High
C. albicans	42	5	20	16	15	20	3
C. glabrata	3	0	2	0	1	1	0
C. krusei	19	5	8	2	1	0	0
C. parapsilosis	1	1	0	0	0	0	0
C. tropicalis	10	2	8	0	2	2	3
G. candidum	1	0	0	0	0	0	0
R. mucilaginosa	17	14	0	0	0	0	0
Total Isolates	93	27	38	18	19	23	6

Results of the present investigation showed also that lipase was produced by most isolates of *C. albicans*, *C. tropicalis* and *C. glabrata.* On the other hand, the majority of *C. krusei* did not show detectable lipase (Table 3). Phospholipase activity was reported in 30 to 100% of *Candida* isolates tested by Price *et al*. (1982). According to Samaranayake *et al.* (1984) 73% of *C. albicans* isolates showed phospholipase activity. Thangam *et al* (1989) also reported high phospholipase activity in *C. tropicalis*. Kantarcioglu and Yucel (2002) reported that the positivity rate for phospholipase activitiy was 62.1%, in samples from patients with invasive *Candida* infection and phospholipase activity was found to be higher in *C. albicans* isolates than in non-*albicans Candida*. Oksuz *et al*. (2007) found that the phospholipase positive isolates comprised 43 *C. albicans* and 7 non-*albicans Candida* isolates. In their review on microbial lipases, Stehr *et al*. (2003) focused on the virulent traits of these enzymes with special emphasis on lipases from *C. albicans.* They suggested the following roles i) during microbial infections lipolysis might provide carbon sources that the micro-organism could use for growth, ii) the released free fatty acids (FFA) due to lipolytic activity could support cell-to-cell and/or cell-to-host tissue adhesion, iii) a lipase might work hand in hand with another enzyme or it might optimize conditions for other enzymes, iv) lipases might possess additional phospholipolytic activity, v) lipases and their catalytical end products may have an effect on different immune cells and might initiate inflammatory processes, and vi) micro-organisms that secrete lipolytic enzymes might have a selection advantage by lysing competing microflora. Lipolytic enzymes have also been implicated in the virulence of fungal pathogens; the contribution of lipases in fungal pathogenesis has been extensively characterized in *Candida* spp. *C. albicans* possesses at least 10 lipase-encoding genes, the expression of which is largely influenced by the stage of infection (Hube *et al*. 2000). In *C. parapsilosis*, lipases are responsible for the destruction of epidermal and epithelial tissues (Gacser *et al*. 2007). Phospholipase enzyme digests the phospholipid constituents of the host cell membrane leading to cell lysis and alterations of surface characteristics that facilitate adherence and subsequent infection and hence phospholipase production may be used as one of the parameters to distinguish virulent invasive strains from non-invasive colonizers (Sachin *et al*. 2012).

Urease test in the present work was negative for all isolates of *Candida* and *Geotrichum* but those belonging to *Rhodotorula* were urease positive. According to Ellis *et al.* (2007), *R. mucilaginosa* is urease positive and the fungus is a known cause of fungal peritonitis in patients on continuous ambulatory peritoneal dialysis.

Sensitivity of yeasts to antifungal agents

In-*vitro* sensitivity test of vaginal yeast isolates to 12 different antifungal therapeutic agents and clove oil revealed that Nystatin and Terbinafine inhibited all isolates of *C. albicans*, *C. glabrata* and *C. parapsilosis.* Cetrimide, Fluconazole, Clotrimazole, Miconazole and Amphotericin-B inhibited 59.5.8%-85.7% of yeast isolates. *R. mucilaginosa* showed intermediate sensitivity to the majority of antifungal agents (Table 4). Data of the present work showed also that some fungal isolates were resistant to one or more of the frequently prescribed antifungal agents such as Tioconazole (24% of isolates), Ketoconazole (23%), Fluconazole (20%), Clotrimazole (17%), and Sertaconazole (13.9%). There are reports on limited clinical response following fluconazole treatment of *C. glabrata* (Horowitz *et al*. 1985 and Redondo-Lopez *et al*. 1990). Houang *et al*. (1990) reported that after oral therapy of administration of a single dose of 150 mg of Fluconazole, concentrations of Fluconazole above the MIC that inhibited the growth of 90% of *Candida* isolates were achieved for 72-96 h in vaginal tissue (2.43 ng/gm) and secretions. However, no clinical correlation was observed between the in-*vitro* Fluconazole susceptibility profile and clinical response both in diabetic and non-diabetic subjects.

Table 4: Sensitivity of yeast isolates to antifungal agents.

Yeast species (No. of isolates)	DS	AP		CC		CET		FU		IT		KT		MIC	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%
C. albicans (42)	S	25	59.5	31	73.8	36	85.7	34	80.9	20	47.6	9	30.9	21	61.8
	I	17	40.4	5	11.9	1	2.3	1	2.3	20	47.6	23	41.8	21	38.2
	R	0	0.0	6	14.2	5	11.9	7	16.6	2	4.7	10	27.3	0	0.0
C. glabrata (3)	S	2	75	2	75	3	100	3	100	2	75	2	75	1	50
	I	1	25	0	0	0	0.0	0	0.0	1	25	1	25	2	50
	R	0	0	1	25	0	0.0	0	0.0	0	0	0	0	0	0.0
C. krusei (19)	S	4	21	9	47.3	9	47.3	9	47.3	3	15.7	8	37.5	7	62.5
	I	10	52.6	8	42.1	4	21	4	21	8	42.1	3	37.5	12	37.5
	R	5	26.3	2	10.5	6	31.5	6	31.5	8	42.1	8	25.0	0	0.0
C. parapsilosis (1)	S	1	100	1	100	0	0.0	1	100	1	100	1	100	1	100
	I	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
	R	0	0.0	0	0.0	1	100	0	0.0	0	0.0	0	0.0	0	0.0
C. tropicalis (10)	S	4	40.0	4	40	8	80	8	80	2	20	0	0	4	40
	I	4	40.0	2	20	2	20	2	20	4	40	6	60.0	6	60
	R	2	20.0	4	40	0	0.0	0	0.0	4	40	4	40.0	0	0.0
G.candidum (1)	S	0	0.0	0	0.0	0	0.0	1	100	0	0.0	0	0.0	0	0.0
	I	1	100	1	100	0	0.0	0	0.0	1	100	0	0.0	1	100
	R	0	0.0	0	0.0	1	100	0	0.0	0	0.0	1	100	0	0.0
R. mucilaginosa (17)	S	2	11.7	0	0.0	10	58.8	10	58.8	4	23.5	0	0.0	1	4.5
	I	15	88.2	13	76.4	0	0.0	0	0.0	9	52.9	17	100.0	8	54.6
	R	0	0.0	4	23.5	7	41.1	7	41.1	4	23.5	0	0.0	8	40.9
Non- *Candida albicans* (33)	S	11	33.30	16	48.48	20	60.60	21	63.63	8	24.24	13	39.39	13	39.39
	I	15	45.4	10	30.30	6	18.18	6	18.18	13	39.39	10	30.30	20	60.60
	R	7	21.21	7	21.21	7	21.21	6	18.18	12	36.36	10	30.30	0	0.0
Total (93)	S	38	40.8	47	50.5	66	70.9	66	70.9	32	34.4	20	21.5	35	37.6
	I	48	51.6	29	31.1	7	7.5	7	7.5	40	43.1	50	53.7	50	53.8
	R	7	7.5	17	18.2	20	21.5	20	21.5	18	19.3	23	24.7	8	8.6

Table 4: Continued

Yeast species (No. of isolates)	DS	NS		SER		TER		TIO		VRC		Clove oil	
		N	%	N	%	N	%	N	%	N	%	N	%
C. albicans (42)	S	42	100	32	76.1	42	100	17	40.4	12	28.5	10	23.8
	I	0	0	8	19	0	0	10	23.8	30	71.4	29	69
	R	0	0	2	4.7	0	0	15	35.7	0	0.0	3	7.1
C. glabrata (3)	S	3	100	3	100	3	100	3	100	1	33.3	3	100
	I	0	0.0	0	0	0	0.0	0	0	1	33.3	0	0
	R	0	0.0	0	0	0	0.0	0	0	1	33.3	0	0.0
C. krusei (19)	S	14	73.6	2	10.5	7	36.8	3	15.7	8	42.1	17	89.4
	I	5	26.3	15	78.9	5	26.3	12	63.1	5	26.3	2	10.5
	R	0	0.0	2	10.5	7	36.8	4	21	6	31.5	0	0.0
C. parapsilosis (1)	S	1	100	0	0.0	1	100	0	0.0	1	100	0	0.0
	I	0	0.0	1	100	0	0.0	1	100	0	0.0	1	100
	R	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
C. tropicalis (10)	S	10	100	6	60	10	100	2	20	6	60	0	0.0
	I	0	0.0	2	20	0	0.0	4	40	2	20	8	80
	R	0	0.0	2	20	0	0.0	4	40	2	20	2	20
G.candidum (1)	S	1	100	0	0.0	0	0.0	0	0.0	0	0.0	1	100
	I	0	0.0	0	0.0	0	0.0	1	100	1	100	0	0.0
	R	0	0.0	1	100	1	100	0	0.0	0	0.0	0	0.0
R. mucilaginosa (17)	S	0	0.0	7	41.1	0	0.0	0	0.0	0	0	0	0.0
	I	17	100	4	23.5	17	100	17	100	17	100	13	76.4
	R	0	0.0	6	35.2	0	0.0	0	0.0	0	0.0	4	23.5
Non- *Candida albicans* (33)	S	28	84.84	11	33.33	21	63.63	8	24.24	16	48.48	20	60.60
	I	5	15.15	18	54.55	5	15.15	17	51.51	8	24.24	11	33.33
	R	0	0.0	4	12.12	7	21.21	8	24.24	9	27.27	2	6.06
Total (93)	S	71	76.3	50	53.7	63	67.7	25	26.8	28	30.1	31	33.3
	I	22	23.6	30	32.2	22	23.6	45	48.3	56	60.2	53	56.9
	R	0	0.0	13	13.9	8	8.6	23	24.7	9	9.7	9	9.6

DS: Degree of sensitivity. **S**: Sensitive, **I: I**ntermediate, **R:** Resistant,
AP: Amphotericine-B, **CC**: Clotrimazole, **CET:** Cetrimide**, FU:** Fluconazole, **IT:** Itraconazole**, KT**: Ketoconazole, **MIC:** Miconazole, **NS:** Nystatin, **SER**: Sertaconazole, **TER**: Terbinafine, **TIO**: Tioconazole, **VRC**: voriconazole**.**

In Turkey, Nawrat *et al*. (2000) found Amphotericin-B effective against 100% of *Candida* isolates. They also noticed that 72.1% of *Candida* strains were sensitive to Ketoconazole, 61.4% to Fluconazole and 47.1% to Itraconazole. The study of Kronvall and Karlsson (2001) showed that all *Candida* isolates were susceptible to Fluconazole except *C. glabrata* and *C. Krusei*. According to Sobel *et al.* (2004), treatment with Fluconazole has been successful based on moderate evidence from a randomized clinical trial. As stated by Khan and Baqai (2010), Clotrimazole was more effective as compared to Fluconazole and Nystatin. Working with 270 isolates of *Candida*, Ellis (2011) recorded that all isolates of *C. albicans* were susceptible to Fluconazole and Clotrimazole but resistance to Fluconazole was manifested by 20% of *C. glabrata* isolates. The in-*vitro* drug sensitivity test performed by Goswami *et al*. (2000) showed no significant difference in the Fluconazole susceptibility pattern between *C. albicans* and non-*C. albicans* candidiasis The same authors mentioned that certain strains of *C. glabrata* are genetically tolerant to Fluconazole.

Conclusion It can be concluded that correct diagnosis of yeast isolates based on phenotypic and genotypic methods is necessary. Antifungal susceptibility testing is also of great value to exclude ineffective antifungal agents and allows better selection of the most active drugs.

References

Al-Hussaini MS, El-Tahtawi NFO and Moharram AM (2013): Phenotypic and molecular characterization of *Candida* species in urine samples from renal failure patients Science. Journal of Clinical Medicine 1: 1-11

Barnett JA, Payne RW and Yarrow D (2000): Yeasts characteristics and identification, 3rd ed., Cambridge University Press, Cambridge, England.

Bernal S, Mazuelos EM, Chváez M, Coronilla J and Valverde A (1998): Evaluation of the new API Candida system for identification of the most clinically important yeast species. Diagnosis of Microbiological Infectious Diseases 32:217-221.

Bohannon NJ (1998): Treatment of vulvovaginal candidiasis in patients with diabetes. Diabetes Care 21: 451-6.

Chakrabarti RN, Dutta K, Sarkhel T and Maity S (1992): Cytologic evidence of the association of different infective lesions with dysplastic change in the uterine cervix. European Journal of Gynecology and Oncology 13: 398-402.

CLSI (2004): Clinical and laboratory standards Institute, methods for antifungal disk diffusion susceptibility testing of yeasts; approved guideline, 2nd ed., Document M44-A2. National Committee for Clinical Laboratory Standards (NCCLS), Wayne, PA.

Ellis D (2011): Antifungal susceptibility profile (Australian antifungal susceptibility data for *Candida* isolates from recurrent volvovaginal candidiasis (2007-2009) using the CLSI M44-A2 disc susceptibility standard for yeasts). Mycology Online.

Ellis D, Davis S, Handke R and Bartley R (2007): Description of medical fungi 2nd edition Mycology Unit Women's and Children's Hospital, North Adelaide 5006 Australia.

Ferrer J (2000): Vaginal candidiasis: epidemiological and etiological factors. International Journal of Gynecology and Obstetrics 71: 521-527.

Gácser A, Schäfer W, Nosanchuk JS, Salomon S, Nosanchuk JD. (2007): Virulence of *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* in reconstituted human tissue models. Fungal Genetics and Biology 44: 1336-1341.

Goswami R, Dadhwal V, Tejaswi S, Datta K, Paul A and Haricharan RN (2000): Species-specific prevalence of vaginal candidiasis among patients with diabetes mellitus and its relation to their glycaemic status. Journal of Infection 41: 162-166.

Horowitz BJ, Edelstein SW and Lippman L (1985): Candida tropicalis vulvovaginitis. Journal of the American Academy of Obstetrics and Gynecology 66:229-232.

Houang ET, Chappatte O, Byrne D, Macrae PV and Thorpe JE (1990): Fluconazole levels in plasma and vaginal secretions of patients after a 150-miligram single oral dose and rate of eradication of infection in vaginal candidiasis. Antimicrobial Agents and Chemotherapy 34: 909-410.

Hube B, Stehr F, Bossenz M, Mazur A, Kretschmar M and Schäfer W (2000): Secreted lipases of *Candida albicans*: cloning, characterization and expression analysis of a new gene family with at least ten members. Archives for Microbiology 174: 362–374.

Jarvis WR (1995): Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. Clinical Infectious Diseases 20: 1526-1530.

Jombo GTA, Opagobi SO, Egah DZ, Banwat EB and Akaa PD (2010): Symptomatic vulvovaginal candidiasis and genital colonization by *Candida* species in Nigeria. Journal of Public Health and Epidemiology 2: 147-151.

Kantarcioglu AS and Yucel A (2002): Phospholipase and protease activities in

clinical *Candida* isolates with reference to the sources of strains. Mycoses 45: 160-165.

Khan F and Baqai R (2010): In-*vitro* antifungal sensitivity of fluconazole, clotrimazole and nystatin against vaginal candidiasis in females of childbearing age. Journal of Ayub Medical College Abbottabad, Pakistan 22: 197-200

Kronvall G and Karlsson I (2001): Fluconazole and voriconazole multidisk testing of *Candida* species for disk test Calibration and MIC estimation. Journal of Clinical Microbiology 39: 1422-1428.

Monjaraz–Rodríguez S, Alvarez–Gutiérrez PE, Vega–Villa VM, Xoconostle–Cázares B and Pérez–Luna YD (2012): A molecular epidemiological study of prevalence of *Candida* spp. in women in the City of Tuxtla Gutierrez, Chiapas. International Biotechnology Color Journal 2: 6-14.

Nawrat U, Grzybek-Hryncewicz K and Karpiewska A (2000): Susceptibilty of *Candida* species to antimycotics determined by microdilution method. Mikologia Lekarska 7:19-26.

Nwadioha SI, Egah DZ, Alao OO and Iheanacho E (2010): Risk factors for vaginal candidiasis among women attending primary health care centers of Jos, Nigeria. Journal of Clinical Medicine and Research 7: 110-113.

Nwokedi EE and Anyiam NN (2003): A study of high vaginal swabs in Kano Teaching Hospital .A preliminary report. Highland Medical Research Journal 1: 57-61.

Nyirjesy P, Seeney SM, Grody MHT, Jordan CA and Buckley HR (1995): Chronic fungal vaginitis: the value of cultures. Journal of the American Acadmy of Obstetrics and Gynecology 173: 820-823.

O'Connor MI and Sobel JD (1989): Epidemiology of recurrent vulvovaginal candidiasis identification and strain differentiation of *Candida albicans*. Journal of Infectious Diseases 154: 358-363.

Odds FC (1994): *Candida albicans*, the life and times of a pathogenic yeast. Journal of Medical and Veterinary Mycology 32: l-8.

Odds FC and Bernaerts R (1994): CHROMagar *Candida*, a new differential isolation medium for presumptive identification of clinically-important *Candida* species. Journal of Clinical Microbiology 32: 1923-1929.

Oksuz S, Sahin I, Yildirim M, Gulcan A, Yavuz T, Kaya D and koc AN (2007): Phospholipase and proteinase activities in different *Candida* species isolated from anatomically distinct sites of healthy adults. Japanese Journal of Infectious Diseases 60: 280-283.

Pakshir K, Yazdani M and Kimiaghalam R (2007): Etiology of vaginal candidasis in Shiraz, Southern Iran. Research Journal of Microbiology 2: 696-700.

Paterson RRM and Bridge PD (1994): Biochemical Methods for Filamentous fungi. IMI Technical Handbooks No, 1, Wallingford, UK: CAB International, 144 pp.

Pfaller MA, Houston A and Coffmann S (1996): Application of CHROMagar *Candida* for rapid screening of clinical specimens for *Candida albicans*, *Candida tropicalis*, *Candida krusei*, and *Candida* (*Torulopsis*) *glabrata*. Journal of Clinical Microbiology 34: 58-61.

Pinjon E, Sullivan D, Salkin I, Shanley D and Coleman D (1998): Simple, inexpensive, reliable method for differentiation of *Candida dubliniensis* from *Candida albicans*. Journal of Clinical Microbiology 36:2093–2095.

Price MF, Wilkinson ID and Gentry LO (1982): Plate method for detection of phospholipase activity in *Candida albicans*. Sabouraudia 20:7-14.

Reed B (1992): Risk factors for Candida vulvovaginits. Obstetrics and Gynecology Survey 47: 551-560.

Redondo-Lopez V, Lynch M, Schmitt C, Cook R, Sobel JD (1990): *Torulopsis glabrata* vaginitis: clinical aspects and susceptibility to antifungal agents. Obstetrics and Gynecology 76: 651-655.

Ribeiro MA, DietzeR, Paula CR, Da Matta DA and Colombo AL (2001): Susceptibility profile of vaginal yeast isolates from Brazil. Mycopathologia 151: 5-10.

Sachin CD, Ruchi K, Santosh S (2012): In-*vitro* evaluation of proteinase, phospholipase and haemolysin activities of *Candida* species isolated from clinical specimens. International Journal of Medicine and Biomedical Research 1: 153-157.

Samaranayake LP, Raeside JM and Max Farlane TW (1984): Factors affecting the phospholipase activitiy of *Candida* spp. in-*vitro*. Sabouraudia 22: 201-207.

Sobel JD (1985): Management of recurrent vulvovaginal candidiasis with intermittent ketoconazole prophylaxis. American Journal of Obstetrics and Gynecology 65: 435-440.

Sobel JD, Brooker D, Stein GE, Thomason JL, Wermeling DP and Bradley B (1995): Single oral dose fluconazole compared with conventional clotrimazole topical therapy of *Candida* vaginitis. Fluconazole Vaginitis Study Group. American Journal of Obstetrics and Gynecology 172: 1263-1268.

Sobel JD, Faro S, Force RW and Fox B (1998): Vulvovaginal candidiasis: Epidemiologic, diagnostic and therapeutic considerations.

American Journal of Obstetrics and Gynecology 178: 203-211.

Sobel JD, Wiesenfeld HC, Martens M, Danna P, Hooton TM and Rompalo A (2004): Maintenance fluconazole therapy for recurrent vulvovaginal candidiasis. The New England Journal of Medicine 351: 876-883.

Stehr F, Kretschmar M, Kröger C, Hube S and Schafer W (2003): Microbial lipases as virulence factors. Journal of Molecular Catalysis B: Enzymatic 22: 347–355.

Thangam M, Smith S and Deivanayagam CN (1989): Phospholipase activity of *Candida* isolates from patients with chronic lung disease. Lung India 7: 125-126.

Ullman U and Blasins C (1974): A simple medium for the detection of different lipolytic activity of microorganisms. Zentralblatt für Bakteriologie und Hygiene 229: 264–267.

Van Dyck E, Meheus AS and Piot P (1999): A manual on laboratory diagnosis of sexually-transmitted diseases. World Health Organization, Geneva.

Vazquez JA and Sobel JD (2003): Candidiasis. In: Dismukes WE, Pappas PG and Sobel JD (eds.) Clinical Mycology, Oxford University Press Inc., 519 pp.

Vidotto V, Kogo-Ito CYand Garramone A (1999): Virulence factors, serotype distribution and adherence in *Candida albicans*. The 5th Congress of the European Confederation of Medical Mycology, Mycoses, Dresden, Germany.

White TJ, Bruns T, Lee S and Taylor J (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A., Gelfand D. H., Sninsky J. J. and White T. J. (eds.) PCR Protocols: a Guide to methods and applications, Academic Press, New York, pp. 315-322.

Wu T, Samaranayake LP, Cao BY and Wang J(1996): In-*vitro* proteinase production by oral *Candida albicans* isolates from individuals with and without HIV infection and its attenuation by antimycotic agents. Journal of Medical Microbiology 44: 311-316.

Journal of Basic & Applied Mycology (Egypt) 4 (2013): 33-45

Preliminary study on soil microbiota degrading plant latices and their protease and lipase enzymes

A. A. M. Shoreit[1], M. A. Ismail[1,*], Nadia H. Mohamed[1,2]

[1]Department of Botany and Microbiology, Faculty of Science, Assiut University, Assiut, Egypt
[2]Biology Department, Faculty of Science and Art, Samtah, Jazan University, Jazan, Saudi Arabia

[*]Corresponding author: e-mail: madyismail@yahoo.com
Receivd 24/6/2013, Accepted 3/12/2013

Abstract. The present study aimed to isolate soil microorganisms-degrading plant latices and to screen their capablilities of producing protease and lipase. Many bacterial and fungal strains of mesophilic, thermotolerant or thermophilic nature were able to grow on sugar-free medium amended with crude latex of either *Euphorbia pulcherrima, Ficus elastica* or *F. nitida* and incubated at 25ºC, 36ºC or 45ºC. This growth revealed that these growing microorganisms were able to make use of the crude latex as a sole carbon source. The total mesophilic bacterial propagules recovered on mineral salt medium amended with F. elastica latex was much higher than those recovered on media amended with either *E. pulcherrima* or *F. nitida* latex. Some of the mesophilic bacteria formed clear zones around their colonies (some *Streptomyces* spp. recovered on *Ficus elastica* and *F. nitida* latices) while the others showed no clear zones. Also one thermophilic and another thermotolerant species related to the genus *Bacillus* were isolated on media amended with the three plant latices. The total numbers of mesophilic fungal propagules recovered on medium amended with either *Ficus* latex were nearly equal, but higher than that amended with *E. pulcherrima* latex. Thirty-one species of fungi were recovered on the three latex media. Of mesophiles, *Aspergillus* followed by *Fusarium, Penicillium* and *Cladosporium* were the most common fungi, while the remaining fungi were less frequent. Three truly thermophilic species, *Humicola grisea* var. *thermoidea, Malbranchea cinnamomea* and *Myceliophthora thermophile*, were isolated. All mesophilic bacterial isolates, except one related to *Streptomyces* sp., were capable of producing protease and lipase, while from the thermophilic/thermotolerant bacterial isolates tested, only one *Bacillus* isolate could produce both enzymes and the other two isolates of *Bacillus* could not. The thirty mesophilic fungal isolates tested were capable of producing lipase, while 25 only were protease producers. On the other hand, the eleven thermophilic fungal isolates tested were capable of producing protease, while 8 only were lipase producers.

Key words: Degradation, lattices of *Ficus* and *Euphorbia* spp., soil fungi and bacteria, production of lipase, protease.

Introduction

Over 2000 species of plants belonging to different families (Euphorbiaceae, Asclepiadaceae, Moraceae, Asteraceae, Caricaceae, Cannabinaceae and Apocyanaceae) can produce latex and natural rubber (Archer and Audley 1973 and Lewinsohn 1991). In addition, at least two fungal genera, *Lactarius* and *Peziza* were also found capable of producing latex and natural rubber with low molecular weights (Ohya and Tanaka 1998).

Latex is the cytoplasm of highly specialized cells known as laticifers, and is produced and stored in the tubular structures known as vessels. The latex vessel is located mainly in the secondary phloem of the trunk, and is intermingled with a sieve tube, although latex is also present in the flowers, fruits and leaves. The average composition of latex milk is as follows (wt/wt): 25 to 35 % polyisoprene, 1 to 1.8 % protein, 1 to 2% carbohydrates, 0.4 to 1.1% neutral lipids, 0.5 to 0.6% polar lipids, 0.4 to 0.6% inorganic components, 0.4% amino acids, amides, etc., and 50 to 70% water (Subramanian 1995). Within the latex milk, natural rubber (NR) occurs in the form of particles as an emulsion of droplets with a predominant size of 0.1 to 2 mm in diameter in water (Pendle and Swinyard 1991). These rubber particles are covered by a layer of proteins and lipids, which separate the hydrophobic rubber molecules from the hydrophilic environment.

Investigations of bacterial degradation of NR revealed two groups of NR-degrading bacteria according to their strategy for substrate utilization. (i) Members of the first group form clear zones on latex overlay-agar plates, indicating an extracellular enzyme activity. Representatives belong to the genus *Streptomyces* (Rose *et al.* 2005, Ibrahim *et al.* 2006 and Bröker *et al.* 2008). (ii) Members of the second group (non-forming clear zone) exhibit adhesive growth with direct contact of the cells with the NR material and extensive disintegration of the substrate. Representatives belong to the genera *Gordonia*, *Mycobacterium*, *Streptomyces* and *Nocardia* (Linos *et al.* 1999, 2002).

This work aimed to isolate and identify soil microorganisms (bacteria and fungi) which have the ability to degrade and metabolize plant

latices of *Euphorbia pulcherrima*, *Ficus elastica* or *Ficus nitida* when added to the carbon-free medium and to assess the ability of these microorganisms to produce protease and lipase enzymes that may enable them to grow on and metabolize such latices.

Materials and Methods

Collection of soil samples

Fifty samples of cultivated soils were collected from different sites in Assiut Governorate, Egypt.

Plant Latices used and their sampling

Latices chosen for this study were obtained from *Euphorbia pulcherrima* Willd. ex Koltzsch (family Euphorbiaceae), *Ficus elastica* Roxb. ex Hornem and *Ficus nitida* Thunb (family Moraceae). Their natural rubber is cis-1, 4 polyisoprene. Crude latices from the above plants growing in the farms of the Faculties of Science and Agriculture, University of Assiut were collected from plants by the tapping method (Wititsuwannakul and Wititsuwannakul 2001). They were obtained in sterile Eppendorf tubes by cutting leaves of *E. pulcherrima* or injuring trunk in case of *F. elastica* and *F. nitida*, thereafter the latex was drawn by sterile disposable syringe.

Media used for isolation of bacteria and fungi degrading crude latex

Sugar-free mineral salt medium (MSM) for bacteria (Heisey and Papadatos 1995) of the following composition (g/l); K_2HPO_4 8, KH_2PO_4 1, $(NH_4)2SO_4$ 0.5, $MgSO_4.7H_2O$ 0.2, NaCl 0.1, Ca $(NO_3)_2$ 0.1, $CaCl_2.2H_2O$ 20 mg, $FeSO_4.7H_2O$ 20 mg, $Na_2MoO_4.H_2O$ 0.5 mg, $MnSO_4$ 0.5 mg, yeast extract 30 mg, agar (for the bottom layer) 20, agar (for the soft top layer) 7, pH 7.5. Sterile crude latex was added at a rate of 2 ml/l of autoclaved soft agar medium.

Sugar-free Czapek agar medium (SFCZ) for fungi (Lynch *et al.* 1981) of the following composition (g/l): $NaNO_3$ 3, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.5, KCl 0.5, agar (for the bottom layer) 20 and agar (for the soft top layer) 7, pH 7. Sterile crude latex was added at a rate of 2 ml/l of autoclaved soft agar medium.

Isolation procedure of bacteria and fungi degrading crude latex

Bacteria and fungi were isolated on sugar-free agar media described above. Overlay technique was used (Braaz *et al.* 2004). About 20 ml of bottom agar media were poured into each of three replicates of sterile Petri-dishes, then overlayed with vortexed soft agar media (0.7%) containing a proper dilution volume of soil suspension plus crude latex (20 µl / 10 ml soft agar). After solidification, the plates were incubated for 15 days at 36ºC and 45ºC for bacteria and 25ºC and 45ºC for fungi. The developing bacterial and fungal colonies were counted, isolated and identified. Pure cultures were deposited in the Culture Collection of Assiut University Mycological Center (AUMC).

Identification of microorganisms

Bacterial isolates were identified on the basis of their phenotypic characters according to Bergey's Manual (2005). Gram staining, motility (Rhodes 1958), catalase test (Wittenberg 1964) and cytochrome C oxidase (Ewing and Johnson 1960) were carried out. Fungal isolates were identified based on their macro- and microscopic features following the keys of Raper and Fennell (1965), Moubasher (1993), Domsch *et al.* (2007), and Salar and Aneja (2007).

