Biologically active fungi recorded for the first time from new reclaimed soil, Egypt

Abdel-Nasser A. Zohri[a], Waill A. Elkhateeb[b], Mohamed B. Mazen[a], Mohamed Hashem[a], Ghoson M. Daba[b]

[a]Department of Botany, Faculty of Science, Assiut University, Assiut, [b]Department of Chemistry of Microbial Natural Products, National Research Center, Giza, Egypt

Correspondence to Waill A. Elkhateeb, Department of Chemistry of Microbial Natural Products, National Research Center, Tahrir Street, 12311, Dokki, Giza, Egypt
Tel: +20 100 498 8670; fax: +20 33 370 931; e-mail: waillahmed@yahoo.com

Received 8 December 2013
Accepted 17 February 2014

Egyptian Pharmaceutical Journal
2014, 13:27–32

Objective
This work was designed to record fungal isolates of known biological activities isolated for the first time from new reclaimed soil in El-Wady El-Assiuty and Assiut Petroleum farm in Assiut, Egypt.

Materials and methods
Soil, phyllosphere, and phylloplane samples were collected from El-Wady El-Assiuty and Petroleum farm, which represent the largest distinctive regions of newly reclaimed soil at Assiut Governorate. Identification of the isolated fungi during our investigation was carried out on the basis of many reported morphological and microscopic features, which was confirmed by Assiut University Mycology Center.

Results and conclusion
Three fungal species, in addition to one unidentified ascomycete, were isolated for the first time from new reclaimed soil in El-Wady El-Assiuty and Assiut Petroleum farm in Assiut, Egypt. They were identified and recorded by the Mycological Laboratories at Assiut University. They were identified as *Arthrinium sacchari* (Speg.) M.B. Ellis, which was isolated from the phylloplane of Guava, *Beltrania querna* Harkn., which was isolated from the soil from Petroleum farm, and *Papulaspora immersa* Hotson, which was isolated from the phyllosphere of Wheat plants; the newly unidentified ascomyceteous fungal species was obtained from the phyllosphere of pomegranate. The endemic mycobiota of these areas is of great significance, as there are no adequate mycological studies that have been performed to describe the fungal flora of these areas.

Keywords:
identification, isolation, phylloplane fungi, phyllosphere fungi, reclaimed soil, soil fungi

Egypt Pharm J 13:27–32

1687-4315

Introduction

Studying the occurrence, diversity, and distribution of mycoflora provides a clear image of the phytosanitarian state of the surrounding environment. Many papers have reported the presence and occurrence of fungi [1–4]. Investigating the mycobiota inhabiting the newly reclaimed areas at Assiut Governorate was very important for many reasons, including achieving a better understanding of the fungal flora existing in these areas; in addition, it presents an opportunity to isolate and identify new fungal species. Most investigations on fungal flora have been carried out on agricultural or forest soils; however, in this study, we investigated soil, phyllosphere, and phylloplane fungi from newly reclaimed localities of El-Wady El-Assiuty and Assiut petroleum Farm. They represent two of the largest newly reclaimed areas at Assiut Governorate, cultivated with different important crops. Many investigations have been carried out worldwide on the occurrence of soil fungal flora [5–12]. In Egypt, numerous investigations have been carried out on soil fungi in the upper Egypt delta area and Sinai Peninsula, as well as in some valleys in the eastern desert of Egypt [13–19].

The term phyllosphere fungi was coined by Abdel-Hafez *et al*. [20] and Last [21] to describe organisms found on leaf surfaces. Phylloplane, as described by Ruinen [22], is the outer skin of the leaf surface and is thus an excellent site for growth of saprophytic and parasitic fungi. Several studies have described the microorganisms on insects or on the leaf surface of plants growing or cultivated worldwide as parasites [23–28].

Materials and methods

Selected areas

Two areas representing the largest and most distinctive regions of newly reclaimed soil in Assiut Governorate were selected. They are El-Wady El-Assiuty, which lies 10 km east of Assiut, and Petroleum farm, which lies 10 km northwest of Assiut.