Enzyme production

Protease production: Bacterial and fungal proteolytic ability was tested using casein hydrolysis media, medium of O'reilly and Day (1983) for bacterial isolates and medium of Paterson and Bridge (1994) for fungal isolates. The media were sterilized by autoclaving at 121ºC for 15 minutes. The cooled medium was then poured into 9 cm Petri-dishes (~20 ml/plate). The inoculated plates were incubated at 36ºC and 45ºC for three days in case of bacteria and 25ºC, and 45ºC for 48 hours in case of fungi. After incubation complete degradation of milk protein was seen as clear zone around the colony. The diameters of the clear zone and fungal colony were measured (in mm). Enzyme index for caseinase (PI) was expressed according to Ho and Foster (1972) as follows:

Enzyme index = Diameter of the outer limit of the clear zone/ Diameter of the fungal colony.

Lipase production: Bacterial and fungal lipolytic capability was determined using the medium of Ullman and Blasins (1974) with slight modification. Tween 80 (sorbitan polyoxyethylene monooleate) was added instead of tween 20. The medium was dispensed aseptically in test tubes. The inoculated tubes were incubated for 48 hours at 36ºC and 45ºC in case of bacteria and at 25ºC and 45 ºC in case of fungi. The enzyme production was detected by measuring the depth of white precipitate formed at the top of the medium, due to the formation of crystals of the calcium salt of the oleic acid liberated by the enzyme.

Results and Discussion

Diversity of soil mesophilic bacteria recovered on carbon-free agar medium amended with crude latex

The total propagules of mesophilic bacteria recovered from the 50 soil samples at 36ºC on mineral salt medium amended with *Euphorbia pulcherrima* latex ranged from 16.66 to 150 colony-forming units (CFUs) per mg soil, however in 10 out of 50 soil samples bacteria were missing on this medium (Table 1). On medium amended with *Ficus elastica* latex, mesophilic bacteria ranged from 8.33 to 135 CFUs per mg soil and in 4 soil samples bacteria were missing. On medium amended with *Ficus nitida* latex, their counts ranged from 5 to 116.66 CFUs per mg soil, however in 7 soil samples no bacteria were recovered. It was noticed that the mean total bacterial propagules recovered on mineral salt medium amended with *Ficus elastica* latex was much higher than those encountered on media supplemented with either *E. pulcherrima* or *F. nitida* latex (Table 1). This may reveal that *E. pulcherrima* and *F. nitida* latex may impose some inhibitory compounds on soil bacteria.

Total mesophilic bacteria recovered from the 50 soil samples at 36°C on media amended each with one of the three types of latices of *E. pulcherrima*, *F. elastica* or *F. nitida* accounted for 523, 636.65 and 388.99 CFUs per mg soil, respectively. The percentage counts of Gram-positive and Gram-negative bacteria were 68.26% and 31.74%, 82.83% and 17.17%, and 80.72% and 19.28% on the above latices respectively (Table 2). It was clear that the percentage CFUs of Gram-positive bacteria was more than that of Gram-negative bacteria.

The Gram-positive and Gram-negative bacteria emerged from 74 %, 50 %; 92 %, 48 %; 68 % and 40 % of the soil samples on media amended with the latices of the three plants respectively (Table 2).

Two *Streptomyces* spp. formed clear zones around their colonies and these were isolated from *F. elastica* latex (one with white colour and the other with grey colour) accounting for 27.9% and 16.61% of the total propagules, and emerging from 86% and 68% of the samples. The third *Streptomyces* sp. with buff colour did not form clear zone and was isolated on the latices of *F. elastica* and *F. nitida* accounting for 25.83% and 53.2% of the total propagules, and emerging from 84% and 68% of the samples, respectively.

In this respect, Heisey and Papadatos (1995) isolated 14 isolates of bacteria from soil on pure natural rubber (pale crepe rubber) that had been surface coated as a thin film on mineral salts agar. Ten isolates were identified as species of *Streptomyces*, *Nocardia* and *Amycolatopsis*. This demonstrates that these bacteria can use the hydrocarbon of natural rubber as well as crude latices as a sole source of carbon and energy (Heisey and Papadatos 1995). Rubber (latex of *Hevea brasiliensis*)-degrading actinobacteria were also recorded from different ecosystems (soil, fresh water and its bottom sediments) by Rifaat and Yosery (2004). These were, in order of dominance, *Streptomyces griseus*, *S. rochei*, *S. coelicolor*, *S. halstedii*, *Micromonospora aurantiaca*, *Actinoplanes italicus*, *Gordonia* sp. and *Nocardia* sp. *Streptomyces* strains degrading natural rubber and forming clear zones on mineral salts agar media containing latex as a sole carbon source were also reported from soil in Germany (Gallert 2000 and Rose *et al.* 2005). They stated that the ability of microorganisms to degrade natural rubber and form translucent halos around their colonies depends on their capability of producing extracellular enzymes. On the other hand, other bacteria could grow on natural rubber latex overlaying agar without showing clear zones around their colonies, e.g. *Pseudomonas aeruginosa* (Linos *et al.* 2000), which exhibited strong rubber-decomposing properties. Also, a *Pseudomonas* sp. was isolated from soil sample through spread plate method on fresh natural rubber latex-agar medium (Roy *et al.* 2006).

Diversity of soil thermophilic and thermotolerant bacteria recovered on carbon-free agar medium amended with crude latex

The total propagules of thermophilic (or thermotolerant) bacteria recovered from 20 soil samples at 45ºC on medium amended with *E. pulcherrima* latex ranged from 0 to 35.33 CFUs per mg soil (Table 1). On medium amended with *F. elastica* latex, counts of soil thermophilic (or thermotolarent) bacteria ranged from 0 to 21.33 CFUs per mg soil and the mean was 8.43 CFUs. In case of *F. nitida* latex, their numbers ranged from 0 to 31.33 CFUs per mg soil. It is worthy to mention that in 6 out of 20 soil samples these bacteria were missing on the three latices. The total numbers of thermophilic and thermotolerant bacteria and percentages of occurrence recovered from the 20 soil samples at 45°C on the latices of *E. pulcherrima*, *F. elastica* and *F. nitida* were 112, 145 and 93.7 CFUs per mg soil respectively (Table 2).

Two species of Gram-positive thermophilic bacteria related to the genus *Bacillus* (one species grew well at 65°C and the other at 55°C) were recovered from 40%-70% of the soil samples on the latices of the above plants (Table 2). In this respect, Ibrahim *et al.* (2006) isolated thermophilic bacteria able to degrade natural

rubber from soil samples collected from Egypt, Germany and Cambodia. These bacteria were identified as *Actinomadura nitritigenes*, *Nocardia farcinica* and *Thermomonospora curvata.*

Diversity of soil mesophilic fungi recovered on carbon-free agar medium amended with crude latex

The total numbers of propagules of mesophilic fungi recovered from 50 soil samples at 25ºC on sugar-free medium supplemented with *E. pulcherrima* latex, ranged from 0 to 53 CFUs per mg of soil, however 2 soil samples were fungi-free on this medium. On the media supplemented with *F. elastica* and *F. nitida*, the counts of fungi ranged from 0.66 to 45 CFUs and 0.33 to 56 CFUs per mg soil respectively (Table 2).

The highest number of CFUs on medium amended separately with the three latices was recorded in a soil sample cultivated with *Triticum aestivum* and possessed the highest pH value (8.4) and nearly the lowest moisture content (3.6%) (data not shown). It was also noticed that the mean total CFUs recovered on medium amended with the latex of the two species of *Ficus* were nearly similar, however higher than those recorded on medium amended with *E. pulcherrima* latex. This indicates that latter latex may impose some antifungal compounds toxic to the soil mycobiota.

The results presented in Table (2) also revealed that 17 genera represented by 27 species were isolated on sugar-free Czapek agar medium amended with latex of either *E. pulcherrima* (21 species related to 14 genera), *F. elastica* (23 species related to 14 genera) or *F. nitida* (25 species related to 11 genera). From these fungi, *Aspergillus* (represented by 7 species) was the only fungus isolated in high frequency on the three media. From *Aspergillus* only *A. niger* and *A. terreus* were isolated in high frequency on the three media. *A. flavus, A. fumigatus, A. sydowii* and *A. ustus* were also isolated on the three media but in low frequency (except *A. ustus* in moderate frequency on *F. elastica* latex). *A. versicolor* was isolated also in low frequency but only on media amended with both *Ficus* species latices. In this respect, Roy *et al.* (2006) isolated an unidentified *Aspergillus* strain from a soil sample through spread plate method on latex-agar medium.

Fusarium (represented by 2 species) was encountered in high frequency on the latices of *E. pulcherrima* and *F. elastica* and in moderate frequency on *F. nitida* latex. *F. solani* was the most common species. It was isolated in moderate frequency on the three latices, while *F. verticillioides* was isolated in moderate frequency only on *E. pulcherrima* latex and in low frequency on both *Ficus* latices. On the other hand, *Penicillium* and *Cladosporium* (2 species each) were isolated in moderate frequency on the three latices in the following order: *P. chrysogenum* > *C. cladosporioides* > *C. sphaerospermum* > *P. aurantiogriseum.* In this respect, attempts to grow *Fusarium solani* upon vulcanized rubber tyres were reported by Faber (1972). Also, Borel *et al.* (1982) isolated some fungal strains from soil samples and deteriorated tyres and rubber by plate cultures on a mineral medium amended with powdered natural rubber and these were identified as *Fusarium solani, F. oxysporum, Cladosporium cladosporioides, C. sphaerospermum, Mucor hiemalis, M. plumbeus, Penicillium variabile, P. vinaceum, Paecilomyces lilacinus* and *Phoma eupyrena*.

Other remaining fungi (14 species) were isolated less frequently as shown in Table (2). Atagana *et al.* (1999) isolated five fungal species from natural rubber waste serum and these included *Mucor racemosus*, *Mucor* sp., *Rhizopus* sp, *Aspergillus niger* and *Aspergillus* sp.

Diversity of soil thermophilic and thermotolerant fungi recovered on carbon-free agar medium amended with crude latex

The total propagules of thermophilic and thermotolerant fungi recovered from 20 soil samples on medium amended with *E. pulcherrima, F. elastica* and *F. nitida* latex at 45ºC ranged from 0.066 to 0.7 CFUs, 0.133 to 0.8 CFUs per mg and 0.133 to 1.033 CFUs per mg soil respectively. It is worthy to mention that in 3 and 2 soil samples no fungi were recovered on medium amended with *E. pulcherrima* or *F. nitida* latex respectively.

Six fungal species were recovered from the 20 soil samples investigated on Czapek agar media at 45 °C coated with latex of *E. pulcherrima* (4 genera and 5 species), *F. elastica* (5 and 6) or *F. nitida* (5 and 6). *Aspergillus* (with *A. terreus* followed by *A. fumigatus*) was the most common fungus recovered on all media. *Emericella nidulans* was reported in high, moderate and low frequency on the three latices of *E. pulcherrima, F. elastica* and *F. nitida* respectively. The three truly thermophilic species, namely *Myceliophthora thermophila* emerged in moderate frequency on both *Ficus* latices only, *Humicola grisea* var. *thermoidea* in moderate frequency on *F. nitida* latex and in low frequency on *E. pulcherrima* and *F. elastica* latices and *Malbranchea cinnamomea* in low frequency on the three latices. In this respect, Allen and Emerson (1949) isolated several species of thermophilic fungi that had temperature limits extending up to 60°C and

Table 1: Minimum, maximum and mean of CFUs/mg dry soil of mesophilic (out of 50 soil samples) and thermophilic (out of 20 samples) bacteria and fungi recovered on sugar-free media amended with plant latices (20 µl latex/plate).

	Counts on media amended with latex of					
	E. pulcherrima		*F. elastica*		*F. nitida*	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
Mesophilic (n=50)						
Minimum	0.0	0.0	0.0	0.66	0.0	0.33
Maximum	150	53	125	45	116.66	56
Mean of total±SD	25.29±36.65	10.67±9.79	64.03±40.44	14.01±11.14	39.46±30.86	14.33±12.33
Thermophilic (n=20)						
Minmum	0.0	0.0	0.0	0.133	0.0	0.0
Maximum	35.33	0.7	21.33	0.8	31.33	1.033
Mean of total ±SD	11.43±10.65	0.32±0.20	8.43±7.63	0.35±0.18	9.57±9.79	0.40±0.27

demonstrated the utilization and reduction in the amount of resin in crude rubber of guayule shrub (*Parthenium argentatum*).

Protease and lipase enzymes production by mesophilic and thermophilic bacteria

Six mesophilic bacterial species represented by 10 isolates, in addition to three thermophilic bacterial isolates related to *Bacillus* were tested for their capabilities of producing protease and lipase enzymes (Table 4). All mesophilic isolates, except the one related to *Streptomyces* sp. ASU3, were capable of producing protease enzyme but with different capabilities. Gram-negative strain ASU13 and *Streptomyces* strain ASU2 and Gram-positive strain ASU6 exhibited high producing abilities (enzyme index >1.5). Also, all mesophilic strains (except *Stroptomyces* sp. ASU3) were capable of producing lipase enzyme but with different rates. From the positive isolates, seven strains assigned to Gram-negative short rods ASU13, ASU9 and ASU4, *Streptomyces* strains ASU5 and ASU8, Gram-positive strains ASU11and ASU12 showed high lipolytic activities (the depth of the visible precipitate was more than 1.5 cm). It was noticed that the low-producing isolates of lipase belong to *Streptomyces* strain ASU2 and Gram-positive strain ASU6 of spore-forming *Bacillus*. In this respect, actinomycetes are known to be good protease producers (Peczynska-Czoch and Mordarski 1988). Rifaat *et al.* (2007) in their screening of 317 isolates of different streptomycetes obtained from several sources and areas in Egypt, found that only 39 isolates produced proteases. Also, Vermelho *et al.* (1996) reported that streptomycetes hydrolyzed preferentially gelatin.

Christen and Marshall (1984) found that lipase and protease were produced simultaneously by *Pseudomonas fluorescens*. On the other hand, many microorganisms are known as good producers of extracellular lipase (Eom *et al.* 2006). It was reported that isolates of bacteria e.g. *Pseudomonas*, *Bacillus*, *Aeromonas* and *Staphylococcus* could produce lipase and the highest producers were *Pseudomonas* sp., *P. aeruginosa* (Haba *et al.* 2000) and *Bacillus stearothermophilus* (Abada 2008).

The present results showed that the three thermophilic isolates tested, only one (*Bacillus* sp. ASU14) exhibited high protease producing ablity (PI>1.94), but low lipase producing ability (LI=0.8). On the other hand, the two strains of *Bacillus* (ASU 7 and ASU 15) were incapable of producing protease and lipase (Table 3). In this respect, there have been many reports on thermophilic proteases produced by different strains of *Bacillus* spp., including the thermolysin from *B. thermoproteolyticus* (Endo 1962), an alkaline protease from *Bacillus* sp. (Takami *et al.* 1989), and a thermostable neutral protease from *B. stearothermophilus* (Fujio and Kume 1991). Protease production has also been manifested by thermophilic *Bacillus* strains (Nascimento and Martins 2004) and *Bacillus licheniformis* (Folasade *et al.* 2008) and a mesophilic *Bacillus* strain (Al-Nahdi 2012). Moreover, Atalo and Gashe (1993) found a thermophilic *Bacillus* species that was high-protease producer. Extracellular lipolytic activity was also shown by *Bacillus* sp. (Wang *et al.* 1995) and *Bacillus thermoleovorans* (Lee *et al.* 2001).

Table 2: Bacteria and fungi (meso & thermo) isolated from soil samples on sugar-free media amended with plant latices from *Euphorbia pulcherrmia*, *Ficus elastica* and *Ficus nitida* (20 µl latex/plate).

Microorganisms	***Euphorbia pulcherrima***				***Ficus elastica***				***Ficus nitida***			
	TC	**TC%**	**F%**	**O**	**TC**	**TC%**	**F%**	**O**	**TC**	**TC%**	**F%**	**O**
Mesophilic bacteria (at 36ºC)												
Gram positive bacteria	357	68.3	74	H	523	82.16	92	H	314	80.7	68	H
Bacilli	357	68.3	74	H	79.7	12.5	42	M	106.3	27.3	64	H
Streptomyces sp. 3 FCz (white)					177.7	27.9	86	H				
Streptomyces sp. 2 FCz (grey)					106	16.61	68	H				
Streptomyces sp. 5 NFCz (buff)					164.7	25.83	84	H	207.7	53.2	68	H
Gram negative short rods	166	31.7	50	H	109.3	17.17	48	M	75	19.3	40	M
Gross total count	523	100	80	H	636.7	100	92	H	389	100	86	H
Thermophilic and themotolerant bacteria (at 45ºC)												
Bacillus sp.	41.3	36.9	55	H	84.3	58.16	70	H	40.7	43.4	70	H
Bacillus sp.	70.7	63.1	55	H	60.7	41.83	70	H	53	56.6	40	M
Gross total count	112	100	70	H	145	100	70	H	93.7	100	70	H
Mesophilic fungi (at 25ºC)												
Acremonium strictum W. Gams					2	0.28	2	L	9.3	1.3	6	L
Alternaria alternate (Fries) Keissler	12.3	2.4	12	L	20.3	2.88	26	M	7	0.98	10	L
Aspergillus	186.7	36.3	86	H	365.3	51.82	84	H	347.7	48.8	98	H
A. flavus Link	9.3	1.8	18	L	12	1.7	18	L	18.7	2.6	20	L
A. fumigatuss Fresenius	1.3	0.3	2	L	1.7	0.23	2	L	1.7	0.2	2	L
A .niger van Tieghem	52.3	10.2	52	H	65.3	9.27	50	H	90.7	12.7	62	H
A. sydowii (Bainier & Sartory) Thom & Church	3.7	0.7	8	L	6.3	0.89	6	L	1.3	0.2	2	L
A .terreus Thom	115.7	22.5	66	H	240.7	34.14	80	H	209.3	29.4	88	H
A .ustus (Bainier) Thom & Church	4.3	0.8	10	L	26.3	3.74	28	M	7.7	1.1	20	L
A .versicolor (Vuillemin) Tiraboschi					13	1.85	2	L	18.3	2.6	4	L
Cladosporium	52.7	10.2	26	M	48.3	6.86	36	M	93	13.1	30	M
C. cladosporioides (Fresenius) de Vries	45.7	8.9	16	L	46.7	6.62	28	M	14.3	2.0	12	L
C. sphaerospermum Penzig	7	1.4	10	L	1.7	0.23	8	L	78.7	11.0	20	L
Cochliobolus lunatus R. Nelson & Haasis	14.3	2.8	16	L	18.7	2.65	20	L	5	0.7	6	L
Fusarium	116	22.6	64	H	104	14.75	50	H	45	6.3	44	M
F. solani (Martius) Saccardo	69.3	13.5	38	M	61	8.65	28	M	21.7	3.0	28	M

F.verticillioides (Saccardo) Nirenberg	46.7	9.1	26	M	43	6.1	22	L	23.3	3.3	20	L
Humicola grisea Traaen	9.3	1.8	4	L	11.3	1.61	10	L	22	3.1	14	L
Mucor circinelloides van Tieghem					4	0.57	8	L	2	0.3	6	L
Myrothecium verrucaria (Albertini & Schweinitz) Ditmar	10.3	2.01	8	L	20	2.84	14	L	26.7	3.7	22	L
Penicillium	55	10.7	36	M	63.3	9	28	M	48.3	6.8	44	M
P. aurantiogriseum Dierckx	16.3	3.2	10	L	25.7	3.64	6	L	2.7	0.4	6	L
P. chrysogenum Thom	38.7	7.5	26	M	37.7	5.34	22	L	45.7	6.4	38	M
Phoma sp.	11.7	2.3	8	L	7	1	6	L	17.3	2.4	6	L
Scopulariopsis candida (Gueguen) Vuillemin					2.7	0.38	6	L	3	0.4	8	L
Setosphaeria rostrata Leonard	1.7	0.3	4	L	1	0.14	4	L	2.3	0.3	4	L
Stachybotrys	43	8.4	26	M	37	5.25	20	L	81.3	11.4	46	M
S. chartarm (Ehrenberg) Hughes	43	8.4	26	M	37	5.25	20	L	79.3	11.1	42	M
S. elegans (Pidoplichko) W. Gams									2	0.3	4	L
Trichderma hamatum (Bonorden) Bainier									2	0.3	4	L
Trichothecium roseum (Persoon: Fries) Link	0.17	0.06	2	L								
Trichurus spiralis Hasselbring	1	0.2	2	L								
Gross total count	514.3	100	96	H	704.93	100	100	H	712	100	100	H
Thermophilic and thermotolerant fungi (at 45ºC)												
Aspergillus	4.57	72.49	85	H	3.37	50.26	85	H	3.73	47.7	85	H
A. fumigatus Fresenius	1.47	23.27	35	M	1.4	20.89	45	M	1.3	16.6	40	M
A. terreus Thom	3.1	49.22	70	H	1.97	29.36	70	H	2.43	31.1	75	H
Emericella nidulans (Eidam) Vuillemin	1.47	23.27	55	H	1.5	22.39	35	M	0.8	10.2	5	L
Humicola grisea var. *thermoidea* Cooney & R. Emerson	0.2	3.18	15	L	0.37	5.463	15	L	0.73	9.37	30	M
Malbranchea cinnamomea (Libert) van Oorschot & de Hoog	0.07	1.05	10	L	0.17	2.477	15	L	0.5	6.4	25	L
Myceliophthora thermophile (Apinis) van Oorschot					1.3	19.4	45	M	2.06	26.3	45	M
Gross total count	6.3	100	85	H	6.7	100	100	H	7.83	100	90	H

TC: Total count of each organism (per 50 samples; TC%: Percentage total count, calculated per total count of all bacteria and fungi; F%: Percentage frequency calculated per total number of samples (50 samples); O: Occurrence remarks: H=high occurrence, >50% (out of 50 samples); M=moderate occurrence, from 25-49%; L=low occurrence, less than 25%.

Protease and lipase production by mesophilic, thermotolerant and thermophilic fungi

Ten mesophilic fungal species represented by 30 isolates (3 isolates for each species were isolated on each of the three types of media), in addition to four thermophilic and thermotolerant species represented by 11 isolates were tested for their capabilities of producing protease and lipase enzymes. Twenty-five mesophilic isolates of the 30 tested organisms were capable of producing protease but with different degrees. Of the positive isolates only 6 were high producers and these were related to *Alternaria alternata* AUMC 4686, *Aspergillus ustus* (three strains AUMC Nos. 4797, 4798 and 4799, *Fusarium verticillioides* AUMC 4801 and *Penicillium chrysogenum* AUMC 4706. It was observed that the 5 non-protease producing strains belong to *Cladosporium cladosporioides* (3 strains) and *Stachybotrys chartarum* (2) (Table 3).

Proteins constitute one of the components of natural rubber serum. Some fungi readily decompose proteins, amino acids and other nitrogenous compounds to liberate ammonia as was reported by Alexopoulus (1962). Fungal genera whose proteolytic enzymes have received extensive studies include *Alternaria, Aspergillus, Mucor, Penicillium* and *Rhizopus* (Atagana *et al.* 1999). Djamel *et al.* (2009) tested a total of 253 *Penicillium* isolates on skimmed milk agar and found only 10 strains (3.9%) with proteolytic activities. Acid proteases are also synthesised by *Mucor miehei* (Fernandez-Lahore *et al.* 1999), *M. hiemalis, M. racemosus* and *M. bacilliformis* (Fernandez-Lahore *et al.* 1997, 1998) and by species of *Aspergillus* (Tremacoldi *et al.* 2004) and *Rhizopus* (Kumar *et al.* 2005).

On the other hand, the 30 mesophilic isolates were lipase-producing but with different capabilities. The high-producing 20 strains were related to *Alternaria alternata* (2 isolates), *Aspergillus flavus* (3), *A. niger* (1) *A. ustus* (2), *Cladosporium cladosporioidies* (3), *Fusarium solani* (3), *F. verticillioides* (2), *Penicillium chrysogenum* (2) and *Stachybotrys chartarum* (2). The low-producing strains of lipase belong to *Alternaria alternata*, *Aspergillus niger*, *A. terreus, A. ustus*, *Fusarium verticillioides*, *Penicillium chrysogenum* and *Stachybotrys chartarum* (Table 3). Cho *et al.* (2007) recorded that *Penicillium chrysogenum* and *Cladosporium cladosporioides* out of 9 strains tested for lipase production showed the highest activity. High lipolytic activity was also observed with *Penicillium citrinum* (Maliszewska and Mastalerz 1992), *P. restrictum* (Gombert *et al.* 1998), and *P. wortmannii* (Costa and Peralta, 1999). Different from our findings that *A. terreus* isolates were of low lipolytic activity, those of Gulati *et al.* (1999) were of high activity when corn oil was used as an inducer.

The eleven thermophilic and thermotolerant isolates tested were capable of producing protease. The three strains of *Aspergillus terreus* followed by the three strains of *Emericella nidulans* showed high protease-producing abilities. The two strains of *Myceliophthora thermophila* (one recovered on *F. elastica* and the other on *F. nitida* latex) showed also high production of protease. On the other hand, the three strains of *Aspergillus fumigatus* tested were with low ability (Table 3). The extracellular protease production by thermophilic moulds was studied by several workers (Ong and Gaucher 1976 and Satyanarayana and Johri 1983). Thermostable alkaline proteases were also identified on casein agar by *Malbranchea sulfurea, Chaetomium thermophile, Humicola lanuginose* (Ong and Gaucher 1976), *Aspergillus fumigatus* (Paneerselvam *et al.* 1978), *Mucor miehei* (Rickert and McBride-Warren 1974), and *Sporotrichum thermophile* (Adams and Deploey 1978).

Eight out of the 11 isolates tested were capable of producing lipase but with different rates while the three isolates of *A. terreus* were not. One strain of each of *Myceliophthora thermophila* AUMC 4652 and *Emericella nidulans* AUMC 4654 were high producers. *Aspergillus fumigatus* (the three strains), *Myceliophthora thermophila* AUMC 4653 and *Emericella nidulans* (two strains) were low producers (Table 3). In relation with our results, Krikstaponis *et al.* (2001) reported that some of the tested strains of *A. fumigatus* were able to produce extracellular lipases and hydrolyze fatty acids of vegetable oil. Others reported also lipase from *A. fumigatus* (Ogundero 1982). Moreover, Arima *et al.* (1972) purified an extracellular lipase from *Humicola lanuginosa* strain. Also, Omar *et al.* (1987) reported that production and thermostability of lipase differed with different strains of *H. lanuginosa*. Cold-adapted lipases were also produced by *Aspergillus nidulans* (Mayordomo *et al.* 2000).

Table 3: Protease and lipase production by most common mesophilic, thermotolerant and thermophilic bacteria and fungi recovered on sugar-free media amended with the lattices of *Euphorbia pulchermmia (*EP*)*, *Ficus elastica* (FE) and *Ficus nitida* (FN).

Bacterial and fungal species	ASU/ AUMC No.	Latex source	Protease			Lipase
			MCD±SD	MDCZ±SD	PI	
Mesophilic bacteria						
Gram positive bacilli	6	EP	1.1±0.11	2.35±0.17	2.13	1.3
	11	FE	4.65±0. 4	6±0	1.29	2.5
	12	FN	4±0.16	5.08±0.15	1.26	1.8
Gram negative short rods	4	EP	1.82±0.12	2.5±0	1.37	1.8
	9	FN	1.22±0.2	2.45±0.1	2	2.6
	13	FE	0.9±0.08	1.47±0.12	1.63	1.9
Stroptomyces spp.	3	FE	1±0	0	0	0
	5	FE	0.9±0	0.98±0.05	1.08	1.95
	8	FN	0.9±0	1.2±0.14	1.33	1.8
	2	FE	0.5±0	1.02±0.05	2.04	1.3
Thermophilic bacteria						
Bacillus spp.	14	EP	0.9±0	1.75±0.05	1.94	0.8
	7	FE	0.8±0	0	0	0
	15	FN	0.9±0	0	0	0
Mesophilic fungi						
Alternaria alternata	4686	EP	1.43±0.09	2.55±0.17	1.79	1.3
	4687	FE	2.6±0.14	3.25±0.2	1.25	1.95
	4684	FN	2.93±0.09	3.5±0	1.19	1.95
Aspergillus flavus	4794	EP	2.78±0.15	4.05±0.1	1.46	1.8
	4795	FE	3.98±0.12	4.45±0.17	1.12	2.25
	4793	FN	3.43±0.17	4.5±0	1.31	1.7
Aspergillus niger	4700	EP	3.4±0.14	4.28±0.32	1.26	1.15
	4702	FE	3.8±0.21	4.2±0.14	1.11	1.9
	4703	FN	3.75±0.13	4.1±0.2	1.09	1.1
Aspergillus terreus	4680	EP	3.1±0.11	4.15±0.28	1.34	0.9
	4682	FE	3±0	4.08±0.09	1.36	0.7
	4683	FN	3.03±0.09	4.2±0.08	1.39	0.8
Aspergillus ustus	4797	EP	2.08±0.09	3.25±0.12	1.57	1.1
	4799	FE	2.15±0.057	3.6±0.08	1.67	1.95
	4798	FN	2.1±0	3.3±0.11	1.57	1.8
Cladosporium cladosporioides	5063	EP	2.55±0.17	0	0	1.5
	4663	FE	3.25±0	0	0	1.7
	4665	FN	2.15±0.057	0	0	1.75
Fusarium solani	5066	EP	5.08±0.05	5.35±0.12	1.05	2
	5073	FE	3.25±0.05	3.55±0.05	1.09	2.3
		FN	3.55±0.05	4±0.18	1.13	2.15
Fusarium verticillioidies	4801	EP	2.8±0.24	4.2±0.08	1.5	1.2
	5065	FE	4.1±0	5.15±0.05	1.26	1.5
	4800	FN	4.35±0.17	5.25±0.17	1.21	1.95
Penicillium chrysogenum	4706	EP	1.53±0.5	2.33±0.17	1.52	1.2
	4704	FE	2.43±0.09	2.58±0.05	1.06	1.8
	4707	FN	2.3±0	2.45±0.057	1.06	1.95
Stachybotrys chartarum	4690	EP	2.4±0.08	2.5±0	1.04	1.15
	4691	FE	2.33±0.17	0	0	2
	4688	FN	2.55±0.17	0	0	2
Thermotolerant and thermophilic fungi						
Aspergillus.fumigatus	4658	EP	1.58±0.05	1.8±0	1.14	0.8

	4657	FE	2.05±0.1	2.23±0.05	1.08	1.1
	4659	FN	1.85±0.057	2.03±0.05	1.09	1
Aspergillus terreus	4662	EP	0.78±0.05	2.65±0.05	3.42	0
	4661	FE	0.65±0.09	2.78±0.18	4.26	0
	4660	FN	0.8±0.11	3.07±0.09	3.83	0
Emericella nidulans	4656	EP	1.08±0.09	2.58±0.05	2.93	1.1
	4654	FE	1.18±0.095	2.45±0.05	2.08	1.75
	4660	FN	0.95±0.057	2.13±0.05	2.24	1.3
Myceliophthora thermophila	4653	FE	1.83±0.09	3.53±0.05	1.93	1.1
	4652	FN	1.7±0.14	3.13±0.05	1.84	1.85

Figures in the table are mean diameters of the clear zones MDCZ ± SD of three replicates for protease (in cm after 5 days), or mean depths of the visible precipitate of two replicates for lipase (in cm after 7 days), >1.5 cm: high producer, <1.5 cm: low producer.
MCD=mean colony diameter, MDCZ= mean diameter of clear zone.
PI= protease index. 0= zero indicates negative result, H >1.5, L<1.5.
ASU: Assiut University bacterial collection numbers, AUMC: Assiut University Mycological center numbers

References

Abada EA (2008): Production and characterization of a mesophilic lipase isolated from *Bacillus stearothermophilus* AB-1. Pakistan Journal of Biological Sciences 11(8): 1100-1106.

Adams PR and Deploey JJ (1978): Enzymes produced by thermophilic fungi. Mycologia 70(4): 906-910.

Alexopoulos CJ (1962): Introductory Mycology. 2nd edition, John Wiley and Sons, Inc., New York, pp. 5-13.

Al-Nahdi HS (2012): Isolation and screening of extracellular proteases produced by new isolated *Bacillus* sp. Journal of Applied Pharmaceutical Science 2(9): 71-74.

Archer BL and Audley BG (1973): Rubber, gutta percha and chicle, in phytochemistry, (Nord,FF and Miller,LP, Eds.) Vol. 2: 310-343. New York: Van Nostrand Reinhold.

Allen PJ and Emerson R (1949): Guayule rubber, microbiological improvement by shrub retting. Industrial, Engineering and Chemistry 41: 346-365.

Arima K, Liu WH and Beppu T (1972): Studies on the lipase of thermophilic fungus *Humicola lanuginosa*. Agricultural Biology and Chemistry 36:893–895.

Atagana HI, Ejechi BO and Ayilumo AM (1999): Fungi associated with degradation of wastes from rubber processing industry. Environmental Monitoring and Assessment 55: 401-408.

Atalo K, Gashe BA (1993): Protease production by a thermophilous *Bacillus* species which degrades various kinds of fibrous proteins. Biotechnology Letters 15: 1151-1156.

Bergey's Manual of Systematic Bacteriology (2005) Volume Two: The proteobacteria Part C, Springer-Verlag, Berlin, Heidelberg New York.

Braaz R, Fischer P and Jendrossek D (2004): Novel type of heme-dependent oxygenase catalyzes oxidative cleavage of rubber (Poly-cis-1, 4-Isoprene). Applied and Environmental Microbiology 70(12): 7388-7395.

Bröker D, Dietz D, Arenskötter M and Steinbüchel A (2008): The genomes of non-clearing-zone-forming and natural rubber-degrading species *Gordonia polyisoprenivorans* and *Gordonia westfalica* harbor genes expressing Lcp activity in *Streptomyces* strains. Applied and Environmental Microbiology 74(8): 2288-2297.

Borel M, Kergomard A and Renard MF (1982): Degradation of natural rubber by Fungi Imperfecti. Agricultural Biology and Chemistry 46: 877-881.

Cho HY, Bancerz R, Ginalska G, Leonowicz A, Cho NS and Ohga S (2007): Culture conditions of psychrotrophic fungus, *Penicillium chrysogenum* and its lipase characteristics. Journal of Faculty of Agriculture, Kyushu University 52(2): 281-286.

Christen GL and Marshall RT (1984): Selected properties of lipase and protease of *Pseudomonas fluorescens* 27 produced in four media. Journal of Dairy Science 67(8): 1680-1687.

Costa MM and Peralta R (1999): Production of lipase by soil fungi and partial characterization of lipase from a selected strain (*Penicillium wortmannii*). Journal of Basic Microbiology 39: 11-15.

Djamel C, Ali T and Nelly C (2009): Acid protease production by isolated species of *Penicillium*. European Journal of Scientific Research 25(3): 469-477.

Domsch KH, Gams W and Anderson TH (2007): Compendium of Soil Fungi. 2nd edition, IHC, Verlag, Eching, pp. 672.

Endo S (1962): Studies on protease by thermophilic bacteria. Hekko Kogaku Zasshi 40: 346-353.

Eom GT, Rhee JS and Song JK (2006): An efficient secretion of type 1 secretion pathway-dependent lipase, TliA, in *Escherichia coli*: Effect of relative expression levels and timing of passenger protein and ABC transporter. Microbiology and Biotechnology 16(9): 1422-1428.

Ewing HW and Johnson JG (1960): The differentiation of *Aeromonas* and C27 cultures from Enterobacteriaceae. International Bulletin of Bacteriological Nomenclature and Taxonomy 10(3): 223-230.

Faber MD (1972): Transformation and Degradation of Synthetic and Natural Polymers, Thesis, Rutgers University, New Jersey.

Fernandez-Lahore HM, Gallego Duaigues MV, Cascone O and Fraile ER (1997): Solid state production of a *Mucor bacilliformis* acid protease. Revista Argentina de Microbiologia 29: 1-6.

Fernandez-Lahore HM, Fraile ER and Cascone O (1998): Acid protease recovery from a solid-state fermentation system. Journal of Biotechnology 62: 83-93.

Fernandez-Lahore HM, Audaz RM, Fraile ER, Biscoglio de Jimenez Bonino M, Pirpignani L and Machalinski C (1999): Purification and characterization of an acid proteinase from mesophilic *Mucor* sp. solid-state cultures. Journal of Peptide Research 53: 599-605.

Folasade M Olajuyigbe and Ajele JO (2008): Some properties of extracellular protease from *Bacillus licheniformis* LBBL-11 isolated from "iru", a traditionally fermented African locust bean condiment. African Journal of Biochemistry Research 2(10): 206-210.

Fujio Y and Kume S (1991): Characteristics of a highly thermostable neutral protease produced from *Bacillus stearothermophilus*. World Journal of Microbiology and Biotechnology 7:12-16.

Gallert C (2000): Degradation of latex and of natural rubber by *Streptomyces* strain La7. Systematic and Applied Microbiology 23: 433-441.

Gombert AK, Pinto AL, Castilho LR and Freire DMG (1998): Lipase production by *Penicillium restrictum* in solid–state fermentation using babassu oil cake as substrate. Process Biochemistry 35: 85-90.

Gulati RRK, Saxena R, Gupta RP, Yadav and Davidson WS (1999): Parametric optimisation of *Aspergillus terreus* lipase production and its potential in ester synthesis. Process Biochemistry 35: 459-464.

Haba E, Bresco O, Ferrer C, Marques A, Busquets M, and Manresa A (2000): Isolation of lipase-secreting bacteria by deploying used frying oil as selective substrate. Enzyme and Microbial Technology 26(1): 40-44.

Heisey RM and Papadatos S (1995): Isolation of microorganisms able to metabolize purified natural rubber. Applied and Environmental Microbiology 61(8):3092-3097.

Ho HH and Foster B (1972): Starch utilization by *Phytophthora* species. Mycopathologia et Mycologia Applicata 46: 335-339.

Ibrahim EMA, Arenskötter M, Luftmann H and Steinbüchel A (2006): Identification of poly (cis-1, 4-isoprene) degradation intermediates during growth of moderately thermophilic Actinomycetes on rubber and cloning of a functional lcp homologue from *Nocardia farcinica* strain E1. Applied and Environmental Microbiology 72: 3375-3382.

Krikstaponis A, Lugauskas A, Krysinska–Traczyk A, Prazmo Z and Dutkiewicz J (2001): Enzymatic activities of *Aspergillus fumigatus* strains isolated from the air at waste landfills. Annals of Agricultural and Environmental Medicine 8: 227-234.

Kumar S, Sharma NS, Saharan MR and Singh R (2005): Extracellular acid protease from *Rhizopus oryzae:* purification and characterization. Process Biochemistry 40: 1701–1705.

Lee DW, Kim HW, Lee KW, Kim BC, Choe EA, Lee HS, Kim DS and Pyuna YR (2001): Purification and characterization of two distinct thermostable lipases from the gram-positive thermophilic bacterium *Bacillus thermoleovorans* ID-1. Enzyme and Microbial Technology 29: 363-371.

Lewinsohn TM (1991): The geographical distribution of plant latex. Chemoecology 2: 64-68.

Linos A, Steinbüüchel A, Sproer C and Kroppenstedt RM (1999): *Gordonia polyisoprenivorans* sp. nov., a rubber-degrading actinomycete isolated from automobile tire. International Journal Systematic Bacteriology 49: 1785-1791.

Linos A, Reichelt R, Keller V and Steinbuchel A (2000): A Gram negative bacterium identified as *Pseudomonas aeruginosa* AL 98, is a potent degrader of NR and synthetic cis 1, 4 polyisoprene. FEMS Microbiology Letters 182(1):155-161

Linos A, Berekaa MM, Steinbüchel A, Kim KK, Sproer C and Kroppenstedt RM (2002): *Gordonia westfalcia* sp. nov., a novel rubber-degrading actinomycete. International Journal of Systematic and Evolutionary Microbiology 52: 1133-1139.

Lynch JM, Slater JH, Bennett JA and Harper SHT (1981): Cellulase activities of some aerobic microorganisms isolated from soil. Journal of General Microbiology 127: 231-236.

Maliszewska J and Mastalerz P (1992): Production and some properties of lipase from *Penicillium citrinum*. Enzyme and Microbial Technology 14: 190-193.

Mayordomo IZ, Randez-Gil F and Prieto JA (2000): Isolation, purification, and characterization of a cold-active lipase from *Aspergillus nidulans*. Journal of Agriculture and Food Chemistry 48(1): 105-109.

Moubasher AH (1993): Soil fungi in Qatar and other Arab Countries. University of Qatar, Centre for Scientific and Applied Research, 566 pp.

Nascimento WCA and Martins MLL (2004): Production and properties of an extracellular protease from thermophilic *Bacillus* sp. Brazilian Journal of Microbiology 35: 91-96.

Ogundero VW (1982): Hydrolysis of vegetable oils and triglycerides by thermotolerant and zoopathogenic species of *Aspergillus* from Nigerian palm produce. Mycopathologia 77(1): 43-46.

Ohya N and Tanaka Y (1998): Structural characterization of rubber from *Lactarius* mushrooms; H-NMR analysis of terminal groups in the rubber chain. Journal of Rubber Research 1: 84-94.

Omar IC, Nishio N and Nagai S (1987): Production of a thermostable lipase by *Humicola lanuginosa* grown on sorbitol-corn steep liquor medium. Agricultural Biology and Chemistry 51: 2145-2151.

Ong PS and Gaucher GM (1976): Production, purification and characterization of thermomycolase, the extracellular serine protease of the thermophilic fungus *Malbranchea pulchella* var. *sulfurea*. Canadian Journal of Microbiology 22: 165-176.

O'reilly T and Day DF (1983): Effect of culture conditions on protease production by *Aeromonas hydrophila*. Applied and Environmental Microbiology 45(3): 1132-1135.

Paneerselvam M, Dhar SC and Santappa M (1978): Factors influencing the production of extracellular proteinases by *Aspergillus fumigatus*. Leather Science 25: 499-506.

Paterson RRM and Bridge PD (1994): Biochemical techniques for filamentous fungi. International Mycological Institute, CAB International, Surrey, UK, P. 21.

Peczynska-Czoch W and Mordarski M (1988): Actinomycetes enzymes. In: Actinomycetes in Biotechnology, Goodfellow M, Williams ST and Mordarski M (eds.), London: Academic Press, pp. 219-283.

Pendle TD, Swinyard PE (1991): The particle size of natural rubber latex concentrates by photon correlation spectroscopy. Journal of Natural Rubber Research 6: 1-11.

Rhodes ME (1958): The ecology of *Pseudomonas* spp. as revealed by silver-plating staining method. Journal of General Microbiology 18: 639-648.

Raper KB and Fennell DJ (1965): The genus *Aspergillus*. Williams and Wilkins, Baltimor, UAS.

Rickert WS and McBride-Warren PA (1974): Structural and functional determinants of *Mucor miehei* protease 111: Isolation and composition of the carbohydrate moiety. Journal of Biochemistry, Biophysics and Molecular Biology 36: 437-444.

Rifaat HM and Yosery MA (2004): Identification and characterization of rubber-degrading actinobacteria. Applied Ecology and Environmental Research 2 (1): 63-70.

Rifaat HM, El-Said OH, Hassanein SM and Selim MSM (2007): Protease activity of some mesophilic streptomycetes isolated from Egyptian habitats. Journal of Culture Collections 5: 16-24.

Rose K, Tenberge KB and Steinbüchel A (2005): Identification and characterization of genes from *Streptomyces* sp. strain K30 responsible for clear zone formation on natural rubber latex and poly (cis-1, 4-isoprene) rubber degradation. Biomacromolecules 6: 180-188.

Roy RV, Das M, Banerjee R and Bhowmick AK (2006): Comparative studies on rubber biodegradation through solid-state and submerged fermentation. Process Biochemistry 41: 181-186.

Salar RK and Aneja KR (2007): Thermophilic fungi: Taxonomy and Biogeography. Journal of Agricultural Technology 3(1): 77-107.

Satyanarayana T and Johri BN (1983): Variation in xylanolytic activity of thermophilic fungi. Bionature 3: 39 - 41.

Subramaniam A (1995): The chemistry of natural rubber latex. Immunology and Allergy Clinics of North America 15:1-20.

Takami H, Akiba T and Horikoshi K (1989): Production of extremely thermostable alkaline protease from *Bacillus* sp. no. AH 101. Applied Microbiology and Biotechnology 30: 120-124.

Tremacoldi CR, Watanabe NK and Carmona EC (2004): Production of extracellular acid proteases by *Aspergillus clavatus*. World Journal of Microbiology and Biotechnology 20: 639-642.

Ulman V and Blasins G (1974): A simple medium for the detection of different lipolytic activity of microorganisms. Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene, Abt A 229: 264-267.

Vermelho AB, Meirelles MNL, Lopes A, Petinate SDG, Chaia AA and Branquinha MH (1996): Detection of extracellular proteases from microorganisms on agar plates. Memórias do Instituto Oswaldo Cruz 91:755-760.

Wang Y, Srivastava KC, Shen GJ and Wang HY (1995): Thermostable alkaline lipase from a newly isolated thermophilic *Bacillus*, strain A30-1 (ATCC 53841). Journal of Fermentation and Bioengineering 79(5): 433-438.

Wititsuwannakul D and Wititsuwannakul R (2001): Biochemistry of natural rubber and structure of latex. In: Koyama T, Steinbuchel A (eds.), Vol. 2, Biopolymers, Polyisoprenoids, Wiley–VCH, Germany, pp. 151-201.

Wittenberg R (1964): Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. Journal of General Microbiology 35: 13-26.

Journal of Basic & Applied Mycology (Egypt) 4 (2013): 47-58

Terrestrial fungi tolerating the hypersaline water of Wadi El-Natrun Lakes, Egypt

A. H. Moubasher, M. A. Ismail*, N. A. Hussein, H. A. A. Gouda

Department of Botany and Microbiology, Faculty of Science & Assiut University Mycological Centre (AUMC), Assiut University, Assiut, Egypt.

***Corresponding author: e-mail: ismailmady60@yahoo.com**
Received 1/5/2013, Accepted 22/6/2013

Abstract: Chemical analysis revealed that water samples collected from Wadi El-Natrun Lakes were highly alkaline, of pH ranging from 8.4 –9.5 and of high levels of total soluble salts, chlorides, sodium and potassium. Water collected from El-Zugm Lake showed the highest levels of organic matter, sodium, calcium, magnesium and chlorides among the 8 lakes investigated. On the other hand, some parameters showed their peak in other lakes e.g. pH (9.4) and total soluble salts (87%) in Fasida. A total number of genera (16) and species (33) were recorded from water samples collected from all lakes during the seasons of study, with the widest spectrum of species being isolated on the control medium (14) and the lowest on 10% NaCl medium (3). *Aspergillus, Acremonium* followed by *Penicillium* were the most dominant genera possessing the highest proportions of propagules on all isolation media except on 10 % NaCl. On the other hand, only species of the genera *Scopulariopsis* and *Acremonium* were isolated on 10% NaCl medium. *Aspergillus* showed its count peak in Al-Beida Lake during winter 2007 on both acidic and alkaline media while in spring 2007 on control medium (from Khadra Lake) and on 40% sucrose (from El-Zugm Lake). From *Aspergillus, A. terreus* followed by *A. flavus* and *A. niger* were the most common on all the isolation media, but *A. ochraceus* was dominant on acidic media only. Other most common species, namely *Penicillium chrysogenum* and *P. puberulum* were encountered on all media but not on 10 % NaCl medium. Some species were isolated on one medium but not on the others: *Scopulariopsis halophilica* (on 10% NaCl), *Emericella quadrilineata* (on 40% sucrose), *Staphylotrichum coccosporum* (on medium adjusted at pH 4) and *Acremonium hyalinulum* (on alkaline media).

Key words: Hypersaline waters, lakes, Wadi El-Natrun, extremophilic fungi, terrestrial.

Introduction

Extreme environments, such as acidic or hot springs, saline and/or alkaline lakes, deserts and the ocean beds are found in nature, which are too harsh for normal life to exist (Satyanarayana, *et al*. 2005). Hypersaline environments can be found in all continents and in most countries. They consist of two primary types: thalassohaline and athalassohaline systems. Thalassohaline systems arose from seawater evaporation and hence contain sodium chloride as the predominant salt. The Great Salt Lake, Utah, is an example of such, but other examples are salt mine drainage waters, playas, natural coastal splash zones and tide pools, brine springs from underground salt deposits, and solar salterns (Litchfield and Gillevet 2002). Athalassohaline systems arose from non-seawater sources and contain different ion ratios. These athalassohaline systems are dominated by potassium, magnesium, or sodium and are frequently the sources of potash, magnesium metal, soda, and even borax if the waters were high in boron. Some examples of these are the alkaline soda lakes of Egypt (e.g., Wadi El-Natrun), the Dead Sea, the soda lakes of Antarctica, and Big Soda Lake and Mono Lake in California (Gunde-Cimerman *et al.* 2000, Ulukanli and Digrak 2002, Litchfield and Gillevet 2002, Oren 2002 and Cantrell *et al*. 2006).

Alkaline environments e.g. East African Rift Valley, Wadi El-Natrun in Egypt, Indian Sambhar Lake (Satyanarayana *et al.* 2005) are considered type of athalassohaline systems. Soda lakes and soda deserts represent the major type of naturally occurring highly alkaline environments. The pH of these environments fluctuates due to their limited buffering capacity and therefore, alkalitolerant microbes are more abundant in these habitats than alkaliphiles. These lakes are often closed basins with no obvious outflow, forming semipermanent standing bodies of water. Surface evaporation rates exceed the rate of inflow of water allowing the dissolved minerals to concentrate with $CO3^{-2}$ and Cl^{-1} as major anions creating a pH 8.5 to > 12 (Grant *et al.* 1990, Jones *et al.* 1994, 1998, Jones and Grant 1999 and Grant 2006).

Wadi El-Natrun Depression is situated at the western side of the Nile Delta of Egypt and includes some water bodies characterized by high salinity. It is a narrow depression located approximately 90 km south of Alexandria and 110 km North West of Cairo. It is about 50 km long, narrow at both ends and wider in the middle (Zahran and Willis 1992). It lies 23 m below sea

level and 38 m below the water-level of Rossetta branch of the Nile (Abdel-Malek and Rizk 1963). It is characterized by a series of twenty small disconnected lakes in the bottom of the Wadi. Ten of these lakes are relatively larger in size and have permanent water in all or some of their parts. Inland saline lakes and salt crusts occupy the area surrounded by contour zero (Abu Zeid 1984). The principal lakes of Wadi El-Natrun are Fasida, Umm-Risha, Al-Razoniya (Rosetta), Abu-Gubara, Hamra, El-Zugm (Zaagig), Al-Beida, Khadra and Al-Gaar (Taher 1999 and Zahran and Willis 1992). The Wadi El-Natrun depression gets its water from two sources: the springs in the bottom (e.g. in Lake Hamra), and seepage into the lakes. Pavlov (1962) attributed the source of the water to the radial inflow of underground water towards the lakes. Shata and El Fayoumi (1967) noted that the main source of water to the depression comes from underground water flowing from the Rosetta branch and its branches. It is believed also that the water originates from the Nile, infiltrating through sands and gravels constituting the main strata separating the wadi from the river (Atia *et al*. 1970). Most lakes reach maximum levels between December and March, with the lowest levels in summer. Shallow saline pools shrink in volume by >60% following evaporation in summer. It is assumed that the underground water from the Nile Delta infiltrating into Wadi El-Natrun has roughly the same relative salt composition when it reaches the lakes. The differences found between the relative composition of different lakes are mainly the result of differing microbial activities in the sediments and brines (Taher 1999). The water of Wadi El-Natrun saline lakes is enriched in dissolved minerals that have accumulated in the brines following solar evaporation. Detailed chemical analysis of the lakes of Wadi El-Natrun depression in Egypt revealed high pH level (> 11.5) and high concentration of carbonate, chloride, phosphate, sodium, potassium and silicon oxide (Grant 2006).

Fungi can also grow in hypersaline waters. Some "terrestrial" fungi had been isolated from sea water or sea water flora and fauna. *Aspergillus* and *Cladosporium* were the most frequent isolates found in samples of sand, wood and mangrove in Puerto Rico (Acevedo-Ríos 1987). Others, namely *Cephalosporium*, *Diplodia*, *Fusarium*, *Helminthosporium*, *Penicillium*, *Trichoderma*, *Scopulariopsis* and *Pleospora* were found particularly in sand (Acevedo-Ríos 1987). Xerophilic and halophilic fungi are able to grow in media with low water activities (a_w) and they can be expected to survive in this type of environment. In 1977, Cronin and Post reported the isolation of halophilic fungi belonging to the genus *Cladosporium* were growing in a submerged piece of pine wood in the Great Salt Lake in Utah. Later, Butinar *et al.* (2005b) reported the occurrence of the yeasts *Debaryomyces hansenii* and *Metschnikowia bicuspidate* in this lake. *Aspergillus versicolor*, *Chaetomium globosum*, *Eurotium herbariorum*, *E. amstelodami* and *E. rubrum* were also isolated from Dead Sea waters (Kis-Papo *et al*. 2001, 2003a). On the other hand, *Gymnascella marismortui* (an obligate halophile), *Ulocladium chlamydosporum* and *Penicilium westlingii* (halotolarant) were recorded in Dead Sea waters by Buchalo *et al*. (1998a). Several culturable fungi such as *Cladosporium* spp., *Alternaria* spp., *Aspergillus sydowii*, *Nigrospora* spp., and *Penicilliun solitum* from deep-sea water samples (below 500 m depth) have been reported from different geographical locations (Roth *et al.* 1964 and Raghukumar *et al*. 1992).

To our knowledge, no report was published up till now about mycobiota inhabiting the hypersaline habitat environment of Wadi El-Natrun lakes. So, this work has been designed to highlight on the extremophilic groups of fungi (including osmophilic/ osmotolerant, halophilic/ halotolerant, acidiphilic/ aciditolerant, and alkaliphilic and alkalitolerant fungi) that may be found in the hypersaline waters of Wadi El-Natrun lakes.

Materials and Methods

Collection of water samples

Water samples were collected during three seasons naming autumn of year 2006, winter and spring of year 2007, from the big eight lakes (Fasida, Umm-Risha, Rosetta, Hamra, El-Zugm, Al-Beida, Khadra, Al-Gaar) of Wadi El-Natrun depression. The 24 water samples were collected in sterile bottles at a depth of about 10-20 cm near the lake shore from different sites. Samples were brought into the laboratory and kept in a cold place (5°C) till chemical and fungal analysis.

Chemical analysis of water samples

PH value: The pH meter (Orior Research Model GOHL Digital Ionalyzer) electrode was immersed directly in water sample for the determination of water pH (Jackson 1958).

Total soluble salts (TSS): The specific electrical conductance (EC expressed in mmhos /cm) was measured in the samples using the conductance meter (YSI, model 35). The percentage total soluble salts in a sample were estimated using the following equation:

% TSS in the dry sample = 0.064 × EC × extract ratio. The conversion factor to percentage salts (0.064) is fairly applied for solutions extracted from alkaline and saline soils (Richards 1954 and Jackson 1958).

Carbonate and bicarbonate: Total carbonate was determined directly in water according to the method described by Jackson (1958).

Chloride (Cl^-): Soluble chloride was estimated by applying the silver nitrate titration method using potassium chromate as an indicator (Jackson 1958).

Calcium and magnesium (Ca^{+2} & Mg^{+2}): The versene (disodium dihydrogen ethylene diamine tetraacetic acid) titration method as recommended by Schwarzenbach and Biedermann (1948) was employed for Ca^{+2} and Ca^{+2} + Mg^{+2} determination.

Sodium and potassium (Na^+ & K^+): Flame photometer method (Williams and Twine 1960) using Carl Zeiss flame photometer was used for the determination of Na^+ and K^+ cations.

Isolation of terrestrial fungi from water samples

Seven agar media types (5 plates each) were used for enumeration and isolation of terrestrial fungi from lakes water. One ml of lake water was transferred into each of Petri-dish and mixed by rotation with agar medium. After solidification of the agar, the plates were incubated at 28ºC for 7-15 days. The developing fungal colonies were counted and calculated per ml water and preserved in agar slants for identification.

The seven media types used for isolation of fungi are: 1. Modified Czapek Dox agar in which glucose (10 g/l) replaced sucrose, of the following composition (g/l) sodium nitrate 3.0, potassium dihydrogen phosphate 1.0, magnesium sulphate 0.5, potassium chloride 0.5, ferrous sulphate 0.01, glucose 10.0, agar 15.0), to which rose bengal (1/15000) and chloramphenicol (25 µg/ml) were used as bacteriostatic agents (Smith and Dawson 1944, Al- Doory 1980). This medium was adjusted to pH7.3 and was used as a control medium; 2. Czapek Dox agar supplemented with 40% sucrose for isolation of osmophilic and osmotolerant fungi; 3. Modified Czapek Dox agar medium (in which glucose, 10g/l, replaced sucrose) supplemented with 10% sodium chloride was used for isolation of halophilic and halotolerant fungi; 4 and 5. Modified Czapek Dox agar media in which pH was adjusted at 4 or 5 using diluted HCl were used for isolation of acidiphilic and aciditolerant fungi; 6 and 7. Modified Czapek Dox agar in which pH was adjusted at 10, 13 using NaOH were used for isolation of alkaliphilic and alkalitolerant fungi.

Identification of fungi

The identification of fungal genera and species (purely morphologically) was based on macroscopic and microscopic features following the keys and descriptions of Ellis (1971, 1976), for Dematiaceous Hyphomycetes, Pitt (1979), for *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*, Raper and Fennell (1965), for *Aspergillus* species, Moubasher (1993), Domsch *et al.* (2007) for fungi in general and Booth (1971), Leslie and Summerell (2006) for *Fusarium* specie.

Results and Discussion

Chemical analysis of water samples

Chemical analysis of water samples collected during spring 2007 from Wadi El-Natrun lakes revealed that pH was highly alkaline in different lakes ranging from 8.4 in El-Zugm to 9.5 in Fasida (Table 1). This is in agreement with previous results obtained by Taher (1999) and Moussa *et al.* (2009) in Wadi El-Natrun Lakes (pH 8.51-9.45) and by Steiman *et al.* (2004) of Mono Lake of California (pH 9.4-9.8); however it is remarkably different from that of the Dead Sea water which is acidic (pH 6.6) (Steiman *et al.* 1995). Total soluble salts (TSS%) was much higher in water than those recorded in both soil and mud samples (Gouda 2009), ranging from 50% in Al-Gaar to 87% in Fasida; however higher amounts were recorded in salt samples (Gouda 2009). In the study of Taher (1999) the concentration of the total soluble salts (TSS) ranged from 283 g/l in Khadra to 557 g/l in Al-Beida Lake. However she reported the lowest salt contents of water in spring (97 g/l), a very low value compared to our results in spring. The higher values of total soluble salts in water of all lakes are due to the high concentrations of both sodium and chloride ions, which is similary reported by Taher (1999). Although the Dead Sea (Steiman *et al.* 1995) and the Great Salt lake (Utah Geological survey, 2001) have similar salinities to those of Wadi El-Natrun lakes water, but their chemical composition is different. Sodium cations (mg/ml) ranged from 13.0 in Fasida to 44.0 in El-Zugm Lake; results agreed with those reported by Moussa *et al.* (2009) who found nearly similar composition of sodium (954.0 mmol/l = 41.5 mg/ml), however, lower than those (30.2 – 295.2 g/l) reported by Taher (1999).

Chloride anions exhibited the highest value in Umm Risha (23.0 mg/ml) and the lowest in Al-Beida (10.5). In agreement with our results Moussa *et al.* (2009) recorded almost similar concentrations of chlorides in Wadi El-Natrun water (920.0 mmol/l = 25.6 mg/ml). However higher concentrations (12.7- 125.7 g/l) were reported by Taher (1999). Also Steiman *et al.* (2004) and Mason (1967) recorded 1.4-11.4 and 17.5 g/l in Mono Lake water respectively. However, far highest amounts of chlorides were obtained from the Dead Sea water (223.3 g/l) (Steiman *et al.* 1997) and in the Great Salt Lake (54.51) (Utah Geological survey, 2001). Potassium cations (K^+, 0.1-0.3 mg/ml), carbonates ($CO3^{-2}$, 0.2- 2.0), bicarbonates ($HCO3^-$, 0.1-1.0), calcium cations (Ca^{+2}, 0.01-0.5) and magnesium cations (Mg^{+2}, 0.02-0.4) showed lower values

Table 1: Chemical analysis of water samples collected from Wadi El-Natrun lakes.

Lake	Al-Gaar	Khadra	Al-Beida	El-Zugm	Hamra	Rosetta	Umm-Risha	Fasida
pH	8.8	9	9.0	8.4	9	9.1	9	9.5
OM	0.05	0.1	1.0	0.1	0.07	0.08	0.05	0.1
TSS	50	80	65	80.2	80	67	70.2	87
Na^{+}	17	22	22	44	40	20	40	13
K^{+}	0.2	0.3	0.2	0.2	0.3	0.3	0.1	0.2
CO_3^{-2}	0.2	1.1	1.3	0.2	0.5	0.3	1.0	0.2
HCO_3^{-}	0.22	2	0.23	0.2	1.0	0.11	1.2	0.19
Ca^{+2}	0.02	0.04	0.1	0.5	0.01	0.1	0.01	0.1
Mg^{+2}	0.03	0.05	0.02	0.4	0.03	0.3	0.03	0.09
Cl^{-}	12	14	10.5	22	20.1	12.5	23	15.2

Figures were obtained during spring 2007 of study; OM and TSS are calculated as % of the water samples analyzed; Na^{+}, K^{+}, CO_3^{-2}, HCO_3^{-}, Ca^{+2}, Mg^{+2} and Cl^{-} are calculated as mg/ml water.

than their respectives in different lakes ranging from 0.01 in calcium to 2.0 in carbonates. In agreement with our results, Moussa *et al.* (2009) found almost similar concentrations of calcium (6.32 mmol/l = 0.15 mg/ml), magnesium (8.76 mmol/l = 0.3 mg/ml), potassium (21.48 mmol/l = 0.6 mg/ml) and bicarbonates (98.5 mmol/l = 1.6 mg/ml).

Wadi El-Natrun Lake water contained extremely lower concentrations of magnesium (0.4 mg/ml) and calcium (0.1 mg/ml) than those reported in the Dead Sea (22.0 and 42.4 for calcium and magnesium respectively) (Steiman *et al*. 1995) and the Great Salt Lake (0.2 for calcium, 3.3 for magnesium g/l) (Utah Geological Survey, 2001). However lower concentrations of calcium and magnesium were reported in Mono Lake water (Mason, 1967; Steiman *et al*. 2004). Comparison of Wadi El Natrun lake water analysis in the current study and the studies of Taher (1999) and Moussa *et al.* (2009) with that of Sothern African lakes studied by Steiman *et al*. (1991) revealed that the latters were much less saline (mostly <50 g/l) and with pH varies between 9.2 and 10.4.

Terrestrial Fungi recovered from water samples

Fifteen species related to 7 genera were recovered on Czapek Dox agar (as control medium) from water collected from the 8 lakes during the 3 seasons of study. *Aspergillus* (5 species), *Acremonium* (3), *Penicillium* (3) were the most common genera. They accounted for 49.0%, 30.2% and 9.9% of total fungi respectively (Table 6).

Aspergillus (with *A. terreus* being the most dominant species, 24.1% of total fungi) was reported from all lakes during winter and spring seasons only. The peak of *Aspergillus* count was recorded from Khadra during spring 2007. The remaining *Aspergillus* species were recorded from 3 lakes: *A. niger* and *A. flavus* (El-Zugm, Umm-Risha and Al-Gaar), or one lake: *A. fumigatus* (Al-Beida, winter 2007) and *A. parasiticus* (Al-Gaar). The current results agree with those reported by Faryal and Hameed (2005) who isolated *A. niger, A. fumigatus* and *A. flavus* from water samples with pH values between 8.06 to 12.44 in Peshawar Road, Rawalpindi. Also, *Aspergillus candidus, A. melleus, A. niger, A. ochraceus, Chaetomium globosum, Cladosporum cladosporioides, C. sphaerospermum, Hortaea werneckii, Myrothecium roridum, Penicillium citrinum* and *P. chrysogenum,* have been reported in hypersaline waters from temperate or tropical regions (Butinar *et al.* 2005a, b, Gunde-Cimerman *et al*. 2004 and Kis-Papo *et al.* 2001, 2003). *Acremonium* and its dominant species *Acremonium furcatum* were isolated from Rosetta and Khadra lakes or from Rosetta only (*A. kiliense, A. strictum*) during winter 2007. *Acremonium persicinum* and *A. terricola* were isolated from Dead Sea water (Buchalo *et al.* 1999, 2000 a,b and Kis-Papo *et al.* 2001).

Penicillium was recorded from 3 lakes (Al-Beida, El-Zugm and Al-Gaar). *P. chrysogenum* and *P. puberulum* were recovered each from 2 lakes during winter or spring season while *P. oxalicum* was recovered from one lake (Al-Gaar, winter 2007). Also, Cantrell *et al.* (2006) isolated *Penicillium citrinum, P. chrysogenum, P. oxalicum* and *P. variabile* from water of salt ponds in hypersaline environments of solar salterns in Puerto Rico.

Fusarium (represented only by *F. solani)* was recorded from 5 lakes (Hamra, Al-Beida, El-Zugm, Rosetta and Umm-Risha) during winter and spring 2007. The remaining 3 species were isolated from 3 lakes: *Trichoderma* sp. (El-Zugm, Umm-Risha and Fasida during winter), or 2 lakes: *Cladosporium cladosporioides* (Hamra and Fasida during autumn and winter

seasons) and *Cochliobolus tuberculatus* (El-Zugm, Al-Gaar, winter 2007 during winter). In this respect, *Cladosporium cladosporioides* was reported from Dead Sea water (Buchalo *et al.* 1999, 2000 a, b and Kis-Papo *et al.* 2001). *C. cladosporioides, C. oxysporum, C. sphaerospermum* and *Emericella nidulans* were isolated from the water of the salt ponds in hypersaline environments in Puerto Rico (Cantrell *et al.* 2006).

Osmophilic and osmotolerant terrestrial fungi from water samples

Six genera represented by 12 species of osmophilic and osmotolerant fungi were recovered from water samples collected from Wadi El-Natrun lakes during 3 seasons on Czapek Dox agar supplemented with 40% sucrose compared to 7 genera and 15 species on the control medium (Table 3).

Aspergillus (5 species), *Penicillium* (2) and *Acremonium* (1) were the most common genera on 40% sucrose medium accounting for 65.8%, 15.0% and 2.8% of total fungi respectively (Table 6).

Aspergillus and its dominant species *A. terreus* (37.1%) were recovered from all lakes during the 3 seasons of study. Their count peaks were recorded from El-Zugm during spring 2007. The remaining *Aspergillus* species were isolated from 3 lakes: *A. niger* (El-Zugm, Khadra and Fasida during the 3 seasons), 2 lakes: *A. flavus* (Al-Gaar and Fasida during winter and spring 2007), or one lake: *A. ochraceus* (Fasida during autumn 2006 and spring 2007) and *Aspergillus* sp. (Umm-Risha during spring 2007). In this respect, *A. flavus* and *Aspergillus* sp. were reported from the water of the salt ponds collected from hypersaline environments in Puerto Rico (Cantrell *et al.*, 2006) and *A. niger* from swash zone interstitial water during June and August 2005 on a Mediterranean beach, Genoa, Italy (Vezzulli *et al.* 2009). Also, Faryal and Hameed (2005) isolated *A. niger, A. fumigatus* and *A. flavus* from water samples at pH values between 8.06 to 12.44 in Peshawar Road, Rawalpindi.

Penicillium was recovered from 5 lakes during 2 seasons of study. It had the highest CFUs in water of Al-Beida Lake**.** *P. chrysogenum* was recovered from 4 lakes during winter and spring 2007 while *P. puberulum* was recorded from 3 lakes during winter 2007 (Hamra, Al-Beida and Umm-Risha). *P. chrysogenum* was also recovered from water of salt ponds in hypersaline environments of Puerto Rico (Cantrell *et al.* 2006) and from Dead Sea water (Buchalo *et al.* 1999, 2000 a, b and Kis-Papo *et al.* 2001).

Acremonium furcatum was recovered from water of Rosetta and Khadra lakes during winter 2007. Other species of *Acremonium* (*A. persicinum* and *A. terricola*) were isolated from Dead Sea water by Buchalo *et al.* (1999, 2000 a,b) and Kis-Papo *et al.* (2001).

Fusarium (represented by 2 species) was recorded from water of 4 lakes during winter and spring 2007. The peak of *Fusarium* count was recorded from Rosetta during spring 2007. *F. solani* was encountered during 2 seasons from 3 lakes (Hamra, El-Zugum and Rosetta) while *F. subglutinans* was isolated only during winter 2007 from Umm-Risha Lake water. Other remaining 2 species were recorded either from 2 lakes: *Cladosporium cladosporioides* (Umm-Risha and Al-Gaar during spring 2007), or one lake: *Emericella quadrilineata* (Al-Beida during winter 2007). Also, *C. cladosporioides, C. oxysporum, C. sphaerospermum* and *Emericella nidulans* were isolated from the water of the salt ponds in hypersaline environments in Puerto Rico (Cantrell *et al.* 2006). *C. cladosporioides* was also reported from Dead sea water by Buchalo *et al.* (1999, 2000 a, b) and Kis-Papo *et al.* (2001), and *Cladosporium* spp. on medium containing 50% and 70% sugar from hypersaline waters throughout the whole season in Sečovlje salterns in the south east part of the Piran bay, at the delta of the Drag-onja river, at the border between Slovenia and Croatia (Gunde-Cimerman *et al.* 2000).

Halophilic and halotolerant terrestrial fungi recovered from water samples

Using Czapek Dox agar supplemented with 10% NaCl, only 3 species related to *Scopulariopsis* (*S. halophilica* and *S. brumptii*) and *Acremonium* (*A. hyalinulum*) were isolated from water samples during one season only. In this respect, *Acremonium persicinum* and *A. terricola* were isolated from Dead sea water (Buchalo *et al.* 1999, 2000 a, b and Kis-Papo *et al.* 2001)**.**

Table 2: Seasons of isolation of terrestrial fungi on control medium from water of different lakes of Wadi El–Natrun.

Fungal taxa	Hamra	Al-Beida	El-Zugum	Rosetta	Umm-Reisha	Al-Gaar	Khadra	Fasida
Acremonium W. Gams				2			1, 2, 3	
A. furcatum Moreau & Moreau ex Gams				2			1, 2, 3	
A. kiliense Gütz				2				
A. strictum W. Gams				2				
Aspergillus P. Micheli ex Link	2, 3	2, 3	2, 3	2, 3	2, 3	2, 3	2, 3	2, 3
A. flavus Link			3		2	2, 3		
A. fumigatus Fresenius		2						
A. niger van Tieghem			2, 3		2	2		
A. parasiticus Speare						2		
A. terreus Thom	3	3	2, 3	2, 3	3		3	2, 3
Cladosporium cladosprioides (Fresenius) de Vries	2							1
Cochliobolus tuberculatus Sivanesan			2			2		
Fusarium solani (Martius) Saccardo	2	2	2	3	2			
Penicillium Link		2, 3	2			2		
P. chrysogenum Thom		2				2		
P. oxalicum Currie & Thom						2		
P. puberulum Bainier		2, 3	2					
Trichoderma sp.			2		2			2
No. of genera (7)	3	3	5	3	3	3	2	1
No. of species (15)	3	5	7	5	5	6	4	4

1 = autumn 2006, 2 = winter 2007 and 3 = spring 2007

Table 3: Seasons of isolation of osmophilic and osmotolerant fungi from water of different lakes of Wadi El–Natrun.

Fungal taxa	Hamra	Al-Beida	El-Zugum	Rosetta	Umm-Reisha	Al-Gaar	Khadra	Fasida
Acremonium furcatum				2			2	
Aspergillus	3	3	2, 3	2	2, 3	2	2, 3	1, 2, 3
A. flavus						2, 3		3
A. niger			1, 2				2	1, 3
A. ochraceus Wilhelm								1, 3
A. terreus	3	3	2, 3	2	2, 3	2	2, 3	1, 2, 3
Aspergillus sp.					3			
Cladosporium cladosporioides					3	3		
Emericella quadrilineata (Thom & Raper) Benjamin		2						
Fusarium	2		2	3	2			
F. solani	2		2	3				
F. subglutinans (Wollenweber & Reinking) Nelson, Toussoun & Marasas					2			
Penicillium	2	2			2, 3	2, 3	2	
P. chrysogenum		2			3	2, 3	2	
P. puberulum	2	2			2			
No. of genera (6)	3	3	2	3	4	3	3	1
No. of species (12)	3	4	3	3	6	4	4	4

The same legends as below Table 2

Acidiphilic and aciditolerant terrestial fungi recovered from water samples

A total of twenty-one species related to 10 genera of acidiphilic and aciditolerant fungi were collected at pH4, 12 species and 6 genera and at pH5, 13 spp. and 6 genera, compared to 15 spp. and 7 genera on Czapek Dox agar (as a control medium, pH 7) (Table 4).

Aspergillus (7 species), *Acremonium* (3) and *Penicillium* (5) comprised the major proportions of the total propagules at both pHs. They accounted for 87% and 65.4%, 8.4%, 28.8%, and 1.5% and 1.0% of total fungi on media adjusted at pH 4 and pH 5 respectively (Table 6). *Aspergillus* was the most common fungus in water from the 8 lakes investigated on both acidic media. The peak of *Aspergillus* was recorded from Al-Beida during winter 2007 at both pHs. *Aspergillus* and its dominant species *A. terreus* (57.4%, 42.2% of total fungi) were recorded from all lakes during winter and spring seasons only. *A. ochraceus* was recovered from 5 lakes while *A. flavus* from 4 lakes, each during 2 seasons. The remaining *Aspergillus* species were recovered from water of 3 lakes: *A. niger* (Al-Beida, Al-Gaar and Fasida during winter 2007), or one lake: *A. fumigatus*, *A. sydowii* (both from Al-Beida) and *A. ustus* (Khadra). In this respect, *Aspergillus candidus, A. melleus, A. niger, A. ochraceus, Chaetomium globosum, Cladosporum cladosporioides, C. sphaerospermum, Hortaea werneckii, Myrothecium roridum, Penicillium citrinum* and *P. chrysogenum,* have been reported in hypersaline waters from temperate to tropical regions (Butinar *et al.* 2005a, b, Gunde-Cimerman *et al*. 2004 and Kis-Papo *et al.* 2001, 2003a, b). Also, Khallil and Abdel-Sater (1992) found that the water and submerged mud directly exposed to industrial effluents of Mankabad superphosphate factory (highly acidic, low content of oxygen and relatively high contents of total soluble salts, phosphate, sulphate, calcium and magnesium) were poor in terrestrial fungi. They also found *Aspergillus fumigatus, A. niger* and *A. flavus*, *Alternaria alternata, Fusarium verticillioides* and *Stachybotrys chartarum* were the most prevalent terrestrial fungi.

Acremonium was recovered in 3 lakes during winter 2007 only. The peak of *Acremonium* was recorded from Umm-Risha Lake at both pHs. *Acremonium furcatum* was identified from 2 lakes (El-Zugm and Umm-Risha) while an unidentified *Acremonium* species was recovered from Hamra Lake. In this respect, *Acremonium persicinum* and *A. terricola* were isolated from Dead Sea water by Buchalo *et al.* (1999, 2000 a, b) and Kis-Papo *et al.* (2001)**.**

Pencillium and its dominant species *P. puberulum* were recorded from 5 lakes during winter and spring seasons. The peak was recorded from Al-Beida at pH4 and from El-Zugm at pH5. The remaining *Pencillium* species were recorded from water of either 3 lakes: *P. chrysogenum* (Al-Beida, El-Zugm and Al-Gaar), or 2 lakes during winter 2007 only: *P. aurantiogriseum* (Al-Beida and El-Zugm), or one lake: *P. expansum* (El-Zugum). In this respect, Cantrell *et al.* (2006) reported several species of *Penicillium: P. citrinum, P. chrysogenum, P. oxalicum* and *P. variabile* from the water of the salt ponds in hypersaline environments of solar salterns in Puerto Rico, most of them are halotolerant.

The remaining fungal species were isolated during winter 2007 from water of either 3 lakes: *Cochliobolus tuberculatus* (El-Zugm, Umm-Risha and Al-Gaar), 2 lakes: *Scopulariopsis brumptii* (Rosetta and Khadra), or one lake: *Alternaria tenuissima*, *Paecilomyces* sp., *Ulocladium botrytis* (from Al-Beida), *Staphylotrichum coccosporum* (from Umm-Risha), *Trichoderma* sp. (Fasida). A wide variety of fungi have been reported from water samples of Korangi Creek and Clifton areas of Karachi, Pakistan and these were *Aureobasidium pullulans, Bispora* sp., *Botrytis* sp., *Cladosporium* sp., *Fusarium solani, Humicola* sp., *Mucor* sp., *Penicillium* sp., *P. expansum, P. brefeldianum, Phoma* sp., *Pythium* sp., and *Rhizopus* sp. (Mehdi and Saifullah 1992) and from deep-sea water samples from different geographical locations (*Cladosporium* spp., *Alternaria* spp., *Aspergillus sydowii, Nigrospora* spp., and *Penicillium solitum*) (Roth *et al.* 1964 and Raghukumar *et al.* 1992).

Alkaliphilic and alkalitolerant terrestial fungi recovered from water samples

Seventeen species related to 8 genera of alkaliphilic and alkalitolerant fungi were recovered from water samples collected from Wadi El-Natrun lakes on Czapek Dox agar adjusted at pH10 (13 species related to 5 genera) and pH13 (7 species related to 3 genera) (Table 5).

Aspergillus (6 species), *Acremonium* (2) and *Penicillium* (5) were the most common genera at both pHs accounting for 64.1%, 31.2% and 1.2%, and 65.6%, 32.4% and 1.4% of total fungi at pH 10 and pH 13 respectively. However, no fungi were isolated from water samples collected from the Mono Lake in California, an alkaline, hypersaline and closed

Table 4: Seasons of isolation of acidiphilic and aciditolerant terrestrial fungi from water in different lakes of Wadi El-Natrun.

Fungal taxa	Hamra	Al-Beida	El-Zugum	Rosetta	Umm-Reisha	Al-Gaar	Khadra	Fasida
Acremonium	2		2		2			
A. blochii (Matruchot) W. Gams								
A. furcatum			2		2			
Acremonium sp.	2							
Alternaria tenuissima (Kunze: Fries) Wiltshire		2						
Aspergillus	2, 3	2, 3	2, 3	2, 3	2, 3	2, 3	2, 3	2, 3
A. flavus		2, 3	2, 3			2, 3		2
A. fumigatus		2						
A. niger		2				2		2
A. ochraceus		2	2, 3	2		2		2
A. sydowii (Bainier & Sartory) Thom & Church		2, 3						
A. terreus	2, 3	3	2, 3	3	2, 3	2	2, 3	2, 3
A. ustus (Bainier) Thom & Church							2	
Cochliobolus tuberculatus			2		2	2		
Paecilomyces sp.		2						
Penicillium		2, 3	2, 3	2	2	2		
P. aurantiogriseum		2	2					
P. chrysogenum		2	2			2		
P. expansum Link			2					
P. puberulum		2, 3	2, 3	2	2	2		
Scopulariopsis brumptii Salvanet-Duval				2			2	
Staphylotrichum coccosporum Meyer & Nicot					2			
Trichoderma sp.								2
Ulocladium botrytis Preuss		2						
No. of genera (10)	2	5	4	3	5	3	2	2
No. of species (21)	2	12	9	4	5	7	3	5

The same legends as below Table 2

basin (Steimen *et al*. 2004) as well as from the Dead Sea water (Guiraud *et al.* 1995 and Steimen *et al.* 1995). These authors stated that this may be attributed to the high salt levels (mostly sodium and potassium) in both waters combined with alkaline pH (9.4-9.8) in the Mono Lake or acidic pH (6.6) in the Dead Sea, which usually not favorable to fungal life. On the other hand, halophilic and halotolerant fungal species can live in some hypersaline waters (Zalar *et al*. 1999, Buchalo *et al.* 1998a, b and Grunde-Cimerman *et al*. 2000). *Aspergillus* was the most common fungus in water of the 8 lakes investigated on both alkaline media and from 6 out of 8 lakes on the control medium. The peak of *Aspergillus* count was recorded from Al Beida during winter 2007 at both pHs. The total CFUs of *Aspergillus* collected from the 8 lakes during the 3 seasons of study was higher at pH10 than at pH13. *Aspergillus* and its dominant species *A. terreus* (47.2% and 43.4% of total fungi on both pHs) were recovered from all lakes during winter and spring seasons. *A. flavus* was found in 4 lakes (Al-Beida, El-Zugm, Umm-Risha and Al-Gaar). The remaining *Aspergillus* species were recorded either from 3 lakes: *A. niger* (Al-Beida, Al-Gaar and Fasida), 2 lakes: *A. ochraceus* (El-Zugm and Rosetta), *A. ustus* (Al-Gaar and Khadra), or from Al-Beida Lake only (*A. fumigatus*). In this respect, several species of *Aspergillus* such as *A. candidus, A. caespitosus, A. flavus, A. flavipes, A. melleus, A. nidulans, A. ochraceus, A. penicillioides* and *A. unguis* most of them are halotolerant were

recovered from the water of the salt ponds in hypersaline environments of solar salterns in Puerto Rico (Cantrell *et al.* 2006). Also, Faryal and Hameed (2005) found that the fungi from water samples of textile industry effluent (pH 8.06 to 12.44) included species of *Rhizopus*, *Aspergillus*, *Penicillium*, *Candida*, *Drechslera*, and *Rhodotorula* with species of *Aspergillus* (*A. niger*, *A. fumigatus* and *A. flavus*) and *Rhizopus* spp. being the most predominant, and *Drechslera* spp. showing the lowest incidence. *Acremonium* was isolated from water collected from 4 lakes during winter 2007 only. It had the highest CFUs in water samples collected from Umm Risha Lake at both pHs**.** *A. hyalinulum* was isolated in Al-Beida, El-Zugm and Umm-Risha lakes and the unidentified *Acremonium* species was isolated from Khadra Lake. Other species of *Acremonium* were reported from Dead Sea water (Buchalo *et al.* 1999, 2000 a, b and Kis-Papo *et al.* 2001).

Penicillium (with *P. puberulum* being the most common) was recorded from 6 lakes. The remaining *Penicillium* species were recorded either from 2 lakes: *P. aurantiogriseum* (Al-Beida and Rosetta), or one lake during winter 2007: *P. chrysogenum* and *P. expansum* (Al-Beida) and *P. verrucosum* (Hamra). In this respect, Cantrell *et al.* (2006) reported *Penicillium citrinum*, *P. chrysogenum*, *P. oxalicum* and *P. variabile* from the water of the salt ponds in hypersaline environments of solar salterns in Puerto Rico.

The remaining fungi were recorded from either 2 lakes: *Trichoderma* sp. (Rosetta and Fasida), or from one lake during winter 2007 only: *Humicola grisea*, *Paecilomyces* sp., yeasts (from Khadra) and *Eurotium chevalieri* (Al-Beida). In this respect, Mehdi and Saifullah (1992) isolated *Humicola* sp. from water samples of Clifton, Pakistan. Butinar *et al.* (2005c) isolated six species of *Eurotium* from hypersaline waters of salterns and determined *in vitro* the adaptive ability of propagules to survive prolonged exposure to hypersaline conditions indicating that *E. amstelodami*, *E. herbariorum* and *E. repens* contribute to the indigenous fungal community in hypersaline water environments, while *E. rubrum*, *E. chevalieri* and *E. halotolerans* are only temporal inhabitants of brine at lower salinities. Moreover, some species were also isolated from surface and deep waters of the Dead Sea: *Aspergillus phoenicis*, *Chaetomium nigricolor*, *Emericella nidulans*, *Gymnascella marismortui*, *Paecilomyces farinosus*, *Penicillium variabile*, *P. westlingii* and *Acremonium* sp., *Stachybotrys chartarum* and *Ulocladium chlamydosporum* (Buchalo *et al*., 1998a, b, 1999, 2000a).

In conclusion: chemical analysis revealed that waters of Wadi El-Natrun lakes were highly alkaline and were high in total soluble salts, chloride, sodium and potassium ions. Water collected from El-Zugm Lake showed the highest levels of organic matter, sodium, calcium, magnesium and chloride among all lakes. On the other hand, the other parameters showed their peak in other lakes e.g. pH and total soluble salts in Fasida. A total number of genera (16) and species (33) were recorded from water samples collected from all lakes and the widest spectrum of species was recorded on the control (14) and the lowest on 10% NaCl media (3). *Aspergillus*, *Acremonium* followed by *Penicillium* were the most dominant genera possessing the highest proportions of propagules on all isolation media except on 10 % NaCl. Only species of the genera *Scopulariopsis* and *Acremonium* were isolated on 10% NaCl medium. From *Aspergillus*, *A. terreus* followed by *A. flavus* and *A. niger* were the most common on all isolation media. On the other hand, *A. ochraceus* was dominant on acidic media only. Other most commonly encountered species, *Penicillium chrysogenum* and *P. puberulum* were encountered on all media but not on 10 % NaCl medium. Some species were isolated on one medium but not on the others.

Table 5: Seasons of isolation of alkaliphilic and alkalitolerant terrestrial fungi from water in different lakes of Wadi El–Natrun.

Fungal taxa	Hamra	Al-Beida	El-Zugum	Rosetta	Umm-Reisha	Al-Gaar	Khadra	Fasida
Acremonium		2	2		2		2	
A. hyalinulum (Saccardo) W. Gams		2	5		2			
Acremonium sp.							2	
Aspergillus	3	2, 3	2, 3	2, 3	2, 3	2, 3	2, 3	2, 3
A. flavus		2, 3	3		3	2, 3		
A. fumigatus		2						
A. niger		2				2		2

A. ochraceus			2, 3	2				
A. terreus	3	3	2, 3	2, 3	2, 3	3	2, 3	2, 3
A. ustus						2	2	
Eurotium chevalieri Mangin		2						
Humicola grisea Traaen							2	
Paecilomyces sp.							2	
Penicillium	2	2	2, 3	2	2	2		
P. aurantiogriseum		2		2				
P. chrysogenum		2						
P. expansum		2						
P. puberulum	2	2	2, 3		2	2		
P. verrucosum Peyronel	2							
Trichoderma sp.				2				2
Yeasts							2	
No. of genera (8)	2	4	3	3	3	2	5	2
No. of species (17)	3	10	5	4	4	5	6	3

The same legends as below Table 2

Table 6: Percentage counts of the most common fungi (of the total) recovered from water samples collected from Wadi El-Natrun lakes on different isolation media.

Fungal taxa	Cz %	pH4 %	pH5	pH10	pH13	40% S	10% Nacl
Aspergillus	49.0	87	65.4	64.1	65.6	65.8	0.0
Acremonium	30.2	8.4	28.8	31.2	32.4	2.8	40
Penicillium	9.9	1.5	1	1.2	1.4	15.0	0.0

References

Abdel-Malek Y and Rizk SG (1963): Bacterial sulphate reduction and the development of alkalinity. III. Experiments under natural conditions. Journal of Applied Bacteriology 26:20–26.

Abu Zeid KA (1984): Contribution to the geology of Wadi El-Natrun area and its surroundings. M. Sc. Thesis, Faculty of Science, Cairo University.

Acevedo-Ríos C (1987): Hongos marinos de arena, madera y mangle de la Parguera, Puerto Rico. Master Thesis. University of Puerto Rico, Mayagüez Campus, 38 pp.

Al-Doory Y (1980): Laboratory Medical Mycology, pp. 240, 357-367. Lea Febiger Philadelphia Kimpton Publishers, London.

Atia AKM, Hilmy ME and Bolous SN (1970): Mineralogy of the encrustation deposits of Wadi El-Natrun. Desert Institute Bulletin, Egypt 2: 301-325.

Booth C (1971): The Genus *Fusarium*. Kew, UK, Commonwealth Mycological Institute, England.

Buchalo AS, Nevo, E, Wasser SP, Oren A and Molitoris HP (1998a): Fungal life in the extremely hypersaline water of the Dead Sea: First records. Proceedings of the Royal Society of London B 265: 1461-1465.

Buchalo AS, Nevo E, Wasser SP, Oren A, Molitoris HP and Volz PA (1998b): Diversity of fungal life in the extremely hypersaline water of the Dead Sea. In: Abstracts of the VIth International Mycological Congress, Jerusalem, Israel: 180.

Buchalo AS, Wasser SP, Molitoris HP, Volz PA, Kurchenko I, Lauer I and Rawal B (1999): Species diversity and biology of fungal life in the extremely hypersaline water of the Dead Sea. In: SP Wasser (ed.): Evolutionary Theory and Processes: Modern Perspectives. Papers in Honour of Eviatar Nevo, Kluwer Academic Publishers, Dordrecht, pp 293–300.

Buchalo AS, Nevo E, Wasser SP, Oren A, Molitoris HP and Volz PA (2000a): Fungi discovered in the Dead Sea. Mycological Research 104:132–135.

Buchalo AS, Nevo E, Wasser SP and Volz PA (2000b): Newly discovered halophilic fungi in the Dead Sea. In: J Seckbach (ed.) Journey to Diverse Microbial Worlds.

Kluwer Academic Publishers, Dordrecht, pp. 239–252.

Butinar L, Santos S, Spencer-Martins I, Oren A and Gunde-Cimerman N (2005a): Yeast diversity in hypersaline habitats. FEMS Microbiology Letters 244: 229–234.

Butinar L, Sonjak S, Zalar P, Plemenitas A and Gunde-Cimerman N (2005b): Melanized halophilic fungi are eukaryotic members of microbial communities in hypersaline waters of solar salterns. Botanica Marina 48: 73-79.

Butinar L, Zalar P, Frisvad JC and Gunde-Cimerman N (2005c): The genus *Eurotium*- members of indigenous fungal community in hypersaline waters of salterns. FEMS Microbiology Ecology 51(2): 155-66.

Cantrell AS, Casillas-Martinez L and Molina M (2006): Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques. Mycological Research 110: 962-970.

Cronin AE and Post FJ (1977): Reports of a dematiaceous hyphomycete from Great Salt Lake, Utah. Mycologia 69: 846-847.

Domsch KH, Gams W and Anderson TH (2007): Compendium of Soil Fungi. 2nd edition, IHC-Verlag, Eching, 672 pp.

Ellis MB (1971): Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England, 608 pp.

Ellis MB (1976): More Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, England, 481pp.

Faryal R and Hameed A (2005): Isolation and characterization of various fungal strains from textile effluent for their use in bioremediation. Pakistan Journal Botany 37(4): 1003-1008.

Gouda HAA (2009): Studies on xerophilic, acidiphilic and alkaliphilic fungi in Wadi El-Natrun. M SC Thesis, Department of Botany, Faculty of Science, Assiut University.

Grant WD (2006): Alkaline environments and biodiversity, in: Extremophilies, Gerday C, and Glansdorff N (eds.), in Encyclopedia of Life Support Systems (EOLSS), Developed under the Auspices of the UNESCO, EOLSS Publishers, Oxford, UK, [http://www.eolss.net].

Grant WD, Jones BE and Mwatha WE (1990): Alkaliphiles: ecology, diversity and applications. FEMS Microbiology Reviews 75:255-270.

Guiraud P, Steiman R, Seigle-Murandi F and Sage L (1995): Mycoflora of soil around the Dead Sea. II- Deuteromycetes (except *Aspergillus* and *Penicillium*). Systematic and Applied Microbiology 18: 318–322.

Gunde-Cimerman N, Zalar P, de Hoog GS and Plemenitas A (2000): Hypersaline water in salterns-natural ecological niches for halophilic black yeasts. FEMS Microbiology Ecology 32: 235-240.

Gunde-Cimerman N, Zalar P, Petrovic U, Turk M, Kogej T, de Hoog GS and Plemenitas A (2004): Fungi in the Salterns. In: Ventosa A (ed.), Halophilic Microorganisms. Springer-Verlag, Heidelberg, pp. 103–113.

Jackson ML (1958): Soil Chemical Analysis. Constable and Co., London.

Jones BE and Grant WD (1999): Microbial diversity and ecology of the soda lakes of East Africa. In: Microbial Biosystems: New Frontiers. Proceedings of the 8th International Symposium Microbial Ecology. In: Bell CR, Brylinsky M and Johnson-Green P (eds.), Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Jones BE, Grant WD, Collins NC and Mwatha WE (1994): Alkaliphiles: diversity and identification. In: Priest FG, Ramos-Cormenzana A, Tindall BJ (eds.), Bacterial Diversity and Systematics, Plenum Press, New York, pp. 195-229.

Jones BE, Grant WD, Duckworth AW and Owenson GG (1998): Microbial diversity of soda lakes. Extremophiles 2: 191-200.

Khallil AM and Abdel-Sater MA (1992): Fungi from water, soil and air polluted by the industrial effluents of Manquabad superphosphate Factory (Assiut, Egypt). Journal of Basic Microbiology 33(2): 83-100.

Kis-Papo T, Grishkan I, Oren A, Wasser SP and Nevo E (2001): Spatiotemporal diversity of filamentous fungi in the hypersaline Dead Sea. Mycological Research 105: 749-756.

Kis-Papo T, Oren A, Wasser SP and Nevo E (2003): Survival of filamentous fungi in hypersaline Dead Sea water. Microbial Ecology 45(2): 183-90.

Kis-Papo T, Oren A, Wasser SP and Nevo E (2003b): Survival of filamentous fungi in hypersaline Dead Sea water. FEMS Microbiology Ecology 45: 183-190.

Leslie JF and Summerell BA (2006): *Fusarium* laboratory workshops- A recent history. Mycotoxin Research 22(2): 73-74.

Litchfield CD and Gillevet PM (2002): Microbial diversity and complexity in hypersaline environments: A preliminary assessment. Journal of Industrial Microbiology and Biotechnology 28: 48-55.

Mason DT (1967): Limnology of Mono Lake, California. University of California, Publications in Zoology, 83 pp.

Mehdi FS and Saifullah SM (1992): Mangrove fungi of Karachi Coast. Journal of the Islamic Academy of Sciences 5: 24-27.

Moubasher AH (1993): Soil fungi of Qatar and other Arab Countries. The Scientific and Applied Research Centre, University of Qatar, Doha, Qatar, 566 pp.

Moussa AB, Kantiranis N, Kontantinos SV, John AS, Mona FA, Vasilios C (2009): Diagnosis of weathered-Coptic wall paintings in the Wadi El-Natrun region, Egypt. Journal of Cultural Heritage 10: 152-157.

Oren A (2002): Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. Journal of Industrial Microbiology and Biotechnology 28: 56-63.

Pavlov M (1962): Preliminary report on the ground water beneath the Wadi El-Natrun and adjacent areas. Report to the General Desert Development Organization of the U. A. R. Desert Institute, Cairo (Cited in: Taher, G.A. (1999): Inland saline lakes of Wadi El-Natrun depression, Egypt. International Journal of Salt Lake Research 8: 149-169.

Pitt JI (1979): The Genus *Penicillium*. Academic Press, London, 635 pp.

Raghukumar C, Raghukumar S, Sharma S and Chandramohan D (1992): Endolithic fungi from deep-sea calcareous substrata: isolation and laboratory studies. In: Deasai BN (ed.), Oceanography of the Indian Ocean. Oxford IBH Publ, New Delhi, pp. 3-9.

Raper KB and Fennell DI (1965): The genus *Aspergillus*. Williams and Wilkins, Baltimore.

Richards LA (1954): Diagnosis and improvement of saline and alkali soils. US Salinity Laboratory, US Department of Agriculture, Handbook 60, 160 pp.

Roth FJ, Orpurt PA and Ahearn DJ (1964): Occurrence and distribution of fungi in a subtropical marine environment. Canadian Journal of Botany 42: 375-383.

Satyanarayana T, Raghukumar C and Shivaji S (2005): Extremophilic microbes: Diversity and perspectives. Current Science 89: 1-10.

Schwarzenbach G and Biedermann W (1948): Complexons. X-Alkaline earth complexes of O, O-dihydroxy azo dyes. Helvetica Chimica Acta 31: 678-687.

Shatta A and El Fayoumi IF (1967): Geomorphological and morphopedological aspects of the region west of the Nile Delta with special reference to Wadi El-Natrun area. Desert Institute Bulletin, Egypt 17(1).

Smith NR and Dawson VT (1944): The bacteriostatic action of rose bengal in media used for the plate count of soil fungi. Soil Science 58: 467-471.

Steinman AD, Mulholland PJ and Kirschtel DB (1991): Interactive effects of nutrient reduction and herbivory on biomass, taxonomic structure, and P uptake in lotic periphyton communities. Canadian Journal of Fisheries and Aquatic Sciences 48: 1951-1959.

Steiman R, Guiraud P, Seigle-Murandi F and Lafond JL (1995): Mycoflora of soil around the Dead Sea. I- Ascomycetes (including *Aspergillus* and *Penicillium*), Basidiomycetes, Zygomycetes. Systematic and Applied Microbiology 18: 310-317.

Steiman R, Guiraud P, Seigle-Murandi F and Lafond JL (1997): Soil mycoflora from the Dead Sea oases of Ein Gedi and Einot Zugim. Antonie van Leeuwenhoek 72: 261-270.

Steiman R, Ford L, Ducros V, Lafond J and Guiraud P (2004): First survey of fungi in hypersaline soil and water of Mono Lake area (California). Antonie Van Leeuwenhoek 85(1): 69-83.

Taher GA (1999): Inland saline lakes of Wadi El-Natrun depression, Egypt. International Journal of Salt Lake Research 8: 149-169.

Ulukanli Z and Diğrak M (2002): Alkaliphilic micro-organisms and habitats. Turkish Journal of Biology 26: 181-191.

Utah Geological Survey (2001): Great Salt Lake. http://geology.utah.gov/online/PI-39.

Vezzulli L, Zotti M, Marin V, Moreno M, Pezzati E and Fabiano M (2009): Swash zone interstitial water is a reservoir of fungal micro-organisms on a Mediterranean beach (Genoa City, Italy). Marine Biodiversity Records 2, e19 doi:10.1017/S1755267208000225.

Williams V and Twine S. (1960): Flame photometric method for sodium, potassium and calcium. In: Paech K, Tracev M: V. (ed.): Modern Methods of Plant Analysis. Vol. V, pp 3-5, Springer-Verlag, Berlin.

Zahran MA and Willis AJ (1992): The Vegetation of Egypt. Chapman and Hall, London, 424 pp.

Zalar P, de Hoog GS and Gunde-Cimerman N (1999): Ecology of halotolerant dothideaceous black yeast. Studies in Mycology 43: 41-52.

Journal of Basic & Applied Mycology (Egypt) 4 (2013): 59-68

Endophytic fungi of three leguminous plant roots in Egypt

O. M. O. El-Maghraby, S. M. Soltan, Rehab M. Mohammed, Maha M. Mohammed

Botany Department, Faculty of Science, Sohag University, Sohag, Egypt

Corresponding author: e-mail: osmanelmaghraby@yahoo.com
Received 3/11/2013, Accepted 18/12/2013

Abstract: Seventy-eight species, in addition to six varieties belonging to twenty-one genera of endophytic fungi were isolated and identified from the roots of three leguminous plants (peanut, alfalfa and broad bean) on PDA and water agar at 28±2˚C. *Fusarium* (15 species), *Penicillium* (16 species) and *Aspergillus* (12 species and 5 varieties) were the dominant genera of which *F. solani, F. subglutinans, F. oxysporum, P. duclauxii, P. funiculosum, A. tubingensis* and *A. flavus* were the most prevalent. The endophytes of peanut and alfalfa were rich in fungal counts (229 & 209 and 230 & 188 colonies/20 samples on PDA and water agar respectively) compared with broad bean (138 & 102 colonies). All isolated fungi belong to Ascomycota and Deuteromycetes.

Key words: Endophytes, peanut, alfalfa, broad bean, roots.

Introduction

The legumes are second to cereal crops in agricultural importance based on area harvested and total production. The legume family (Fabaceae) is the third largest family of higher plants (Anuradha *et al*. 2006). Seeds of legumes provide about one third of all dietary protein nitrogen and one-third of processed vegetable oil for human consumption (Graham and Vance 2003).

Peanut (*Arachis hypogaea* L.) is an important cash crop of farmers particularly in the semiarid tropics (Iqbal *et al*. 2011). In Egypt, it is grown in 589740 ha mainly for direct consumption than for oil extraction (Ahmad and Mohamed 2009). In addition, peanuts are a rich source of edible oil (43-55%), and protein (25-28%) (Gohari and Niyaki 2010). Also, it contains 20% carbohydrate and 5% fiber and ash which make a substantial contribution to human nutrition (Ahmad and Rahim 2007).

Alfalfa (*Medicago sativa* L.) is a widely cultivated, environmentally tolerant forage crop. It is grown in Egypt in arid and semiarid regions, and provides high quality forage and green manure (Kamel and Shoukry 2001). It is famous for its excellent nutritive value, high digestibility and a high biomass yield (Stajkovic-Srbinović *et al*. 2012). Alfalfa contributes to the incorporation of nitrogen in agriculture systems, with a consequent economic benefit, helping to reduce the application of synthetic N fertilizers (Jensen and Hauggaard-Nielsen 2003).

Broad bean (*Vicia faba* L.) sometimes referred to as horse bean or field bean. It is the fourth important pulse crop in Egypt and many countries. It occupies the greatest area planted to legume crops in the Arab countries (Amin 1988). Nutritionally, mature seeds of broad bean are a good source of protein (about 25% in dried seeds), starch, cellulose, vitamin C and minerals (FAO 1995 and Hamilton 2005). Also, it is an excellent candidate crop to provide nitrogen input into temperate agricultural systems; moreover, it makes a significant contribution to soil fertility restoration as a suitable rotation crop that fixes atmospheric nitrogen (Samuel *et al*. 2008).

Fungal endophytes are microfungi that colonize living tissues of plants without producing any apparent symptoms or obvious negative effects (Hirsch and Braun 1992). Many endophytes produce unusual secondary metabolites of industrial importance (Hawksworth *et al*. 1995). They have a protective role against insect herbivory and many are producer of novel antimicrobial secondary metabolites (Arnold *et al*. 2003, Malinowski and Belesky 2006 and Shiomi *et al*. 2006). Additionally, endophytic fungi are a fascinating source of new natural products which are of great potential for medicinal and agricultural applications (Aly *et al*. 2010 & 2011). Also, endophytic fungi represent an important and quantified component of fungal biodiversity, and are known to affect on diversity and structure of plant community (Gonthier *et al*. 2006 and Krings *et al*. 2007).

The current work aimed for studying the diversity (frequency and counts) of endophytic fungi associated with roots of three leguminous plants in Egypt.

Materials and Methods

Collection of plant samples

Roots of three economical plants (*Arachis hypogaea*, *Medicago sativa* and *Vicia faba*) were collected during the flowering stage of each plant,

from different locations in Sohag Governorate. The plants (20 samples each) were chosen to isolate endophytic fungi. The collected plant materials were stored in separate plastic bags at 4°C in an ice box until isolation of fungi (Strobel and Daisy 2003).

Fungal isolation and identification

Isolation of endophytic fungi was done according to the method described by Hallmann *et al.* (2007) with minor modifications. The plant roots were rinsed gently in running water to remove adhered dust and debris. Samples were surface sterilized with 75% ethyl alcohol for 1 min, soaked in 5% sodium hypochlorite solution for 3 min, and then rinsed with 75% ethyl alcohol for 30 sec. They were finally rinsed with sterile distilled water and dried between sterilized filter papers under laminar air flow chamber and the roots were cut into segments (1 cm).

Forty sterilized segments from each root were placed on both PDA (potato extract, 200 ml; glucose, 20.0 g; agar, 15.0 g; distilled water, to 1.0 L) and WA (agar, 15.0 g; distilled water, to 1.0 L) media. The plates (4 plates for each medium type, 5 segments for each) were incubated at 28°C for 15 days and were periodically observed for fungal growth. The growing fungi were then sub-cultured on PDA and glucose-Czapek's agar medium plates (glucose, l0.0 g; $NaNO_3$, 2.0 g; KH_2P0_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; KC1, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; agar, 15.0 g; distilled water, to 1.0 L; pH, 6.5-7) for purification and identification purposes. The endophytic fungal isolates were identified microscopically on the basis of their critical morphological structures such as hyphal features, arrangement of spores and reproductive structures (Ellis 1971 & 1976, Raper and Fennell 1965, Pitt 1979, Nelson *et al.* 1983, Moubasher 1993 and Leslie and Summerell 2006). Isolates that failed to produce reproductive structures after 3-4 weeks of incubation were referred to as sterile mycelium, and divided into their color (Lacap *et al*. 2003).

Results and Discussion

In the current study, endophytic fungi were isolated from roots of three economic plants. Most isolates were recorded in the first two weeks of incubation. These results correspond with other results obtained for the rate of isolation of endophytic fungi from other hosts (Abdel-Wahab 2000 and Shebany 2008).

Seventy-eight species and six varieties which belong to twenty-one genera were isolated and identified from 60 plant samples of peanut, alfalfa and broad bean roots (each, 20 samples) on PDA and WA media at 28±2˚C (Tables, 1 & 2). Most species isolated belong to genera which have already been described as endophytes (Abdel-Wahab 2000, Shebany 2008 and Nath *et al*. 2012). The endophytic fungi belong to Ascomycetes and Deuteromycetes. The authors who worked in this field indicated that members of the Ascomycotina and Deuteromycotina have been isolated as endophytes (Petrini 1986, Siegal *et al*. 1987, Clay 1991 and Abd-Elaah and Soliman 2005).

A total of 1096 isolates were isolated from the three plants using PDA (597 isolates) and WA (499 isolates); (229 & 209 from peanut, 230 & 188 isolates from alfalfa and 138 & 102 from broad bean on PDA and WA respectively). The differences in the number of isolates rely on the nature, age and other factors of the plants. Hoff *et al*. (2004) mentioned that endophytic fungi usually occur in above ground plant tissues but, are also found in root. Unlike mycorrhizal fungi, fungal endophytes of roots lack extra radical (outside the root) hyphal networks and mantles (sheaths around the roots).

Of the three economic plants studied, the most frequently-occurring genera were *Fusarium*, *Aspergillus* and *Penicillium* in the counts (7.25-44.35%, 8.5- 22.5% and 8.5- 45% of total fungi) and in the number of cases of isolation (30- 95%, 30-80% and 40-90% of the total samples in both PDA and WA media, respectively). These genera were previously isolated as endophytic fungi by several researches from different plants such as *Fraxinus excelsior*, *Gossypium* sp., *Gynoxis oleifolia*, *Manilkara bidentata*, *Picea abies* and *Taxus* sp. (Fisher *et al*. 1995, Redlin and Carris 1996, Strobel *et al*. 1997, Caruso *et al*. 2000 and Wang *et al*. 2007), twigs of *Kandelia candel* and *Avicennia marina* (Abdel-Wahab 2000), different parts of *Althea rosea*, *Calotropis procera* and *Nerium oleander* (Shebany 2008) and roots, stems and leaves of *Hyoscyamus muticus* (Abdel-Motaal *et al*. 2010).

Fusarium (13 species) was the most common genus regarding the number of cases of isolation and total fungal count from peanut and alfalfa, (each, 95% of the samples and 28.4 and 44.35% of total fungi respectively) on PDA, and 75 and 80% of the samples and 22.5 and 34.6% of total fungi, respectively on WA medium (Table 1). These results are in agreement with those obtained by Shebany (2008) who reported that *Fusarium* was the most common genus based on number of cases of isolation and fungal count from *Althea rosea*, *Calotropis procera* and *Nerium oleander*. *Fusarium* spp. have been recorded as endophytes from *Amomum siamense* (Bussaban *et al*. 2001) and was the most dominant genus in *Dracaena cambodiana* and *Aquilaria sinensis* (Jiang *et al*. 1995 and Tian *et al*. 2004). In the present investigation, *F. solani*,

F. subglutinans, F. oxysporum, F. nygamai and *F. anthophilum* were the dominant species recovered from the three economical plants (5-55% and 5-45% of the samples, and 0.48-13.4% of total fungi on both PDA and WA media respectively). Most of these species were recovered by Shebany (2008) from *Althea rosea* and *Nerium oleander*. Weber *et al*., (2007) isolated *F. solani* from healthy leaves of *Quercus ilex* as endophytic fungus on 2% malt extract agar medium and used extract of this fungus against pathogenic and non-pathogenic yeasts and filamentous fungi.

Aspergillus was the second most prevalent genus based on the counts from the three plant roots (Table 1). Caruso *et al*. (2000) recovered *Aspergillus* spp. from woody and herbaceous tissues of *Taxus* sp. The genus was represented by 12 species in addition to 5 varieties of which the most dominant species were *A. tubingensis* and *A. terreus* in the 3 plants. They accounted for 0.87-5% and 2.7-4.9% on PDA and WA respectively. The previous species were recorded in high quality and quantity from *Citrus limon*, *Ocimum basilicum*, *Morus rubra* and *Psidium guajava* (Mohammed 2010).

Penicillium was isolated from the three plants and ranked first in frequency of occurrence (90% of the samples) in broad bean comprising 45% of total fungi and third in peanut and alfalfa (70 and 55% of the samples and 17.9 and 11% of total fungi respectively). The above genus was previously isolated by several researchers (Sinclair and Backman 1989, Fisher *et al*. 1995, Caruso *et al*. 2000, Ananda and Sridhar 2002 and Sasaki *et al*. 2005). *Penicillium* spp. have been commonly recorded as endophytes from leaves and roots of various hosts such as soybean leaves (Larran *et al*. 2002), roots of *Alnus glutinosa* (Cappellano *et al*. 1987 and Fisher *et al*. 1991), *Picea marina*, *Sorbus* spp. (Suryanarayanan *et al*. 2000) and from *Amomum siamense* (Bussaban *et al*. 2001). Sinclair and Backman (1989) recovered *Penicillium* sp. from surface-disinfested soybean seeds. Caruso *et al*. (2000) isolated *Penicillium* sp. from herbaceous tissues of *Taxus* sp. Of the genus, 16 species were collected and identified of which *P. funiculosum* and *P. duclauxii* were the most predominant species in the 3 plant roots (Table 1). The former species was recovered from three medicinal plants as an endophyte (Shebany 2008). Also, Mohammed (2010) isolated *P. duclauxii* from leaves of *Ocimum basilicum* and *Psidium guajava* which comprised 15.6 and 3.3% of total fungi and 15 and 5% of the samples, respectively.

Four genera, namely *Cylindrocarpon* (2 species), *Humicola* (2), *Drechslera* (6) and *Scopulariopsis* (2) were recorded in the 3 tested plants in low counts (0.72-14% of total fungi) and frequencies (5-50% of the samples). Also, four genera were observed in two plants in low counts (0.43-3.9%) and frequencies (5-20%) and these were *Cladosporium* (2 species), *Alternaria* (4), *Macrophomina* (1) and *Curvularia* (4) on PDA (Table 1).

On the other hand, *Cylindrocarpon* was recorded in all plants tested with moderate counts (9.8-19.6% of total fungi) and frequencies (20-45% of the samples), while, *Humicola*, *Drechslera* and *Macrophomina* were observed in two plants with low counts (0.96-9% of total fungi) and frequencies (5-40% of the samples) on WA medium (Table 1). These species were recovered as endophytic fungi from many plants (Rubini *et al*. 2005, Ganley and Newcombe 2006 and Weber *et al*. 2007). *Cladosporium* sp. was collected from 30 trees samples throughout the trees limited range in Northern Florida (Lee *et al*. 1995). Caruso *et al*. (2000) isolated *Alternaria* from woody tissues and herbaceous tissues of *Taxus* sp. In particular, *Alternaria* was isolated from all the analysed plant materials and can be considered a resident genus of *Taxus* tissues.

Sterile mycelia were observed in high diversity of colour (1.3- 8.2% and 3.4-10.1% of counts on PDA and WA respectively) from the three plant roots tested, where alfalfa had the best frequencies and counts (65 and 55% of the samples and 8.2 and 10.1% of the counts on PDA and WA respectively) as shown in Table (1). Brown or blackish sterile fungi isolated from conifer roots were referred to as *Mycelium radicis atrovirens* Melin (MRA) (Melin, 1922 & 1923), but very little is known what comprises MRA, because the name has since been applied to any sterile, dark and septate fungus isolated from roots or soil (Jumpponen and Trappe 1998). Shebany (2008) recovered sterile mycelia from different organs of *Althea rosea*, *Calotropis procera* and *Nerium oleander* with low counts (10.8% of total fungi). Also, Caruso *et al*. (2000) isolated sterile mycelium from woody and herbaceous tissues of a *Taxus* sp. Moreover, dark septate endophytes may indeed function physiologically as mycorrhizas in natural conditions, since some dark septate endophytes have been found to enhance host mineral nutrition and growth (Shivanna *et al*. 1994, Fernando and Currah 1996 and Jumpponen *et al*. 1998).

In conclusion: There are no specific fungal genera and species for each plant tested (*Arachis hypogaea*, *Medicago sativa* and *Vicia faba*) and most of the species were isolated and identified from Egyptian sources. Fusaria were the most prevalent based on frequency and count. Sterile mycelia had varieties of colour and varied in counts in different plants tested.

Table 1: Total counts (TC, calculated per 400 root segments) and number of case of isolation (NCI, out of 20 samples) of fungal genera and species isolated from peanut, alfalfa and broad bean recovered on potato dextrose agar (PDA) and water agar (WA) at 28±2˚C.

Genera and species	Peanut				Alfaalfa				Broad bean			
	PDA		WA		PDA		WA		PDA		WA	
	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI
Fusarium	65	19	47	15	102	19	65	16	10	6	27	9
F. solani (Mart.) Sacc.	32	9	28	9	23	9	13	7			5	2
F. subglutinans (Wollenweber & Reinking) Nelson, Toussoun & Marasas	12	6			24	11	1	1				
F. oxysporum Schlecht.	7	6	8	4	9	3	11	4	3	2		
F. udum Butler					11	6	7	4				
F. nygamai L. W. Burgess & Trimboli	6	3	1	1	6	3	6	4	3	1	4	2
F. semitectum Berkeley & Ravenel	3	3			4	4			1	1		
F. xylarioides Steyaert					4	3	3	1				
F. decemcellulare Brick					3	2						
F. anthophilum (A. Braun) Wollenweber	2	2	2	1	2	2	1	1	1	1	2	2
F. thapsinum Klittich, Leslie, Nelson & Marasas					2	2	8	2	1	1	1	1
F. chlamydosporum Wollenweber & Reinking					3	1	6	4			11	5
F. camptoceras Wollenweber & Reinking	2	1										
F. verticillioides (Saccardo) Nirenberg	1	1			11	5	8	5	1	1	4	2
F. poae (Peck) Wollenweber			8	3								
F. fusarioides (Gonz, Fragoso & Ciferri) C. Booth							1	1				
Aspergillus	44	14	39	13	33	11	16	6	31	16	15	7
A. eburneo-cremeus Sappa	8	6	4	2								
A. tubingensis (Schöber) Moss	11	5	10	5	6	4	5	2	7	6	5	5
A. flavus link	6	4	7	5	7	3			11	5	6	3
A. sulphureus (Fres.) Thom & Church					5	4	2	1				
A. ficuum (Reich.) Hennings	7	3			2	1			1	1		
A. terreus Thom	3	2	8	2	2	2	7	3	2	2	3	2
A. flavus var. *columnaris* Fennell & Raper					4	2						
A. flavo-furcatis Batista & Maia					4	1	1	1				
A. terreus var. *aureus* Thom & Raper	5	1	6	3	2	2					1	1
A. ochraceus Wilhelm					1	1	1	1				
A. terricola var. *americana* Marchal	2	1	3	2								

Table 1: Continued

Genera and species	Peanut				Alfaalfa				Broad bean			
	PDA		WA		PDA		WA		PDA		WA	
	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI
A. terricola var. *indicus* (Mehrotra & Agnihotri) Raper & Fennell			1	1								
A. niveus Blochwitz	1	1										
A. speluneus Thom & Raper	1	1										
A. phoenicis (Cda.) Thom									8	7		
A. janus Raper & Thom									1	1		
A. oryzae (Ahlb.) cohn var. *effuses* (Tiraboschi) Ohara									1	1		
Emericella nidulans var. *echinulata* (Fennell & Raper) Subramanian	2	2	4	2								
Penicillium	41	14	29	10	25	11	16	8	62	18	34	13
P. duclauxii Delacroix	26	7	14	6	9	4	9	4	16	10	2	1
P. piscarium Westling	7	3	3	1								
P. funiculosum Thom	3	3	6	4	15	9	6	4	33	17	10	7
P. baarnense van Beyma	2	1	5	2								
P. kapuscinskii Zaleski	2	1										
P. verruculosum Peyronel	1	1										
P. resticulosum Birkinshaw, Raistrick & Smith					1	1						
P. lavendulum Raper & Fennell									4	4		
P. rubrum Stoll									3	2	2	1
P. chrysogenum Thom									2	1		
P. citrinum Thom									1	1	2	1
P. corylophilum Dierckx									1	1	1	1
P. pulvillorum Turfitt									1	1		
P. variabile Sopp			1	1			1	1	1	1		
P. islandicum Sopp											13	5
P. pulvillorum Turfitt											4	2
Cylindrocarpon	32	10	41	6	13	6	28	9	2	1	10	4
C. radicicola Wollenweber	14	6	21	4	8	4			2	1	1	1
C. didymum (Hartung) Wollenweber	18	5	20	5	5	2	28	9			9	4
Humicola	11	5	5	2	7	3	2	2	3	2		
H. grisea Traaen	10	6	5	2	6	2			3	2		
H. fuscoatra Traaen	1	1			1	1	2	2				

Table 1: Continued

Genera and species	Peanut				Alfaalfa				Broad bean			
	PDA		WA		PDA		PDA		WA		PDA	
	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI
Cladosporium					3	2			3	3	3	3
C. cladosporioides (Fresen.) de Vries					3	2			1	1	2	2
C. oxysporum Berk. & Curt									2	2	1	1
Alternaria	5	4	15	4	1	1						
A. tenuissima (Kunze: ex Pers.) Wilshire	3	2										
A. sonchi J. J. Davis & apud J. A. Elliott					1	1						
A. alternata (Fr.) Keissler	1	1	15	4								
A. radicina Meier, Drechsler & Eddy	1	1										
Drechslera	4	4	3	1	7	3	17	8	2	2		
D. biseptata (Sacc. & Roum.) Richardson & Fraser	1	1					1	1	1	1		
D. dematioidea (Bubák & Wróblewski) Subram & Jain	1	1										
D. hawaiiensis (Bugnicourt) Subram. & Jain ex Ellis, Subram. & Jain	1	1					2	1				
D. rostrata (Drechsler) Richardson & Fraser	1	1										
D. halodes (Drechsler) Subram. & Jain			3	1	3	2	1	1	1	1		
D. bicolor Paul & Parbery					4	1	11	5				
D. papendorfii (van der Aa) M. B. Ellis							2	1				
Macrophomina phaseolina (Maublanc) Ashby	8	3	2	1	3	1	15	6				
Trichoderma ghanense Doi, Y. Abe & J. Sugiyama									9	3		
Curvularia	5	3	5	4	9	4						
C. clavata Jain	3	2	3	2								
C. brachyspora Boedijn	1	1	1	1								
C. lunata (Wakker) Boedijn	1	1	1	1	7	4						
C. ovoidea (Hiroe & Watan.) Muntañola					2	1						
Scopulariopsis	5	2	2	2	2	2			1	1		
S. brumptii Salvanet-Duval	4	1	2	2					1	1		
S. brevicaulis (Sacc.) Bainier	1	1			2	2						
Botryotrichum	1	1									5	2
Botryotrichum atrogriseum van Beyma	1	1										
Botryotrichum piluliferum Saccardo & Marchal											5	2
Stachybotrys atra Corda					1	1						

Table 1: Continued

Genera and species	Peanut				Alfaalfa				Broad bean			
	PDA		WA		PDA		WA		PDA		WA	
	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI	TC	NCI
Torula			1	1	1	1			1	1	2	2
Torula graminis Desm			1	1	1	1			1	1		
T. herbarum (Pers.) Link ex Fries											2	2
Mucor hiemalis Wehmer	1	1							2	1		
Paecilomyces variotii Bainier									2	1	1	1
Trimmatostroma salicis Corda	1	1										
Paecilomyces marquandii (Massee) S. Hughes			9	3								
Gilmaniella humicola Barron							2	1				
Pleospora infectoria Fuckel							1	1				
Unknown 1	1	1										
Unknown 2									3	1		
Unknown 3							1	1				
Unknown 4					3	2						
Unknown 5					1	1	6	1				
Sterile mycelium (white)	2	1	6	2	3	2	2	1	1	1		
Sterile mycelium (buff)			1	1			2	1			1	1
Sterile mycelium (white-gray)	1	1			1	1			1	1		
Sterile mycelium (gray)					2	2					3	2
Sterile mycelium (gray red)							1	1				
Sterile mycelium (white-brown)					3	1			2	1		
Sterile mycelium (white beige)					3	1						
Sterile mycelium (white gray orange)					2	1						
Sterile mycelium (white red)							1	1				
Sterile mycelium (olive-brown)					1	1	13	7	1	1	1	1
Sterile mycelium (gray green)					1	1						
Sterile mycelium (white gray blue)					1	1			2	1		
Sterile mycelium (violet gray)					1	1						
Sterile mycelium (versicolor)					1	1						
Total count	229		209		230		188		138		102	
No. of genera	14		13		13		9		12		8	
No. of species + varieties	41+3		26+4		36+2		29		32+1		23+1	

References

Abd-Elaah AG and Soliman AS (2005): Effect of fluconazole on mycelial growth and protein profiles of some fungal species isolated from molasses. Assiut University Journal of Botany 34: 131-145.

Abdel-Motaal FF, Nassar MSM, El-Zayat SA, El-Sayed MA and Ito S-I (2010): Antifungal activity of endophytic fungi isolated from Egyptian henbane (*Hyoscyamus muticus* L.). Pakistan Journal of Botany 42: 2883-2894.

Abdel-Wahab MA (2000): Diversity of fungi in subtropical mangroves. Ph. D. Thesis, Faculty of Science, Sohag, South Valley University, Egypt.

Ahmad MSH and Mohamed SMS (2009): Improvement of groundnut (*Arachis hypogaea* L.) productivity under saline condition through mutation induction. World Journal of Agricultural Sciences 5: 680-685.

Ahmad N and Rahim M (2007): Evaluation of promising groundnut (*Arachis hypogaea* L.) varieties for yield and other characters. Journal of Agricultural Research 45: 185-189.

Amin ANM (1988): Principles of Field Crops. Basra University Press, Iraq, pp. 442-452.

Ananda k and Sridhar KR (2002): Diversity of endophytic fungi in the roots of mangrove species on the West Coast of India. Canadian Journal of Microbiology 48: 871-878.

Anuradha TS, Jami SK, Datla RS and Kirti PB (2006): Genetic transformation of peanut (*Arachis hypogaea* L.) using cotyledonary node as explant and a promoterless *gus::npt*II fusion gene based vector. Journal of Bioscience 31: 235-246.

Arnold AE, Maynard Z, Gilbert GS, Coley PD and Kursar TA (2003): Are tropical fungal endophytes hyperdiverse? Ecology Letters 3: 267-274.

Bussaban B, Lumyong S, Lumyong P, McKenzie EHC and Hyde KD (2001): Endophytic fungi from *Amomum siamense*. Canadian Journal of Microbiology 47: 943-948.

Cappellano A, de Quartre B, Valla G and Moiroudo A (1987): Root nodule formation by *Penicillium* sp. on *Alnus glutinosa* and *Alnus incana*. Plant and Soil 104: 45-51.

Caruso M, Colombo AL, Fedeli L, Pavesi A, Quaroni S, Saracchi M and Ventrella G (2000): Isolation of endophytic fungi and actinomycetes taxane producers. Annals of Microbiology 50: 3-13.

Clay K (1991): Fungal endophytes, grasses, and herbivores. In: Microbial mediation of plant-herbivore interaction. Barbosa P, Krischik VA and Jones CG (eds.), John Wiley and Sons, Inc., New York, pp. 199-252.

Ellis MB (1971): Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England, 608 pp.

Ellis MB (1976): More Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England, 507 pp.

FAO (1995): Production Year Book. Food and Agricultural Organization of United Nations, Rome, Italy, Volume 38.

Fernando AA and Currah RS (1996): A comparative study of the effects of the root endophytes *Leptodontidium orchidicola* and *Phialocephala fortinii* (Fungi Imperfecti) on the growth of some subalpine plants in culture. Canadian Journal of Botany 74: 1071-1078.

Fisher PJ, Petrini O and Webster J (1991): Aquatic hyphomycetes and other fungi in leaving aquatic and terrestrial roots of *Alnus glutinosa*. Mycological Research 95: 543-547.

Fisher PJ, Graf F, Petrini LE, Sutton BC and Wookey PA (1995): Fungal endophytes of *Dryas octopetala* from a high arctic polar semi- desert and from the Swiss Alps. Mycological Research 87: 319-323.

Ganley RJ and Newcombe G (2006): Fungal endophytes in seeds and needles of *Pinus monticola*. Mycological Research 110: 318-327.

Gepts P, Beavis WD, Brummer EC, Shoemaker RC, Stalker HT, Weeden NF and Young ND (2005): Genomics for food and feed report of the cross-legume advances through genomics conference. Plant Physiology 137: 1228-1235.

Gohari AA and Niyaki SAN (2010): Effects of iron and nitrogen fertilizers on yield and yield components of peanut (*Arachis hypogaea* L.) in Astaneh Ashrafiyeh, Iran. American-Eurasian Journal of Agricultural and Environmental Sciences 9: 256-262

Gonthier P, Gennaro M and Nicolotti G (2006): Effect of water stress on endophytic mycota of *Quercus robur*. Fungal Diversity 21: 69-80.

Graham PH and Vance CP (2003): Legumes importance and constraints to greater use. Plant Physiology 131: 872-877.

Grusak MA (2002): Enhancing mineral content in plant food products. Journal of the American College of Nutrition 21: 178-183.

Hallmann J, Berg G and Schulz B (2007): Isolation procedures for endophytic microorganisms. New York: Springer Brelin Heidelberg, pp. 299-319.

Hamilton D (2005): Broad bean. Available from http://www.Selfsufficientdsh.com.

Hawksworth DFL, Kirk PM, Sutton BC and Pegler DN (1995): Dictionary of the Fungi. CAB International, Surrey, England, 616 pp.

Hirsch GU and Braun U (1992): Communities of parasitic microfungi. In: Handbook of

Vegetation Science, Winterhoff W (ed.), Kluwer, Dordrecht, Volume 19: 225-250.

Hoff JA, Klopfenstein NB, Tohn JR, McDonald GI, Zambina PJ, Rogers JD, Peever TL and Carris LM (2004): Roles of woody root-associated fungi in forest ecosystem processes: Recent advances in fungal identification. USDA Forest Service RMRS-RP.47.

Iqbal MM, Nazir F, Iqbal J, Tehrim S and Zafar Y (2011): In-*vitro* micropropagation of peanut (*Arachis hypogaea*) through direct somatic embryogenesis and callus culture. International Journal of gricultural and Biological Engineering 13: 811-814.

Jensen ES and Hauggaard-Nielsen H (2003): How can increased use of biological N2 fixation in agriculture benefit the environment. Plant and Soil 252: 41-54.

Jiang DF, Ma P, Wang XH, Zhang LQ, Li QD, Wang JL, Cheng ZY and Yang CR (1995): The studies of fungal population and relationship between fungi and forming of dragon's blood resin in Dracaena cochinchinensis. Acta Botanica Ynannanica 17(1): 79-82.

Jumpponen A and Trappe JM (1998): Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. New Phytologist 140: 295-310.

Jumpponen A, Mattson KG and Trappe JM (1998): Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter. Mycorrhiza 7: 261-265.

Kamel S and Shoukry A (2001): Alfalfa and Clover Pollinators in Egypt. Agricultural Engineering Research Institute, Egypt. 83: 547-550.

Krings M, Taylor TN, Hass H, Kerp H, Dotzler N and Hermsen EJ (2007): Fungal endophytes in a 400-million-yr-old land plants: infection pathways, spatial distribution, and host response. New Phytologist 174: 648-657.

Lacap DC, Hyde KD and Liew ECY (2003): An evaluation of the fungal "morphotype" concepts based on ribosomal DNA sequence. Fungal Diversity 12: 53-66.

Larran S, Rollan C, Angeles HB, Alippi HE and Urrutia MI (2002): Endophytic fungi in healthy soyabean leaves. Investigation Agraria, Production Y Protection Vegetables 17(1): 173-178.

Lee JC, Yang X, Schwartz M, Strobel G and Clardy J (1995): The relationship between an endangered North American tree and an endophytic fungus. Chemistry and Biology 2: 721-727.

Leslie JF and Summerell BA (2006): The *Fusarium* Laboratory Manual. Blackwell Publishing, Ames, Iowa, USA, 388 pp.

Malinowski DP and Belesky DP (2006): Ecological importance of *Neotyphodium* sp. Grass endophytes in agroecosystems. Grassland Science 52: 23-28.

Melin E (1922): On the mycorrhizas of *Pinus sylvestris* L. and *Picea abies* Karst: A preliminary note. Journal of Ecology 9: 254-257.

Melin E (1923): Experimentelle untersuchungen über die konstitution und ökologie den mykorrhizen von Pinus sylvestris und Picea abies. Mykologische Untersuchungen und Berichte Von R Falck 2: 330-334.

Mohammed MM (2010): Studies on endophytic microorganisms of some medicinal plants. M. Sc. Thesis, Faculty of Science, Sohag University, Sohag, Egypt.

Moubasher AH (1993): Soil Fungi in Qatar and other Arab Countries. Center for Scientific and Applied Research, University of Qatar, Qatar, pp. 566.

Nath A, Raghunatha P and Joshi SR (2012): Diversity and biological activities of endophytic fungi of *Emblica officinalis*, an ethnomedicinal plant of India. Mycobiology 40: 8-13.

Nelson PE, Toussoun TA and Marasas WFO (1983): *Fusarium* species, an illustrated manual for identification. The Pennsylvania State University press, USA, 193 pp.

Petrini O (1986): Taxonomy of endophytic fungi of aerial plant tissue. In: Microbiology of the phyllosphere. NJ Fokkema, J Van den Henvel (eds.), Cambridge University press, Cambridge, Uk, pp. 175-187.

Pitt JI (1979): The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces*. Commonwealth Scientific and Industrial Research Organization, Division of food Research, North Ryde, N. S. W. Australia, Academic Press, INC. Ltd., London, 634 pp.

Raper KB and Fennell DJ (1965): The Genus *Aspergillus*. Williams and Wilkins, Baltimore, USA, 686 pp.

Redlin SC and Carris LM (1996): Endophytic Fungi in Grasses and Woody Plants; Systematics, Ecology and Evolution. Amereican Phytopathological Society press, pp. 216-220.

Rubini MR, Silva-Ribeiro RT, Pomella AWV, Maki CS, Araújo WL, Santos DR and Azevedo JL (2005): Diversity of endophytic fungal community of cacao (*Theobroma cacao* L.) and biological control of *Crinipellis perniciosa*, causal agent of Witches Broom Disease. International Journal of Biological Science 1: 24-33.

Samuel S, Ahmed S, Fininsa C, Abang MM and Sakhuja PK (2008): Survey of chocolate spot (*Botrytis fabae*) disease of faba bean (*Vicia faba* L.) and assessment of factors influencing

disease epidemics in northern Ethiopia. Crop Protection 27: 1457-1463.

Sasaki M, Tsuda M, Sekiguchi M, Mikami Y and Kobayashi J (2005): Perinadine A, a novel tetracyclic alkaloid from marine-derived fungus *Penicillium citrinum*. Organic Letters 7: 4261-4264.

Shebany YM (2008): Endophytic fungi of three medicinal plants and effect of plant extracts against pathogenic bacteria and fungi. Ph. D. Thesis, Faculty of Science, South Valley University, Qena, Egypt.

Shiomi HF, Silva HAS, De Melo IS, Nunes FV and Bettiol W (2006): Bioprospecting endophytic bacteria for biological control of coffee leaf rust. Scientia Agricola 63: 32-39.

Shivanna MB, Meera MS and Hyakumachi M (1994): Sterile fungi from zoysia grass rhizosphere as plant growth promoters in spring wheat. Canadian Journal of Microbiology 40: 637-644.

Siegal MR, Latch GCM and Johnson MC (1987): Fungal endophytes of grasses. Annual Review of Phytopathology 25: 293-315.

Sinclair JB and Backman PA (1989): Compendium of Soybean Diseases. 3rd ed., American Phytopathological Society, Saint Paul, MN, USA, 106 pp.

Stajković-Srbinović O, De Meyer SE, Miličić B, Delić D and Willems A (2012): Genetic diversity of rhizobia associated with alfalfa in Serbian soils. Biology and Fertility of Soils 48: 531-545.

Strobel GA and Daisy B (2003): Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Biology Reviews 67: 491-502.

Strobel GA, Torczynski R and Bollon A (1997): *Acremonium* sp. aleucinostatin: A producing endophyte of European yew (*Taxus baccata*). Plant Science 128: 97-108.

Suryanarayanan TS, Senthilarasu G and Muruganandam V (2000): Endophytic fungi from *Cuscuta reflexa* and its host plants. Fungal Diversity 4: 117-123.

Tian XL, Cao LX, Tan HM, Zeng QG, Jia YY, Han WQ and Zhou SN (2004): Study on the communities of endophytic fungi and endophytic actinomycetes from rice and their antipathogenic activities in vitro. World Journal of Microbiology and Biotechnology 20: 303-309.

Wang B, Priest MJ, Davidson A, Brubaker CL, Woods MJ and Burdon JJ (2007): Fungal endophytes of native *Gossypium* species in Australia. Mycological Research 3: 347-354.

Weber RWS, Kappe R, Paululat T, Mosker E and Anke H (2007): Anti-*Candida* metabolites from endophytic fungi. Phytochemistry 68: 886-892.

Journal of Basic & Applied Mycology (Egypt) 4 (2013): 69-76

Incidence of mycobiota and toxigenic fusaria in corn grain samples from Sohag, Egypt

M. B. Aboul-Nasr*, Sabah S. Mohamed and Marwa R. A. Obied-Allah

Department of Botany, Faculty of Science, University of Sohag, Sohag, Egypt

***Corresponding author: e-mail: mbaboulnasr@yahoo.com**
Received 27/11/2013, Accepted 25/12/2013

Abstract: A total of 41 species and one variety related to 11 genera of filamentous fungi were isolated from 80 corn grain samples. The total fungal units emerged from non-surface disinfected (NSD) corn grains were much higher than those emerged from surface disinfected (SD) corn grains. However, the number of isolated species in case of SD corn grains exceeded those in case of NSD corn grains. *Aspergillus* and *Penicillium* were the most predominant genera. *Fusarium* was recovered as the third most common genus of which *F. verticillioide* was the most prevalent species. Thin layer chromatography (TLC) showed that of all corn samples tested only one was contaminated by T-2 toxin while the other 79 samples were completely free of any detectable levels of mycotoxins. Methanol extracts of *Fusarium* isolates grown on corn medium showed that 59 out of the 90 *Fusarium* extracts contained at least one of the following *Fusarium* toxins: fumonisins B_1 and B_1 & B_2, zearalenone and deoxynivalenol. Most of *F. verticillioides* isolates (44 out of 76) were fumonisins producers. The HPLC analysis of crude extracts confirmed the results achieved by TLC plates. The highest yield of fumonisin B_1 (2015 µg/kg corn medium) was obtained from an isolate of *Fusarium verticillioides*.

Key words: Corn, *Fusarium verticillioides*, fumonisins B_1 & B_2, zearalenone, deoxynivalenol.

Introduction

Corn (*Zea mays* L.) is the most widely grown cereal crop and one of the four basic food staples consumed as food or feed in the world as well as in Egypt (Anderson *et al.* 2004 and Naqvi *et al.* 2011). It is also at the forefront of a new green revolution in which plants have a number of high value industrial applications to produce valuable molecules as recombinant pharmaceuticals, industrial enzymes, nutrients, chemical precursors and fuels (Naqvi *et al*. 2011). A variety of toxigenic fungi, including *Aspergillus*, *Penicillium* and *Fusarium* are frequently reported as the most dominant fungal genera isolated from corn (Chulze *et al*. 1996, 1999). *Fusarium* could colonize or infect corn grains during pre- and post-harvest periods (Abdel-Mallek *et al*. 1993, Chulze *et al*. 1996, 1999 and Fandohan *et al*. 2005). *Fusarium verticillioides* (Sacc.) Nirenberg and *F. proliferatum* (Matsushima) Nirenberg were recorded as the highest prolific fumonisin producers (Fandohan *et al*. 2003). Fumonisins B_1, B_2 and B_3 were found in naturally-contaminated foods (Soriano *et al.* 2005). These compounds are structurally similar to sphingoid bases such as sphingosine, which is a component of the sphingolipid molecule, and are able to inhibit ceramide synthase. The free amino group appears to play a specific role in the biological activity of FB_1 (Bolger *et al*. 2001 and Soriano *et al*. 2005). The occurrence of fumomisins in corn had been assocaited with the outbreaks of equine leucoencephalomalacia (ELM), pulmonary edema (PE) and hepatocarcinoma (Harrison *et al*. 1990, Ross *et al*. 1990 and Thiel *et al*. 1992). Due to the little information on the natural occurrence of fumonisins in corn and fumonisin-producing *Fusarium* species in Egypt, this study was conducted.

Materials and Methods

Collection of corn samples

A total of 80 corn grain samples (one Kg each) were collected from different regions of Sohag Governorate. None of the samples was visibly damaged or moldy infected. Samples were transferred into the laboratory, kept in a sterilized polyethylene bag and stored in a refrigerator at 4°C till mycological and mycotoxin analysis.

Isolation and identification of fungi

Two conventional methods were used to isolate fungal species colonizing corn grain samples; direct-plate non-surface disinfected (NSD) method (Pitt *et al.* 1992) and surface disinfected (SD) method (Müllenborn *et al.* 2008). Dichloran-rose-Bengal-chloromphenicol (DRBC) agar medium was used for culturing (Tournas *et al*. 2006). Purified fungal isolates were identified morphologically based on macro- and microscopic characteristics using relevant references (Booth 1977, Nelson *et al.* 1983, Moubasher 1993, Leslie and Summerell 2006 and Moustafa 2006). All *Fusarium* isolates were cultured on potato sucrose agar medium (PSA) after single spore isolation (Booth 1977)

and were re-identified at Assuit University Mycological Center (AUMC).

Mycotoxin extraction of corn samples

Fifty grams of corn grains were ground and transferred into a 250 ml Erlenmeyer flask. One hundred ml of 96% methanol was added and the contents were shaken on a rotary shaker (200 rpm for 24 h) and filtered through filter paper (Whatman No.1). The residues were washed twice with the solvent (50 ml each) and the methanol extracts were combined, dried over anhydrous sodium sulphate, and evaporated to near dryness under vacuum. The residues were transferred quantitatively to a small vial with low amount of methanol and evaporated to near dryness (Sydenham *et al*. 1993).

Mycotoxins production by *Fusarium* spp.

A total of 70 different isolates of *Fusarium* obtained by NSD and other 20 isolates of the same genus obtained by SD methods were examined for the production of *Fusarium* toxins. Each isolate was cultivated on corn substrate medium. One hundred grams of corn were moistened overnight with 50 ml distilled water. Excess water was decanted and flasks containing corn were autoclaved at 121°C for 1 hr for 2 consecutive days. Three replicates of autoclaved corn cultures were inoculated with 5 ml of aqueous conidial suspensions (10^7 spores) of each isolate tested. Inoculated flasks were incubated at 25°C for two weeks in darkness. Flasks were shaken once every day to prevent corn adhering and to distribute the inoculum. At the end of the incubation period all flasks were transferred into a refrigerator for 2 more weeks. Control sample was not inoculated by conidial suspensions. Fifty grams of corn cultures of each isolate including the control were transferred from flasks and dried at room temperature, ground using an analytical electric mill and transferred into three 125-ml Erlenmeyer flasks containing 50 ml 96% methanol. Flasks were shaken on a rotatory shaker and filtered through filter paper (Whatman No.1). The residues were washed twice with the same solvent (25 ml each). The methanol extracts were combined, dried over anhydrous sodium sulphate and evaporated to near dryness under vacuum. The residues were transferred quantitatively to a small vial with 96% methanol and evaporated to near dryness.

Thin layer chromatographic analysis

For qualitative determination of mycotoxins other than fumonisins, TLC technique adopted by El-Kady and Moubasher (1982) was employed. For fumonisins detection, 10 µl crude extracts were spotted on TLC plate (Aluminium sheet, silica gel, Merck) along with 5 µg of FB_1 standard (purchased from SIGMA-ALDRICH). Spots were dried during application with a flow of warm air and developed using 96% methanol: water (80: 20, v/v). The developed plates were viewed after spraying with 50% concentrated sulphuric acid in methanol under short wave (254 nm) and long wave (365 nm) ultra-violet irradiation. Fumonisins B_1 and B_2 appear as slight bluish green spots under both short and long UV (Mubatanhema *et al*. 1999) at Rf 0.7 and 0.61 respectively.

HPLC analysis

Twenty-four crude extracts of *F. oxysporum* (1), *F. proliferatum* (6), *F. sterilihyphosum* (1), *F. subglutinans* (6) and *F. verticillioides* (10) were tested. Fumonisins were analyzed using Waters Binary Model 1525 HPLC equipped with Waters 2475 multi-wavelength flourescence detector and data workstation software Breeze 2. A phenomenex C18 (250 x 4.6 mm i.d), five um from Waters Corporation (USA), were used along with a mobile phase of Methanol: 0.1M NaH_2PO_4 (75:25, v/v) isocratic system adjusted to pH 3.35 by the addition of phosphoric acid and was filtered through membrane filter. The separation was performed at ambient temperature at a flow rate of 1.0 ml/min. The injection volume was 20 µl for both standard solutions and sample extracts. The fluorescence detector was operated at an excision of 335 nm and emission of 440 nm wavelength at the National Research Centre (NRC) in Cairo**.** The purified dry film residue of sample extracts were dissolved in 200 µl methanol of which 50 µl were transferred to a vial and 225 µl O-phithaladehyde reagent (OPA) were added. The mixed solutions of standard as well as sample extracts after derivatization were filtered through a 0.22 nm membrane filter and 10 µl were injected to HPLC apparatus within 1 min of adding OPA reagent. Fumonisin B_1 concentration was calculated from chromatographic peak areas (Horwitz and Latimer 2007).

Results and Discussion

A total of 41 species and only one species variety belonging to 11 fungal genera were isolated from the 80 corn samples collected from Sohag Governorate (Table 1). Although the total fungal units emerged from non-surface disinfected (NSD) corn grains (3224 CFU/ 2000 corn grains) was much higher than those emerged on surface disinfected (SD) corn grains (906 CFU), the number of isolated species in case of SD corn grains (37 species belong to 10 genera) exceeded those isolated in case of NSD

corn grains (33 species belong to 9 genera). Taxa isolated were assigned to three taxonomic classes. Hyphomycetes were represented by 37 species and one species variety representing 8 genera. Zygomycetes were represented by 3 species of 2 genera and Ascomycetes were represented by 1 species of *Neosartorya*. A view on the diversity at the generic level revealed that *Aspergillus* showed the greatest spectrum of species (19 species and one species variety) followed by *Penicillium* (9 species), *Fusarium* (5 species) and *Rhizopus* (2 species). The remaining 6 genera were represented only by one species for each. *Aspergillus* was the most common genus in both NSD and SD corn grains (97.5% and 90% of the samples, respectively). Of the genus 14 and 17 species and one variety (*A. flavus* var. *columnaris*) were isolated and identified from NSD and SD corn samples respectively. *Penicillium* was the second dominant on NSD and SD corn samples recovered from 77.5% and 75% of the samples accounting 15.5% and 23% of total fungi respectively. *Fusarium* occupied the third position (67.5% and 28.8% of the samples, comprising 8.7% and 6% of the total fungi respectively). It was represented by five species (*F. oxysporum, F. proliferatum, F. sterilihyphosum, F. subglutinans* and *F. verticillioides*) of which *F. verticillioides* was the most dominant. Adebajo *et al*. (1994), Kpodo *et al*. (2000), Schneweis *et al*. (2000) and Oudeelferink *et al*. (2001) recorded *Aspergillus* and *Penicillium* followed by *Mucor* and *Fusarium* spp. as the most dominant fungi isolated from corn and other seed and grain samples. Most of these species, except *F. sterilihyphosum* which was isolated for the first time in our mycology laboratory of Sohag, were previously isolated from various types of seeds and grains in Egypt (Abdel-Hafez *et al*. 1992, Abdel-Mallek *et al*. 1993 and Abdel-Sater *et al*. 1995). Kpodo *et al*. (2000) recorded *F. verticillioides* as the predominant *Fusarium* species in 15 corn samples from Ghana. In the present study, *F. proliferatum* and *F. subglutinans* were recorded in 6.25% & 2.5% and 5% and 7.5% of the NSD and SD grains respectively. Only one isolate of *F. oxysporum* was obtained from SD corn grains with a frequency of 1.25% of the samples. Fandohan *et al*. (2005) reported that *F. verticillioides* and *F. proliferatum* were the two *Fusarium* species commonly isolated from the corn samples during the 3-year survey in Benin. As shown in Table (1), *Rhizopus, Cladosporium, Mucor, Acremonium, Alternaria* and *Trichothecium* appeared with moderate and low frequencies ranging between 13.75% and 1.25% of the samples. The previous findings can be correlated with those obtained by Abdel-Mallek *et al*. (1993), Adebajo *et al*. (1994), El-Maghraby *et al*. (1994), Abdel-Sater *et al*. (1995) and El-Shanawany *et al*. (2005).

Natural occurrence of mycotoxins was determined in the 80 corn samples. Only one sample was found to be contaminated with T-2 toxin while the remaining 79 samples were free of any detectable amounts of this mycotoxin. T-2 toxin was widely produced by Egyptian *Fusarium* isolates (El-Maghraby 1984, El-Maghraby *et al*. 1992a & b, El-Maghraby 1996 and Soliman 1999).

Table 1: Colony-forming units (CFU/ 2000 corn grins), percentage fungal density (%D), and frequency (%F) of fungal genera and species recovered from 80 corn grain samples by non-surface disinfected (NSD) and surface-disinfected (SD) grain plate techniques.

Fungal genera & species	NSD		SD	
	% D	% F	% D	% F
Acremonium strictum W. Gams	0.155	3.75	0.22	1.25
Alternaria alternate (Fries) Keissler	0.496	8.75	0.22	2.5
Aspergillus	71.03	97.5	66.89	90
A. aculeatus Lizuka	9.71	70	0.44	2.5
A. awamorii Nakazawa	0.093	1.25	2.3	8.75
A. carbonarius (Bainier) Thom	1.58	15	0.99	6.25
A. clavatus Desmazieres	-	-	0.33	3.75
A. ficuum (Reich.) Hennings	-	-	4.64	21.25
A. flavus Link	12.34	72.5	9.8	53.75
A. flavus var. *columnaris* Raper & Fennell	7.54	62.5	3.42	27.5
A. fumigatus Fresenius	2.11	28.75	21.3	53.75
A. japonicus Saito	1.396	13.75	-	-
A. melleus Yukawa	0.96	5	-	-
A. niger van Tieghem	24.26	95	11.59	53.75
A. ochraceus Wilhelm	-	-	1.43	11.25

Table 1: Continued.

Fungal genera & species	NSD		SD	
	% D	% F	% D	% F
A. oryzae (Ahlburg) Cohn	1.78	15	1.99	7.5
A. parasiticus Speare	7.196	62.5	3.64	21.25
A. sulphureus (Fresenius) Thom & Church	-	-	0.55	2.5
A. sydowii (Bainier & Sartory) Thom & Church	0.22	3.75	1.88	3.75
A. tamarii Kita	1.77	23.75	1.2	7.5
A. terreus Thom	0.093	2.5	0.11	1.25
A. versicolor (Vuillemin) Tiraboschi	-	-	0.44	3.75
Cladosporium cladosporioides (Fresenius) de Vries	0.62	13.75	1.55	12.5
Exserohilum rostratum (Drechsler) Leonard & Suggs	-	-	0.11	1.25
Fusarium	8.68	67.5	6.1	28.75
F. oxysporum Schlechtendal	-	-	0.11	1.25
F. proliferatum (Matsush.) Nirenberg	0.47	6.25	0.66	2.5
F. sterilihyphosum Britz, Marasas & Wingfield	0.06	1.25	-	-
F. subglutinans (Wollenweber & Reinking) Nelson *et al.*	0.53	5	1.2	7.5
F. verticillioides (Sacc.) Nirenberg	7.63	60	4.08	21.25
Mucor racemosus Fresenius	0.155	3.75	0.33	3.75
Neosartorya fisherii (Wehmer) Malloch & Cain	-	-	0.66	5
Penicillium	15.48	77.5	22.96	75
P. brevicompactum Dierckx	1.74	18.75	3.64	20
P. chrysogenum Thom	1.33	15	1.2	6.25
P. citrinum Thom	1.09	16.25	0.11	1.25
P. corylophilum Dierckx	3.38	36.25	1.43	10
P. duclauxii Delacroix	3.94	33.75	9.7	45
P. funiculosum Thom	0.465	5	-	-
P. implicatum Biourge	1.12	12.5	1.77	11.25
P. jensenii Zaleski	0.84	10	0.99	6.25
P. pupurogenum Stoll	1.89	17.5	3.2	22.5
Rhizopus	2.7	27.5	1.66	7.5
Rhizopus oryzae Went & Prinsen-Geerligs	1.43	15	1.32	7.5
Rhizopus stolonifer (Ehrenberg) Vuillemin	1.30	13.75	0.33	2.5
Trichothecium roseum (Persoon: Fries) Link	0.34	6.25	-	-
Total CFUs	3224	-	906	-

TLC of the crude extracts of *Fusarium* isolates grown on corn medium showed that 59 out of 76 isolates of *F. verticillioides* had the ability to produce at least one of the following toxins: fumonisins (B_1 and B_2), zearalenone and deoxynivalenol. All tested isolates of *F. proliferatum*, *F. sterilihyphosum* and *F. oxysporum* were non-fumonisin producers and only one out of six isolates of *F. subglutinans* in this study had the ability to produce zearalenone (Table 2). Results obtained in this study were consistent with those reported earlier by other researches who found that many strains of *F. verticillioides* isolated from corn produced fumonisins (Ross *et al*. 1992, Soo *et al*. 1994 and Fadl Allah 1998). Other researchers reported that *F. proliferatum* produced FB_1 (Sanchis *et al*. 1995 and Castella *et al*. 1999). Also, Abbas *et al*. (1999) reported that 15 isolates of *F. proliferatum* isolated from 20 rough rice samples produced fumonisins, moniliformin and beauvericin.

Table 2: Mycotoxins detected by TLC analysis of different *Fusarium* isolates.

Fusarium species	No. of isolates tested		No. of mycotoxigenic isolates			
	NSD	SD	F(B_1)	F (B_1& B_2)	DON	ZEA
F. oxysporum	-	1	-	-	-	-
F. proliferatum	4	2	-	-	-	-
F. sterilihyphosum	1	-	-	-	-	-
F. subglutinans	1	5	-	-	-	1*
F. verticillioides	64*	12**	13*&3**	22*&7**	13*&5**	12*&1**

**Fusarium* isolates collected from non-surface-disinfected corn samples.
*** Fusarium* isolates collected from surface-disinfected corn samples.

Figures (1 & 2) showed the HPLC Chromatograms of fumonosin B1 standard and the crude extracts of fumonosin-producing and non-producing isolates. The HPLC analysis of 24 crude extracts of *F. oxysporum* (one isolate), *F. proliferatum* (6), *F. sterilihyphosum* (1), *F. subglutinans* (6) and *F. verticillioides* (10) proved that none of the selected *Fusarium* isolate extracts but *F. verticillioides* produced FB_1 ranged between 128 to 2015 µg//kg corn medium (Figs. 1 & 2). Many researches in Asian and European countries reported that strains of *F. verticillioides* produced FB_1, FB_2, and FB_3 (Lee *et al*. 1994, Visconti and Doko 1994 and Ghiasian *et al*. 2005). Also, a Taiwanese study reported that 66% of isolated *F. verticillioides* strains produced FB_1 and/or FB_2 (Tseng *et al*. 1995). Rheeder *et al.* (2002) reported that *F. verticillioides* and *F. proliferatum* had been recognized as the highest fumonisin producers although a few *F. verticillioides* isolates from Nepal (Nelson *et al*. 1991) do not produce any fumonisins. Several isolates of this species from Southeast Asia (Miller *et al*. 1993) produced fumonisins at low levels, whereas some isolates from Australia (Nelson *et al*. 1991) produced only trace quantities of FB_1.

In conclusion: although no detectable amounts of fumonisins were found in the corn grain samples tested, *F. verticillioides* was isolated as one of the most prevalent fungal species that contaminated these collected samples and proved to produce fumonisins for the first time in our laboratory and thus further investigation is needed.

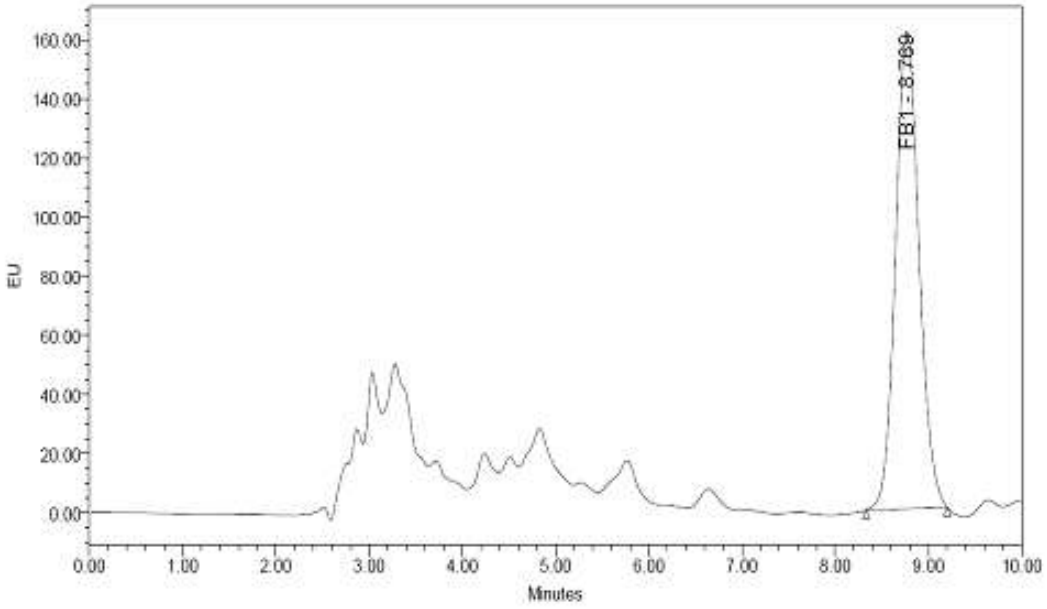
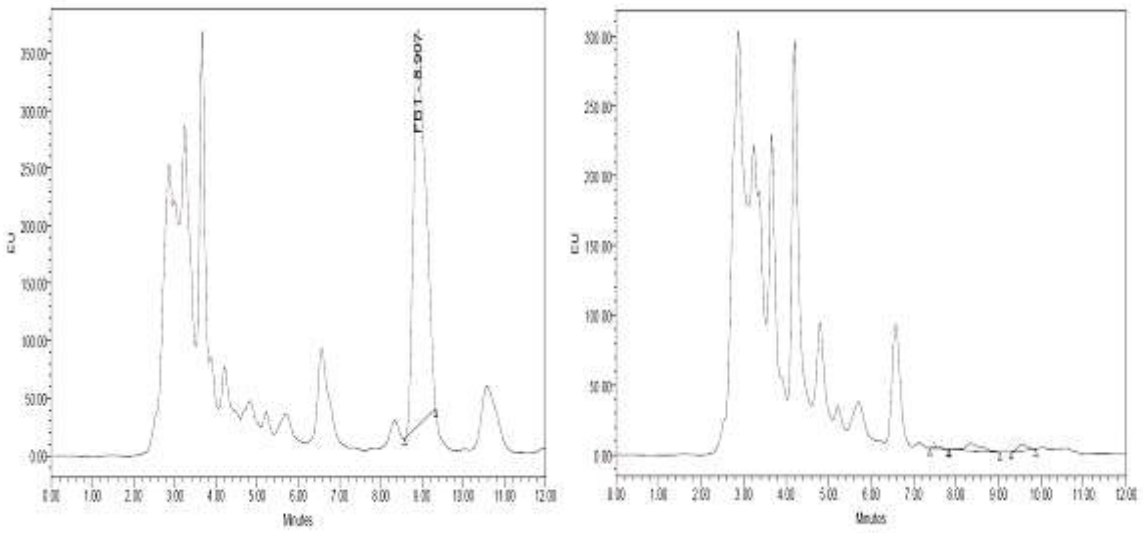


Figure 1: Chromatograms of FB_1 standard solution derivatized with OPA reagent



(a) (b)

Figure 2: Chromatograms of crude extracts of fumonisin-producing (a) and non-producing (b) isolates derivatized with OPA reagent.

References

Abbas HK, Cartwright RD, Xie W, Mirocha CJ, Richard JL, Dvorak TJ, Sciumbato GL and Shier WT (1999): Mycotoxin production by *Fusarium proliferatum* isolates from rice with *Fusarium* sheath rot disease. Mycopathologia 147: 97-104.

Abdel-Hafez SII, El-Kady I, Mazen M and El-Maghraby O (1992): Effect of temperature and moisture content on germination capacity and paddy grain-borne fungi from Egypt. Abhath Al-Yarmouk; Pure Science and Engineering 1: 91-105.

Abdel-Mallek AY, El-Maraghy SSM and Hasan HAH (1993): Mycotoxin-produing potential of some *Aspergillus*, *Penicillium* and *Fusarium* isolates found on corn grains and sun-flower seeds in Egypt. Islamic Academy of Science 6(3): 189-192.

Abdel-Sater MA, Hemida SK, Eraky SA and Nasser MM (1995): Distribution of fungi on two mite species and their habitats in Egypt. Folia Microbiologica 40(3): 304-313.

Adebajo LO, Idown AA and Adensy OO (1994): Mycoflora and mycotoxins production in Nigeria corn and corn based snacks. Mycopathologia 126(3): 183-192.

Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR and Daszak P (2004): Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends in Ecology and Evolution 19:535-544.

Bolger M, Coker RD, DiNovi M, Gaylor D, Gelderblom W and Olsen M (2001): Safety evaluation of certain mycotoxins in food, Edited by the Fifty-sixth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), FAO, Rome, pp. 103-280.

Booth C (1971): The genus *Fusarium*. Commonwealth Mycological Institute, Key, Surrey, England.

Booth C (1977): *Fusarium*: Laboratory Guide to the Identification of Major Species. Commonwealth Mycological Institute, Kew, Surrey, England.

Castella G, Bragulat MR, Cabanes FJ (1999): Fumonisin production by *Fusarium* species isolated from cereals and feeds in Spain. Food Protection 62: 811-813.

Chulze S, Ramírez ML, Farnochi MC, Pascale M, Visconti A and March G (1996): *Fusarium* and fumonisin occurrence in Argentinian corn at different ear maturity stages. Journal of Agricultural and Food Chemistry 44: 2797-2801.

Chulze S, Etcheverry MG, Lecumberry SE, Magnoli C, Dalcero A, Ramírez ML, Pascale M and Rodríguez MI (1999): Fumonisin production on irradiated corn kernels: effect of inoculum size. Food Protection 62: 814-817.

El-Kady IA and Moubasher MH (1982): Toxigenicity and toxins of *Stachybotrys chartarum* isolates from wheat straw samples in Egypt. Experimental Mycology 6: 25-31.

El-Maghraby OMO (1984): Mycoflora and trichothecene toxins of paddy (*Oryza sativa*

L.) grains in Egypt. Ph. D. Thesis, Botany Deptartment, Faculty of Science, Assuit University, Egypt.

El-Maghraby OMO (1994): Transformation of trichoverrols A and B to macrocyclic trichothecenes by *Stachybotrys* spp. Bulletin of the Faculty of Sceince, Assiut University 23: 71-84.

El-Maghraby OMO (1996): Mycotoxins and mycoflora of rice in Egypt with special reference to trichothecenes production and control. Natural Toxins 5(1): 49-59.

El-Maghraby OMO, El-Sawi NM and Mohamed MM (1992a): T-2 toxin control and toxigenicity on adrenal gland. 4th National Conference on Biochemistry, Cairo, Egypt, pp. 149-163

El-Maghraby OMO, Saber SM and Aboul-Nasr MB (1992b): Contamination of pea (*Pisum sativum* L.) seeds by fungi, mycotoxins and resistance of cultivars to mycotoxins accumulation. Sohag Pure and applied Science Bulletin, Faculty of Science, Egypt 8: 137-155.

El-Shanawany AA, Mostafa ME and Barakat A (2005): Fungal populations and mycotoxins in silage in Assuit and Sohag Governorates in Egypt, with a special reference to characteristic aspergilli toxins. Mycopathologia 159: 281-289.

Fadl Allah EM (1998): Occurrence and toxigenicity of *Fusarium moniliforme* from freshly-harvested maize ears with special refrences to fumonisin production in Egypt. Mycopathologia 140: 99-103.

Fandohan P, Gnonlonfin B, Hell K, Marasas WFO and Wingfield MJ (2005): Natural occurrence of *Fusarium* and subsequent fumonisin contamination in preharvest and stored maize in Benin, West Africa. Food Microbiology 99:173-183.

Ghiasian SA, Rezayat SM, Kord-Bacheh P, Maghsood AH, Yazdanpanah H, Shephard GS, van der Westhuizen I, Vismer HF and Marasas WFO (2005): Fumonisin production by *Fusarium* species isolated from freshly harvested corn in Iran. Mycopathologia 159: 31-40.

Harrison LR, Colvin BM, Green JT, Newman LE and Cole JRJr (1990): Pulmonary edema and hyfrotorax in swine produced by fumonisin B_1, a toxic metabolite of *Fusarium moniliforme*. Journal of Veterinary Diagnostic Investigation 2: 217-221.

Horwitz W and George LGJr (2007): Official methods of analysis of the Association of Analytical Chemists AOAC. 18th ed., AOAC Int., Gaithersburg, MD, USA.

Kpodo K, Thrane U and Hald B (2000): Fusaria and fumonisins in maize from Ghana and their co-occurrence with aflatoxins. Food Microbiology 61: 147-157.

Lee U-S, Lee M-Y, Shin K-S, Min Y-S, Cho C-M, Ueno Y (1994): Production of fumonisin B_1 and B_2 by *Fusarium moniliforme* isolated from Korean corn kernels for feed. Mycotoxin Research 10: 67-72.

Leslie JF and Summerell BA (2006): The *Fusarium* Laboratory Manual. Blackwell Publishing Ames, Iowa, USA, 388 pp.

Miller JD, Savard ME, Sibilia A, Rapior S, Hocking AD, and Pitt JJ (1993): Production of fumonisins and fusarins by *Fusarium moniliforme* from Southeast Asia. Mycologia 85: 385-391.

Moubasher AH (1993): Soil Fungi in Qatar and Other Arab Countries. Scientific and Applied Research Center, University of Qatar, 566 pp.

Moustafa AF (2006): Zygomycetes of Egypt. In: Fungi of Egypt, AUMC Descriptions No. 1.

Mubatanhema W, Moss MO, Frank MJ and Wilson DM (1999): Prevalence of *Fusarium* species of the *Liseola* section on Zimbabwean corn and their ability to produce the mycotoxins zearalenone, moniliformin and fumonisin B_1. Mycopathologia 148: 157-163.

Müllenborn C, Steiner U, Ludwig M, and Oerke EC (2008): Effect of fungicides on the complex of *Fusarium* species and saprophytic fungi colonizing wheat kernels. European Journal of Plant Pathology 120: 157–166.

Naqvi S, Ramessar K, Farré G, Sabalza M, Miralpeix B, Twyman RM, Capell T, Zhu C and Christou P (2011): High-value products from transgenic maize. Biotechnology Advances 29: 40-53.

Nelson PE, Plattner RD, Shackelford DD and Desjardins AE (1991): Production of fumonisins by *Fusarium moniliforme* strains from various substrates and geographic areas. Applied and Environmental Microbiology 57: 2410-2412.

Nelson PE, Toussoum TA and Marasas WFO (1983): *Fusarium* species an illustrated manual for identification. The Pennsylvania State University Press, London.

Oude Elferink SJWH, Driehuis F, Gottsschal JC and Spoelstra SF (2001): Silage fermentation processes and their manipulation. FAO Corporate Document

Repository, produced by Agriculture and Consumer Protection Department.

Pitt JI, Hocking AD, Samson RA and King AD (1992): Recommended methods for mycological examination of foods. In: Samson RA, Hocking AD, Pitt JI and King AD (eds.), Modern Methods in Food Mycology. Elsevier, Amsterdam, pp. 365-368.

Rheeder JP, Marasas WFO, Vismer HF (2002): Production of fumonisin analogs by *Fusarium* species. Applied and Environmental Microbiology 68: 2101-2105.

Ross PF, Nelson PE, Richard JL, Osweiler GD, Rice LG, Plattner RD and Wilson TM (1990). Production of fumonisins by *Fusarium moniliforme* and *Fusarium proliferatum* isolates associated with equine leukoencephalomalacia and pulmonary edema syndrome in swine. Applied and Environmental Microbiology 56:3225-3226.

Ross PF, Rice LG, Osweiler GD, Nelson PE, Richard JL and Wilson TM (1992): A review and update of animal toxicoses associated with fumonisin-contaminated feeds and production of fumonisins by *Fusarium* isolates. Mycopathologia 117: 109-111.

Sanchis V, Abadias M, Oncins L, Sala N, Vinas I and Canela R (1995): Fumonisin B_1 and B_2 and toxigenic *Fusarium* strains in feeds from the Spanish market. Food Microbiology 27: 37-44.

Schneweis I, Meyer KM, Rmansdorfer S and Bauer J (2000): Mycophenolic acid in silage. Applied and Environmental Microbiology 66(8): 3639-3641.

Soliman S (1999): Mycoflora and mycotoxin of sorghum grains in Egypt. Ph. D. Thesis, faculty of Science at Sohag, South Valley University.

Soo LU, Yur LM, Sop SK, Sik MY, Min CC and Ueno Y (1994): Production of fumonisin B_1 and B_2 by *Fusarium moniliforme* isolated from Korean corn kernels for feed. Mycotoxin Research 10: 67-72.

Soriano JM, Gonzálea L and Catalá AI (2005): Mechanism of action of sphingolipids and their metabolites in the toxicity of fumonisin B1. Progress in Lipid Research 44: 345-356.

Sydenham EW, Shephard GS, Thiel PG, Marasas WFO, Rheeder JP, Peralta Sanhueza CE, Gonzalez HHL and Resnik SL (1993): Fumonisins in Argentinian field trial corn. Journal of Agricultural and Food Chemistry 41: 891–895.

Thiel PG, Marasas WFO, Sydenham EW, Shepard GS and Gelderblom WCA (1992): The implications of naturally-occurring levels of fumonisins in corn for human and animal health. Mycopathologia 117: 3-9.

Tournas VH, Heeres J and Burgess L (2006): Moulds and yeasts in fruit salads and fruit juices. Food Microbiology 23(7): 684-688.

Tseng TC, Lee KL, Deng TS, Liu CY and Huang JW (1995): Production of fumonisins by *Fusarium* species in Taiwan. Mycopathologia 130: 117-121.

Visconti A and Doko B (1994): Survey of fumonisin production by *Fusarium* isolated from cereals in Europe. Journal of the Association of Official Agricultural Chemists 77: 546-550.

Journal of Basic & Applied Mycology (Egypt) 4 (2013): 77-83

Marine-derived fungus, *Penicillium aurantiogriseum* AUMC 9757: a producer of bioactive secondary metabolites

M. A. Abo-Kadoum, N. F. Abo-Dahab, M. F. Awad and A. M. Abdel-Hadi*

Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Assuit Branch

***Corresponding author: e-mail: ahmed_alhadi2000@yahoo.com**
Received 27/8/2013, Accepted 20/10/2013

Abstract: Thirty-six isolates comprising 23 species of fungi belonging to 8 genera isolated from five regions in Mediterranean Sea (Alexandria) were screened for production of indole alkaloids. Twenty-two isolates gave positive reactions (blue spots on TLC) with Van Urk's reagent and were regarded as indole alkaloids producers. *Penicillium aurantiogriseum* AUMC 9757 was isolated from sea sediment, was selected as the most active producer of indole alkaloids for biological evaluation (antimicrobial and antitumor activities). The crude extract of the strain exhibited high activities against four bacterial strains (*Staphylococcus aureus*, *Bacillus cereus*, *B. subtillus* and *Salmonella* sp.), four fungal strains (*Fusarium solani*, *Alternaria alternata*, *Aspergillus flavus* and *A. ochraceus*) and liver carcinoma cell line (HEPG2). The maximum concentration (100 µg/ml) killed 82.76% of the viable cells, while 50 µg/ml killed 80.52% of the viable cells. The cytotoxicity bioassay using brine shrimp eggs revealed that, there was no mortality in the tested samples at different concentrations. The present study identified *P. aurantiogriseum* from marine sediment as a potential producer of safe bioactive compounds which can be used as antimicrobial and anticancer compounds.

Key words: Marine fungi, *P. aurantiogriseum*, alkaloids, antimicrobial and antitumor.

Introduction

Many fungi are adapted to the presence of salt even up to quite high concentrations. Marine fungi are recognized as a prolific source of biologically active secondary metabolites. A number of reviews, describing the importance and attractiveness of marine fungal metabolites, have been published (Proksch *et al*. 2002, Bugni and Ireland 2004, Somei and Yamada 2005, Blunt *et al*. 2006, Pivkin *et al*. 2006 and Smetanina *et al*. 2007). Potential of marine fungi in providing biologically-active metabolites and new compounds is greater than that of terrestrial counter parts (Cuomo *et al*. 1995).

Alkaloids are a large group of secondary metabolites produced by many organisms, which contain almost 40% metabolites of microorganisms (Blunt *et al*. 2011). These N-containing metabolites usually biosynthesized by a convergence of multiple biosynthetic pathways from many kinds of amino acids (Khadem and Marles 2012) and they have the novelty and diversity of structures.

Screening members of the genus *Penicillium* for the alkaloids production revealed that most of them produce alkaloids belonging to various structural groups, mainly clavines, diketopiperazines, benzodiazepines and quinolines (Kozlovsky *et al*. 2000, Rundberget and Wilkins 2002 and Xin *et al*. 2007). In particular, they have a variety of biological activities such as antimicrobial, antitumor, antipredator, antiinflammatory, and antiviral (Zhuravleva *et al*. 2012). From this point and due to biological activity of alkaloids, we conduct this work to explore new strains which have the ability for indole alkaloid production and determining the biological activity of these compounds.

Materials and Methods

Samples collection

A total of 10 samples of the sediments and sea water were collected from five regions from the Mediterranean Sea (Alexandria). These samples were collected in sterile containers and kept in refrigerator at 4°C for further processing.

Fungal survey

The collected samples were screened for isolation fungal strains on Czapek-Dox agar. The medium composition was (per liter): Sucrose 30.0g, Agar 15.0g, $NaNO_3$ 3.0g, K_2HPO_4 1.0g, KCl 0.5g, $MgSO_4 \cdot 7H_2O$ 0.5g, $FeSO_4 \cdot 7H_2O$ 0.01g and Chloramphenicol 50 mg/l as bacteriostatic agent. All medium ingredients were dissolved in sea water then autoclaved at 121°C for 20 minutes. Afterwards, the collected samples were diluted at 10 and 100-fold with autoclaved filtered sea water, aliquots of 0.1 ml of diluted samples (Awad and Kraume 2011) put into Petri-dish followed by 20 ml of Czapek-Dox agar medium (3 replicates). Plates were incubated at 30 °C for 1-2 weeks to allow for development of pigment on colonies to facilitate complete differentiation of fungal types. Repeated sub-culturing on Czapek-Dox agar was done to obtain pure cultures. Isolates were identified according to morphological features, cultural characteristics such as pigmentation of the mycelium. Identification was accomplished using

appropriate taxonomic techniques (Moubasher 1993 and Pitt and Hocking 2009).

Screening of fungal isolates for indole alkaloids production

All the fungal strains isolated from marine water and sediment were maintained on Czapek-Dox agar medium (Table 1). Spores from a five to seven days old culture were transferred into 250 ml Erlenmeyer flask containing Abe 's medium (per liter) (Manitol 50g, Succinic acid 5.4g, $MgSO_4.7H_2O$ 0.3g, KH_2PO_4 1g). The pH of medium was adjusted to 5.4 with concentrated ammonia. After inoculation, the flasks were agitated on rotary shaker (160 rpm) at 25^{o}C for 10 days. Thereafter the content of each flask was filtered (El-Shanawany *et al*. 2005).

Detection of indole alkaloid production

Indole alkaloids were detected by using Van Urk's reagent (P. dimethylaminobenzaldhyde 0.125 g, 5% $FeCl_3$ 1 ml, 65% v/v sulphuric acid 100 ml). One ml of the filtrate was heated with 2 ml of reagent which gives blue color with positive result (Van Urk 1929).

Extraction of bioactive compounds

The filtrate was extracted two times using organic solvent (chloroform) in a separation funnel. The extract was evaporated to near drying by using rotary evaporator.

Antimicrobial activity

Screening for antibacterial activity

The antibacterial activity of the marine-derived fungus, *P. aurantiogriseum* crude extract was tested against four different pathogenic bacterial strains. Three of them are Gram-positive (*Staphylococcus aureus*, *Bacillus cereus*, *B. subtillus*) and one Gram-negative (*Salmonella* sp.). Aliquots of the tested bacterial cultures were introduced into sterile Petri-dishes then poured with sterilized nutrient agar medium then allowed to solidify; filter paper discs were saturated with crude extract and placed on the surface of agar medium. All plates were incubated at 37^{o}C for 24 h, and were observed for the formation of inhibition zones around the discs.

Screening for antifungal activity

The antifungal activity of the marine-derived fungus (crude extract) was tested against four different fungal strains which belong to *Aspergillus ochraceus*, *A. flavus*, *Fusarium solani*, and *Alternaria alternata*. Aliquots of the tested fungal cultures were introduced into sterilized Petri-dishes then poured with sterilized Czapek's medium and then allowed to solidify. Wells of 0.5 cm were made in the medium by sterilized cork borer, and 150 µl of crude extract were transferred into each well. All plates were incubated at 28^{o}C for 3-5 days, and were observed for the formation of inhibition zones around the wells.

Antitumer activity

The crude extract of marine-derived fungus, *Penicillium aurantiogriseum* was tested against hepatic cellular carcinoma (HEPG2).

A) Cell seeding for sulforhodamine B (SRB) assay (original monolayer 96-well plate seeding)

Potential cytotoxicity of the compound (s) was tested using the method of Skehan *et al.* (1990). Cells under investigation were trypsinized and proper dilution in the compatible media was made. Aliquots of 100 µl cell suspension containing 1000 cells were seeded into flat bottom 96-well plate (according to the cell line doubling time, and operator handling usually ranges from 500-2000 cell per well). Negative control: a lane that only contains media, while positive control is a lane that contains cells but not treated. Each plate must accommodate at least 24 peripheral wells filled with PBS; to guard against media drying. Plates were incubated in a humidified 37ºC, 5% CO_2 chamber for 24 hr. Another aliquots of 100µl media containing the crude extract concentration ranging from 1 ng/ml to 100 µg/ml were added to treated lanes, and blank media to the +ve, and –ve control lanes (this is considered the zero time for treatment). Plates were incubated in a humidified 37ºC, 5% CO2 chamber for another 72 hrs (for the long incubation period the media might need to be changed, and some time PBS washing is recommended according to the treatment under investigation.

B) SRB (sulforhodamine B) assay procedure (for simple 96-well plate format)

On the day of analysis, the 96 well-plates were centrifuged at 1000 rpm, and 4 ºC for 5 min. The media containing the crude extract solution were removed. Fixation was performed by 150 µl of 10% TCA, added and incubated at 4 ºC for 1 hr. The fixative solution was removed and washed 5 times with double distilled water. Aliquots of 70 µl of 0.4 % SRB solution were added and incubated for 10 min at room temperature in a dark place. Plates were washed 3 times with 1% acetic acid, and left to air-dry overnight. To dissolve SRB-bound protein, aliquots of 150 µl of 10 mM Tris- HCl were added and shaken for 2 min. The absorbance was measured at 540 nm (Skehan *et al.* 1990).

Cytotoxicity bioassay

Brine shrimp lethality bioassay (McLaughlin 1991, Krishnaraju *et al*. 2005 and Al-Bari *et al*. 2007) was carried out to investigate the cytotoxicity

of the crude extract of *P. aurantiogriseum*. Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical shaped vessel (1L), filled with sterile artificial seawater (prepared using sea salt 38 g L^{-1}) under constant aeration at 30°C for 48 hrs. After hatching, active nauplii (larvae, matured shrimp) free from egg shells were collected from the brighter portion of the hatching chamber and used for the assay. The extract was dissolved in Dimethyl Sulfoxide (DMSO) (not more than 50 µl in 5 ml solution) plus sea water (3.8% NaCl in water) to attain concentrations of 1, 5, 10, 20, 40 µg ml^{-1}. A tube containing 50 µl DMSO diluted in 5 ml was used as a control. Ten nauplii were drawn through a glass capillary and placed in each tube. The number of the nauplii that died after 24 hrs at room temperature was counted. Experiments were conducted together with a control and different concentrations of the test substances in a set of three tubes per dose. The findings are presented graphically by plotting log concentration versus percentage mortality of nauplii from which lethal dose, 50% (LD50) was determined by extrapolation (Goldstein *et al*. 1974).

Results and Discussion

Biodiversity of marine derived fungi

Diverse filamentous fungi were recovered from the water and sediment samples of five coastal environs along Mediterranean Sea (Alexandria). Thirty-six isolates comprising 23 species of fungi belonging to 8 genera were isolated and identified in this study (Table 1). Of all these, *Aspergillus* was the most dominant genus with 8 species (36.11 % of total isolates), followed by *Penicillium* with 4 species (19.4%), *Fusarium* with 3 species (19.4 %), *Scopulariopsis* with 3 species (8.33 %) and others with 5 species (13.9 %). In this respect, Mabrouk *et al.* (2010) isolated 88 fungal isolates from different algae, sea grasses and decaying wood samples collected from Abou-keer, Alexanderia, Egypt. They found that the marine fungal genera were *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Dendryphiopsis*, *Fusarium*, *Moniliella*, *Penicillium*, *Scopulariopsis*, and *Verticillium.* Saravanan and Sivakumar (2013) isolated a total of 41 fungal species from marine systems of East Coast of Tamil Nadu, India by plating techniques. They recovered 24 species of fungi from sediment samples whereas water samples yielded 30 species and natural substrates with 24 species. *Aspergillus* was the most common genus represented by 14 species followed by *Penicillium* and *Cladosporium*.

Screening of marine derived isolates for indole alkaloids

Thirty-six isolates belonging to 23 marine fungal species were screened for their potentiality to synthesize indole alkaloids. Twenty-two isolates gave positive reactions (blue spots on TLC) with Van Urk's reagent and were regarded as indole-alkaloid producers, namely *Alternaria chlamydospora* (1 isolate), *Aspergillus aegyptiacus* (1), *A. flavus* (1), *A. tamarii* (2), *A. ustus* (1), *A. versicolor* (4), *Fusarium solani* (1), *Penicillium aurantiogriseum* (2); *P. citrinum* (3), *P. corylophilum* (1), *Scopulariopsis brevicaulis* (1), *S. candida* (1), *S. halophilica* (1), *Trichoderma viride* (1) and *Verticillium* sp. (1) (Table 1 and Fig 1). Vinokurova *et al*. (2003) studied the ability of 13 strains belonging to 10 species of the genus *Penicillium* to produce alkaloids. Most of these strains produced identical ranges of alkaloids when grown on wheat and synthetic Abe's medium. A family of prenylated indole alkaloids derived from tryptophan, proline and two isoprene units are produced by various fungi of the genera *Aspergillus* and *Penicillium*. These alkaloids are characterized by a diketopiperazine or a bicyclo[2,2,2]diazaoctane ring and a 1,7-dihydropyrano-[2,3-g]indole ring system. Among them notoamides were produced from a marine-derived *Aspergillus* sp. (Kato *et al*. 2007, Tsukamoto *et al*. 2008, 2010), stephacidins A & B, from *Aspergillus ochraceus* (Qian-Cutrone *et al*. 2002), versicolamide B from *Aspergillus versicolor* (Greshock *et al*. 2008) and aurantiomides A–C from *Penicillium aurantiogriseum* (Xin *et al*. 2007).

Screening for antimicrobial efficacy

The screening of fungal strains for indole alkaloids production revealed that *P. aurantiogriseum* was regarded as a producer of mixture of indole alkaloids and exhibited many blue spots on TLC. A strain of *P. aurantiogriseum* isolated from sediment was selected as the most active producer of indole alkaloids to test its antimicrobial and antitumor activity (Figs 2 & 3). The culture filtrate of this strain was subjected to extraction using chloroform. The crude extract exhibited activities against four bacterial strains (*Staphylococcus aureus, Bacillus cereus, Bacillus subtillus, Salmonella* sp.) and against four fungal strains (*Fusarium solani*, *Alternaria alternata*, *A. flavus*, *A. ochraceus*). It is interesting to observe that the crude extract of *P. aurantiogriseum* achieved the best inhibition when tested against fungi. Fungi of the genus *Penicillium* are promising objects in the search for new biologically active compounds (Dreyfuss and Chapela 1994). Tsuda *et al*. (2005) attained three pyrrolidine alkaloids, scalusamides A-C (1-3), from the cultured broth of *Penicillium citrinum*, each of 1-3 was found to be a mixture of epimers at C-7. Scalusamide A (1) exhibited antifungal and antibacterial activities. Song *et al*. (2012) isolated three new alkaloids, including auranomides A and B, a new scaffold containing quinazolin-4-one substituted with a pyrrolidin-2-iminium moiety, and auranomide C,

Table 1: Screening of active producer isolates for indole alkaloids

Genera and species	No. of isolates	Source	Indole alkaloids production
Alternaria alternata (Fries) Keissler	1	Sediment	-ve
Alternaria chlamydospora Mouchacca	1	Sediment	+ve
Aspergillus aegyptiacus Moubasher & Moustafa	1	Sea water	+ve
Aspergillus flavus Link	1	Sediment	+ve
Aspergillus niger van Tieghem	2	Sea water (1), Sediment (1)	-ve -ve
Aspergillus parasiticus Speare	1	Sediment	-ve
Aspergillus tamarii Kita	2	Sediment	+ve
Aspergillus terreus Thom	1	Sediment	-ve
Aspergillus ustus (Bainier) Thom & Church	1	Sediment	+ve
Aspergillus versicolor (Vuill.) Tirab.	5	Sea water (3), Sediment (2)	+ve (4) -ve (1)
Fusarium dimerum Penzig	1	Sediment	-ve
Fusarium moniliforme Sheld.	1	Sediment	-ve
Fusarium solani (Martius) Saccardo	5	Sediment	+ve (1) -ve (4)
Penicillium aurantiogriseum Dierckx	2	Sea water (1), Sediment (1)	+ve +ve
Penicillium citrinum Thom	3	Sediment	+ve
Penicillium corylophilum Dierckx	1	Sediment	+ve
Penicillium purpurogenum Stoll	1	Sea water	-ve
Scopulariopsis brevicaulis (Saccardo) Bainier	1	Sediment	+ve
Scopulariopsis candida Vuill.	1	Sediment	+ve
Scopulariopsis halophilica Tubaki	1	Sediment	+ve
Talaromyces sp.	1	Sediment	-ve

as well as two known metabolites auranthine and aurantiomides C from the marine-derived fungus *P. aurantiogriseum*. The quinazolin-4-one ring system has been consistently recognized as a promising pharmacophore because of its broad spectrum pharmacological activities as antibacterial and antifungal agents (Pandey *et al*. 2009 and Mohamed *et al*. 2010).

Screening for antitumor activity

The obtained crude extract of *P. aurantiogriseum* was evaluated for potential cytotoxicity, against hepatic cellular carcinoma (HEPG2). The cell line was treated with serial concentrations of 6.25, 12.5, 25, 50 and 100 µg/ml. Results in Table (2) showed that the maximum concentration 100 µg/ml killed 82.76 % of the viable cells, while the minimum concentration 6.25 % killed 52.54% of the viable cells. Similarly, Song *et al*. (2012) isolated Auranomide B from marine-derived fungus *Penicillium aurantiogriseum* which exhibited the most potent inhibitory effect against human myelogenous leukemia HEPG2 cells, with an inhibitory effect 73.28. Mabrouk *et al*. (2011) extracted 11 compounds from *Penicillium brevicompactum* which was isolated from the associated marine alga *Pterocladia* sp. They reported that the maximum concentration of compound 9 (100 µg/ml) killed approximately 40% of the viable infected liver cells and also killed approximately 50% of the viable infected lung cells at concentration equal to 91.6 µg/ml. They concluded that compound 9 can be recommended as an anticancer compound.

In conclusion: The results of this study revealed that *P. aurantiogriseum* is a good producer of safe bioactive compounds which can be used as antimicrobial and an anticancer compound. Further studies are required to separate and identify these compounds in a pure state and determine which compound has the biological activity to use in application.

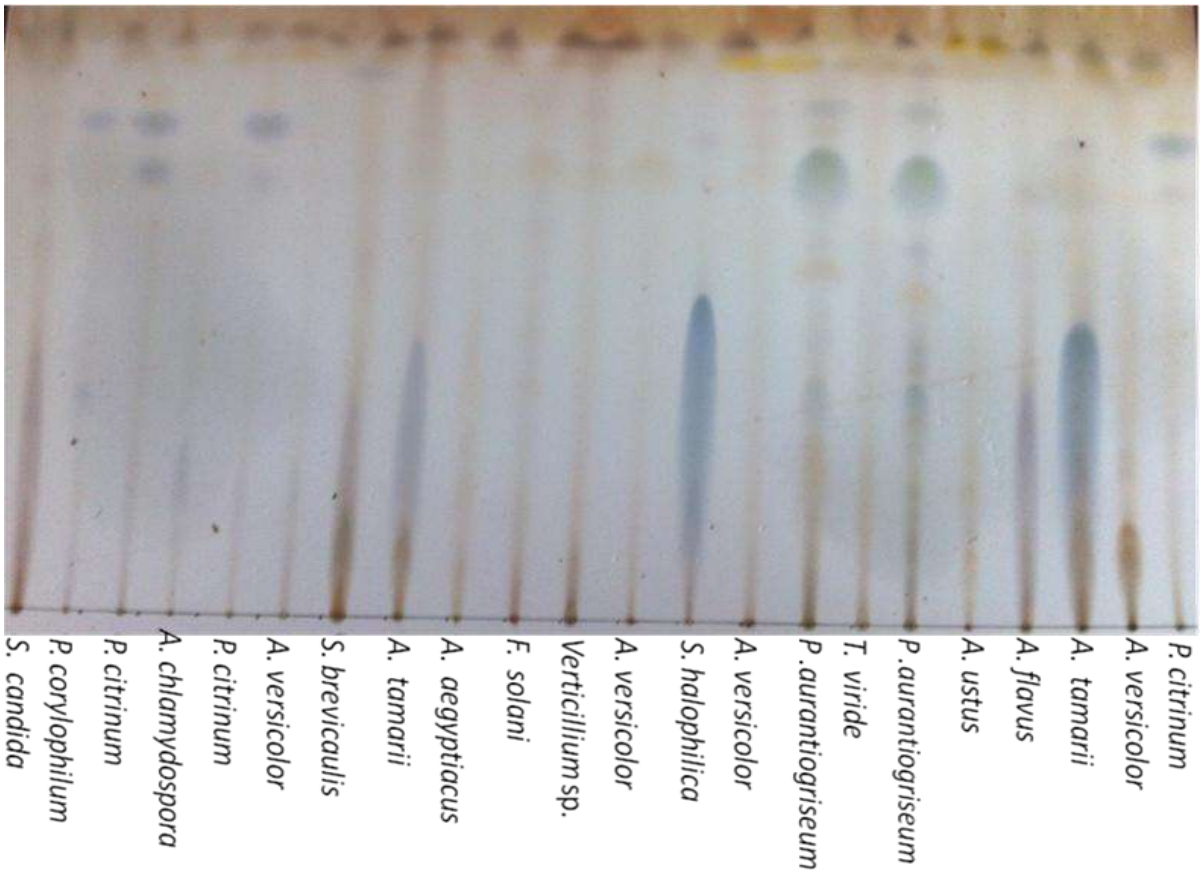


Fig 1: Running extract of tested isolates on TLC Kieselgure using system (Chloroform: Methanol: 25% ammonia; 90:10:0.1)

Table 2: Sensitivity of cancer liver cells to different concentrations of crude extract of *P. aurantiogriseum* AUMC 9757 (AUMC = Assiut University Mycological Center).

Concentration µg/ml	Viability %
Control (0)	100
6.25	47.46396
12.5	41.27069
25	36.99947
50	19.48745
100	17.24506

Acknowledgements: We are very grateful to Prof. Ahmed Moharram Prof. of Mycology, Botany & Microbiology Department, Faculty of Science, Assiut University for help in identification of *Penicillium aurantiogriseum* strain AUMC 9757. Also, we are grateful to Dr Serage Ebraheem, the Holding Company for Biological Products and Vaccines (VACSERA) for doing antitumor assay.

References

Al-Bari MA, Sayeed, MA, Khan A, Islam MR, Khondokar MP, Rahman MS and Islam MAU (2007): In-*vitro* antimicrobial activities and cytotoxicity of ethyl acetate extract from *Streptomyces maritimus*. Biotechnology 6: 81-85.

Awad, MF and Kraume M (2011): Mycoflora of Activated Sludge with MBRs in Berlin, Germany. World Academy of Science, Engineering and Technology 54: 566-570.

Blunt JW, Copp BR, Munro MHG, Northcote PT and Prinse MR (2006): Marine natural products. Natural Product Reports 23: 26-78.

Blunt JW, Copp BR, Munro MHG, Northcote PT and Prinsep MR (2011): Marine natural products. Natural Product Reports 28(2): 196-268.

Bugni TS and Ireland CM (2004): Marine-derived fungi: a chemically and biologically diverse group of microorganisms. Natural Product Reports 21: 143-163.

Cuomo V, Palomba I, Perretti A, Guerriero A, D'Ambrosio M, and Pietra F (1995): Antimicrobial activities from marine fungi. Journal of Marine Biotechnology 2: 199-204.

Dreyfuss MM and Chapela IH (1994): Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. In: The Discovery of Natural Products with Therapeutic Potential, Gullo VP (ed.), Boston, London, Butterworth-Heinemann, pp. 49-79.

El-Shanawany A, Barakat AA and Abdel-Hadi AM (2005): Ergot alkaloids agroclavine-1 and epoxyagroclavine-1 from *Penicillium citrinum* MU-534. The African Journal of Mycology and Biotechnology 13(1): 71-82.

Goldstein A, Arnow L and Kalkan SM (1974): Principles of Drug Action. 2nd edn., Wiley Biomedical Health Publication, pp. 376-381.

Greshock TJ, Grubbs AW, Jiao P, Wicklow DT, Gloer JB and Williams RM (2008): Isolation, structure elucidation, and biomimetic total synthesis of versicolamide B, and the isolation of antipodal (-)-stephacidin A and (+)-notoamide B from *Aspergillus versicolor* NRRL 35600. Angewandte Chemie International Edition 47: 3573–3577.

Kato H, Yoshida T, Tokue T, Nojiri Y, Hirota H, Ohta T, Williams RM and Tsukamoto S (2007): Notoamides A–D: prenylated indole alkaloids isolated from a marine-derived fungus, *Aspergillus* sp. Angewandte Chemie International Edition 46: 2254–2256.

Khadem S and Marles RJ (2012): Chromone and flavonoid alkaloids: occurrence and bioactivity. Molecules 17: 191-206

Kozlovsky A, Vinokurova NG, Adanin VM and Gräfe U (2000): Piscarinines, new polycyclic diketopiperazine alkaloids from *Penicillium piscarium* NKM F-691. Natural Product Letters 14: 333-340.

Krishnaraju AV, Rao TV, Sundararaju D, Vanisree M, Tsay H, and Subbaraju GV (2005): Assessment of bioactivity of Indian medicinal plants using brine shrimp (*Artemia salina*) lethality assay. International Journal of Applied Science and Engineering 3(2): 125-134.

Mabrouk AM, Kheiralla ZH, Hamed ER, Youssry AA and Abd El Aty AA (2011): Physiological studies on some biologically active secondary metabolites from marine-derived fungus *Penicillium brevicompactum*. www.Gate2Biotech.com. 1(1): 1-15.

Mabrouk AM, Kheiralla ZH, Hamed ER, Youssry AA and Abd El Aty AA (2010): Screening of some marine-derived fungal isolates for lignin degrading enzymes (LDEs) production. Agriculture and Biology Journal of North America 1(4): 591-599.

McLaughlin JL (1991): Bench-top bioassays for the discovery of bioactive compounds in higher plants. Brenesia 34: 1-14.

Mohamed MS, Kamel MM, Kassem EM, Abotaleb N, Abd El-moez SI and Ahmed MF (2010): Novel 6,8-dibromo-4(3H) quinazolinone derivatives of anti-bacterial and anti-fungal activities. European Journal of Medicinal Chemistry 45: 3311-3319.

Moubasher AH (1993): Soil fungi of Qatar and other Arab Countries. The Scientific and Applied Research Centre, University of Qatar, Doha, Qatar, 566 pp.

Pandey SK, Singh A and Singh A (2009): Nizamuddin antimicrobial studies of some novel quinazolinones fused with [1,2,4]-triazole, [1,2,4]-triazine and [1,2,4,5]-tetrazine rings. European Journal of Medicinal Chemistry 44: 1188-1197.

Pitt JI and Hocking AD (2009): Fungi and Food Spoilage. 3rd ed, Springer, 519 pp.

Pivkin MV, Kuznetsova TA and Sova VV (2006): Marine Fungi and their Metabolites; Dalnauka: Vladivostok, 133 pp.

Proksch P, Edrada RA and Ebel R (2002): Drugs from the seas: current status and microbiological implications. Applied Microbiology and Biotechnology 59:125-134.

Qian-Cutrone J, Huang S, Shu YZ, Vyas D, Fairchild C, Menendez A, Krampitz K, Dalterio R, Klohr SE and Gao Q (2002): Stephacidin A and B: two structurally novel, selective inhibitors of the testosterone-dependent prostate LNCaP cells. Journal of the American Chemical Society 124: 14556-14557.

Rundberget T and Wilkins AL (2002): Thomitrems A and E, two indole alkaloid isoprenoids from *Penicillium crustosum* Thom. Phytochemistry 61: 979-985.

Saravanan R and Sivakumar T (2013): Biodiversity and biodegradation potentials of fungi isolated from marine systems of East Coast of Tamil Nadu, India. International Journal of Current Microbiology and Applied Science 2: 192-201

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR (1990): New colorimetric cytotoxicity assay for anti-cancer drug screening. Journal of National Cancer Institute 82: 1107-1117.

Smetanina OF, Kalinovsky AI, Khudyakova YV, Pivkin MV, Dmitrenok PS, Fedorov SN, Ji H, Kwak JY and Kuznetsova TA (2007): Indole alkaloids produced by a marine fungus isolate of *Penicillium janthinellum* Biourge. Journal of Natural Products 70: 906-909.

Somei M and Yamada F (2005): Simple indole alkaloids and those with a non-rearranged

monoterpenoid unit. Natural Product Reports 22: 73-103.

Song F, Ren B, Yu K, Chen C, Guo H, Yang N, Gao H, Liu X, Liu M, Tong Y, Dai H, Bai H, Wang J and Zhang L (2012): Quinazolin-4-one coupled with pyrrolidin-2-iminium alkaloids from marine-derived fungus *Penicillium aurantiogriseum*. Marine Drugs 10: 1297-1306.

Tsuda M, Sasaki M, Mugishima T, Komatsu K, Sone T, Tanaka M, Mikami Y and Kobayashi J (2005): Scalusamides A-C, new pyrrolidine alkaloids from the marine-derived fungus *Penicillium citrinum*. Journal of Natural Products 68: 273-276.

Tsukamoto S, Kato H, Samizo M, Nojiri Y, Onuki H, Hirota H and Ohta T (2008): Notoamides F–K, prenylated indole alkaloids isolated from a marine-derived *Aspergillus* sp. Journal of Natural Products 71: 2064-2067.

Tsukamoto S, Umaoka H, Yoshikawa K, Ikeda T, and Hirota H (2010): Notoamide O, a structurally unprecedented prenylated indole alkaloid, and notoamides P–R from a marine-derived fungus, *Aspergillus* sp. Journal of Natural Products 73: 1438-1440.

Van Urk HW (1929): A new sensitive reaction for the ergot alkaloids, ergotamine, ergotoxine and ergotinine and its adaptation to examination and colorimetric determination of ergot preparations. Pharmaceutisch Weekblad 66: 473-481.

Vinokurova NG, Khmel nitskaya II, Baskunov BP and Arinbasarov MU (2003): Occurrence of indole alkaloids among secondary metabolites of soil *Aspergillus* spp. Applied Biochemistry and Microbiology 39: 192-196.

Xin ZH, Fang Y, Du L, Zhu T, Duan L, Chen J, Gu QQ, and Zhu WM (2007): Aurantiomides A–C, quinazoline alkaloids from the sponge-derived fungus *Penicillium aurantiogriseum* SP0-19. Journal of Natural Products 70: 853-855.

Zhuravleva OI, Afiyatullov SS, Denisenko VA, Ermakova SP, Slinkina NS, Dmitrenok PS and Kim NY (2012): Secondary metabolites from a marine-derived fungus *Aspergillus carneus* Blochwitz. Phytochemistry 80: 123-131.

Journal of Basic & Applied Mycology (Egypt) 4 (2013): 85-87

Contribution to the mycobiota of Egypt
Emericella stella-maris Zalar, Frisvad & Samson 2008, a new record to Egypt

A. H. Moubasher*, M. A. Abdel-Sater and Zeinab Soliman

Department of Botany and Microbiology, Faculty of Science and Assiut University Mycological Centre (AUMC), Assiut University, Assiut, Egypt

***Corresponding author: e-mail: ahamaumc@yahoo.com**
Received 3/4/2013, Accepted 12/6/2013

Abstract: Two isolates of *Emericella* with stellate-shaped ascospores were isolated during surveys of filamentous fungi of *Citrus* plantations in Assiut area, Egypt. The isolates were examined for its macroscopic and microscopic features and they were identified as *Emericella stella-maris*. This species is being recorded here for the first time in Egypt and propably the second record after Zalar *et al.* (2008). *E. stella-mais* is different from *Emericella variecolor* by its septate, long conidiophore and its inability to grow at 37^{0}C.

Key words: *Emericella stella-maris*, *Citrus* plantations, mycobiota, Egypt.

Introduction

Emericella species with stellate-shaped ascospores has been described for the first time in 1857 as *Emericella variecolor* by Berkeley & Broome and still represented by one species for 140 years. In 1997, Stchigel & Guarro described a second species, *Emericella pluriseminata* from Indian soil. A third species, *Emericella venezuelensis* was described in 2004 by Frisvad & Samson from a sponge in red mangrove surface water in Mochima Bay, Venezuela. Zalar *et al.* (2008) described two more species: *Emericella olivicola* from decaying fruits of *Olea europaea* in Italy and *Emericella stella-maris* from *Eucalyptus* leaf litter in Tunisia and hypersaline saltern water of Adriatic coast in Slovenia.

In Egypt, *E. variecolor*, the only stellate-shaped ascosporic species of *Emericella* reported, was isolated from soil and salt marsh soils, wheat and corn grains and the air at Assiut (refer to Moubasher 1993). A second stellate-shaped ascosporic *Emericella* species, *E. stella-maris* was isolated from the air of orange plantations and this is the first record in Egypt and propably the second record after Zalar *et al.* (2008). It was recovered in October 2008 and February 2009 on DYM (dichloran yeast extract malt extract) agar, in June and August 2008 and February 2009 on DRBC (dichloran rose Bengal chloramphenicol) agar. Representative isolates were deposited in the Culture Collection of the Assiut University Mycological Centre as AUMC nos. 5778 and 6239.

Description of *Emericella stella-maris* Zalar, Frisvad & Samson 2008
Anamorph: *Aspergillus stella-maris* Zalar, Frisvad & Samson 2008

Colonies attaining 50–70 mm after 14 d at 25ºC on MEA (malt extract agar), 60–65 mm on CYA (Czapek yeast extract agar), ascomata arranged in concentric circles. Conidiophores developing mainly in colony center, with smooth, 200–500 µm long, 3.5–7.0 µm wide, hyaline to brownish septate stipes. Vesicles clavate to globose, hyaline to brownish, 9.0–22.0 µm wide; metulae hyaline to brownish, 5.0–9.5 x 3.0–4.5 µm, bearing whorls of up to four adpressed flask-shaped phialides, 5.5–9.5 x 2.0–3.0 µm. Conidia globose to subglobose, 2.0–3.5 µm diam., smooth-walled to echinulate. Ascomata cleistothecial, oblong, 630–880 µm long x 300–630 µm wide. Hülle cells globose to ovoid, 12–22 µm diam. Asci stellate consisting of globose to subglobose, 10–14 um diam. wide body and 2.5–6.5 µm long equatorial spikes, 8-spored. Ascospores orange-red, in surface view stellate, 10.0–16.0 µm; spore body smooth, subglobose, 3.0–4.5 x 2.5–4.5 µm, in side view broadly lenticular, with two stellate equatorial crests; undissected part of crests 1.0–1.5 µm broad, with 3.0–4.5 µm long extensions; crests regularly ornamented with longitudinal pleats. *Emericella stella-maris* is clearly distinct from *E. variecolor* by its septate, long stipe and its inability to grow at 37^{0}C.

Species	Growth at 37ºC	Stipe length	Vesicle diam.	Conidia size	Hülle cells size	Ascomata size	Ascospores size
Emericella variecolor	Good growth	140-200 µm	8-10 µm	2.5-3.5 µm	Up to 30 µm	300-600 x 1000-1500 µm	3.6-4.0 µm, crest up to 4.0 µm wide
Emericella stella-maris	No growth	200-500 µm	9-22 µm	2.0-3.5 µm	12-22 µm	630-880 x 300-630 µm	3-4.5 x 2.5-4.5 µm, crests up to 11 µm wide

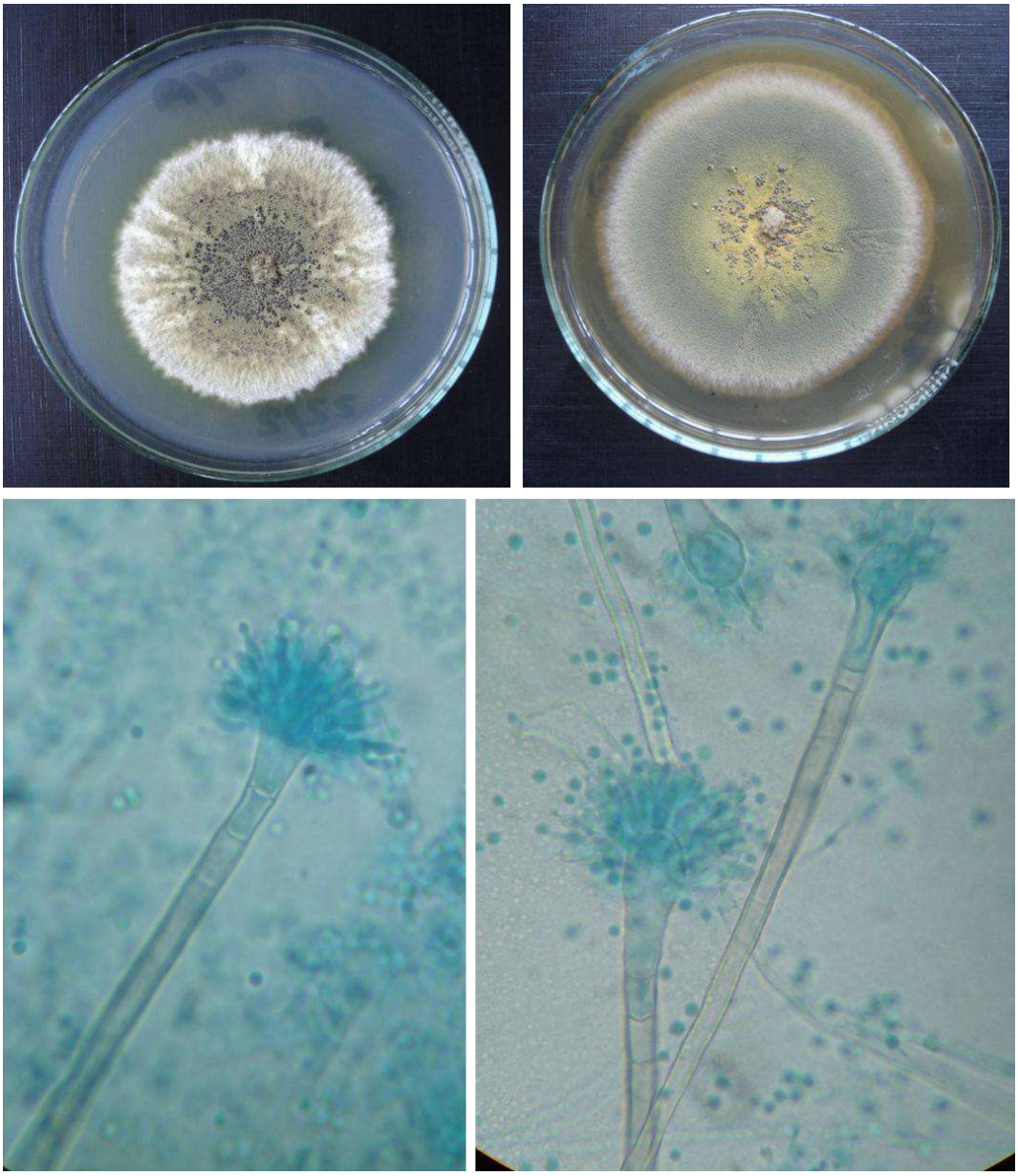


Figure 1: 14 days old colonies on CYA (top left) and MEA agar (top right); conidiophores and conidia of *Emericella stella-maris* (bottom).

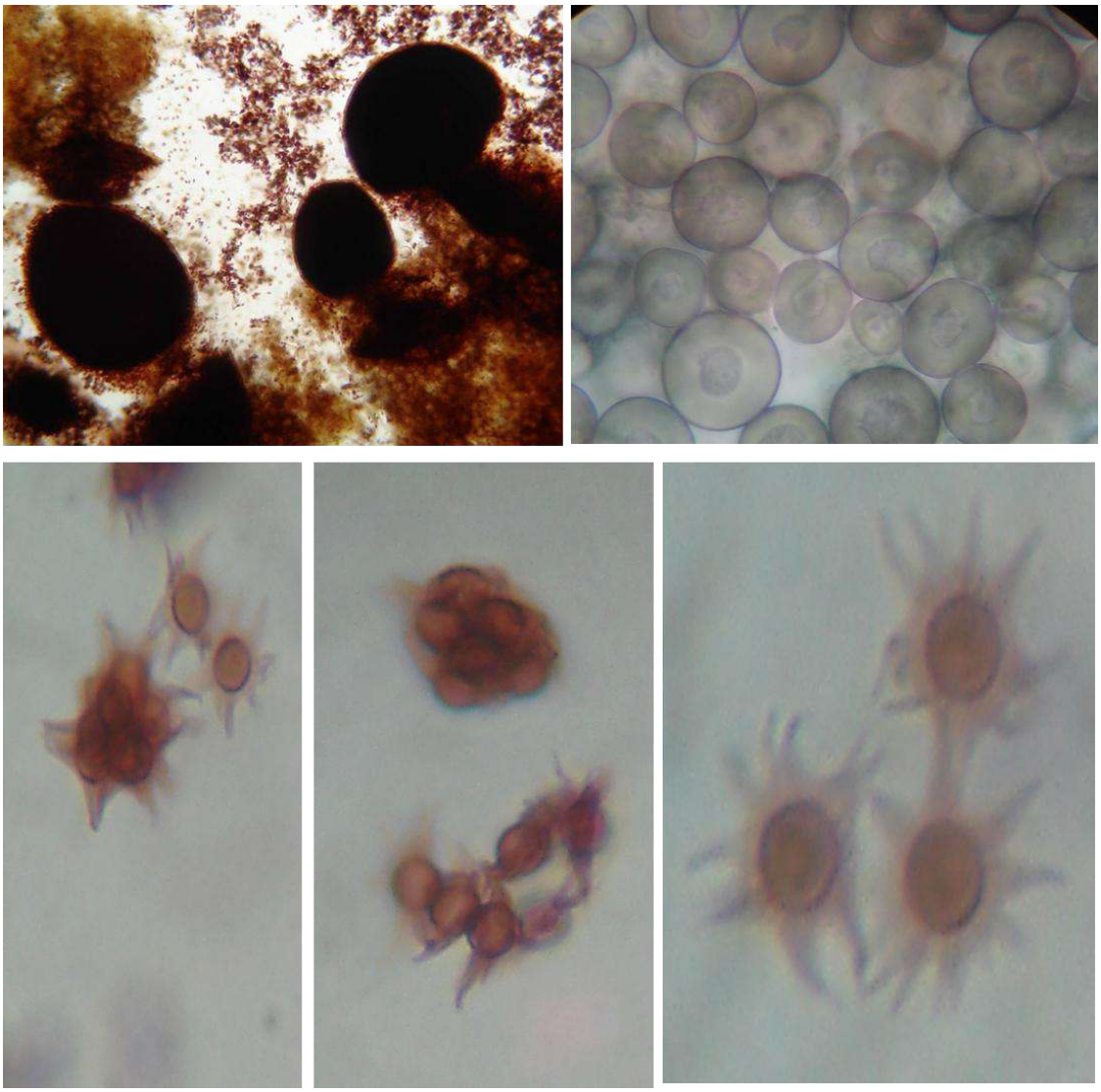


Figure 2: Ascomata (top left), Hülle cells (top right), asci, and ascospores (bottom) of *E. stella-maris.*

References

Frisvad JC and Samson RA (2004): *Emericella venezuelensis*, a new species with stellate ascospores producing sterigmatocystin and aflatoxin B1. Systematic and Applied Microbiology 27: 672-680.

Moubasher AH (1993): Soil fungi in Qatar and other Arab countries. The Scientific and Applied Research Center, University of Qatar, Doha, 566 pp.

Raper KB and Fennell DI (1965): The genus *Aspergillus*. Williams and Wilkins Co., Baltimore.

Stchigel AM and Guarro J (1997): A new species of *Emericella* from Indian soil.Mycologia 89: 937-941.

Zalar P, Frisvad JC, Gunde-Cimerman N, Varga J and Samson RA (2008): Four new species of *Emericella* from the Mediterranean region of Europe. Mycologia 100(5): 779-795.