Collection of samples

Soil samples

Soil samples were collected from the two selected reclaimed regions in Assiut Governorate. Soil samples were collected from patches free from roots according

to the method described by Johnson *et al.* [29], which could be summarized as follows:

(1) A sample tube was used, which was washed thoroughly before starting the sampling procedure. Samples were taken from a depth of 5 inches, and the soil was transferred directly into clean and sterilized plastic bags (at least five samples were taken at random for each replication).
(2) Five or more samples from each replication were combined to form a composite sample, which was mixed thoroughly.
(3) Finally, 10 g (on an oven-dry basis) of the mixed composite sample was used to determine fungal counts by the dilution plate method, as recommended by Johnson and colleagues [10,29].

Phyllosphere and phylloplane samples

Leaves of plants were collected from the above-investigated localities, packed directly into polyethylene bags, and kept in a refrigerator until use.

Isolation and identification of fungi

Soil fungi

The dilution plate method was used to identify soil fungi [10,29], as follows:

(1) Soil to be diluted was sifted through a nine-mesh sieve. Three aliquots each (5–10 g) of soil were placed in a previously weighed metal container and dried overnight in an oven at 105°C. The aliquots were then reweighed, and the moisture content of the soil sample was calculated.
(2) Ten grams of soil particles (oven-dry weighed soil) was taken in a graduated cylinder; sterilized distilled water was added to the soil to obtain a total volume of 100 ml. The suspension was stirred and poured into a 1000 ml Erlenmeyer flask. The flask containing the suspension was shaken on mechanical shaker for 30 min.
(3) Of the suspension, 10 ml was immediately drawn (while in motion) using a sterile Menzies dipper [30] and mixed with a known volume of sterile distilled water blank until the desired final dilution was reached. Each suspension was shaken by hand for a few minutes and was in motion while being drawn using the dipper.
(4) Of the desired dilution, 1 ml was transferred directly into each of the sterilized Petri dishes; thereafter, 12–15 ml of glucose–Czapek's agar medium, cooled to just above solidifying temperature, was added to each dish. The dishes were rotated by hand in a broad swirling motion so that the diluted soil was dispersed in the agar.
(5) Five plates were used for each sample and incubated at 28 ± 1°C for 7 days, during which the developing colonies were identified and counted [expressed as colony forming units (cfu) per gram dry soil]. The average number of colonies per plate was multiplied by the dilution factor to obtain the number per gram in the original soil samples.
(6) Glucose–Czapek's agar [10,31] was used throughout the present investigation for isolation and identification of fungi. This media was supplemented with rose bengal (66 mg/ml) and chloramphenicol (30 mg/ml) as bacteriostatic agents [32]. The composition of this medium is as follows: agar, 15.0 g; $NaNO_3$, 3.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \times 7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4 \times 7H_2O$, 0.01 g; glucose, 10.0 g; 1000 ml distilled water.
(7) For further investigation, growing fungal species were obtained on slants of glucose–Czapek's agar medium and kept in a refrigerator at 4°C.

Phyllosphere fungi

Ten grams of plant leaf segments was taken in a sterile conical flask containing 100 ml sterile distilled water. The flask was shaken by hand in a rotating motion for 10 min; 10 ml of the suspension was transferred into another flask (250 ml) containing 90 ml sterile distilled water; thereafter, the flask was shaken for 5 min. Final dilutions were made in the same way to obtain a series of dilutions. Of the final dilution, 1 ml was transferred to a sterile Petri-dish and agar medium was poured into the dish. Five replicates were prepared for each sample and they were incubated at 28 ± 1°C for 7 days. Developing colonies were identified and counted (expressed as cfu/g fresh leaves).

Phylloplane fungi

Plant leaves were subjected to a series of washing with sterile distilled water. They were thoroughly dried between sterile filter paper and cut into equal segments (about 1 cm each). One hundred segments were selected per sample. Five segments were placed on the surface of the agar medium in each plate; thereafter, the plates were incubated at 28 ± 1°C for 7–10 days, and the developing fungi were counted, identified, and calculated as cfu/100 segments of fresh leaves.

Identification of fungal genera and species

Identification of the isolated fungi during our investigation was carried out on the basis of morphological and microscopic features, according to the following references:

(1) Ainsworth [33] as a dictionary of the fungi.
(2) Domsch *et al.* [34] for fungi in general.

(3) Ellis [35,36] for dematiaceous hyphomycetes.
(4) Kendrick [37] for the genera of imperfect fungi.
(5) Moubasher [38] for fungi in general.

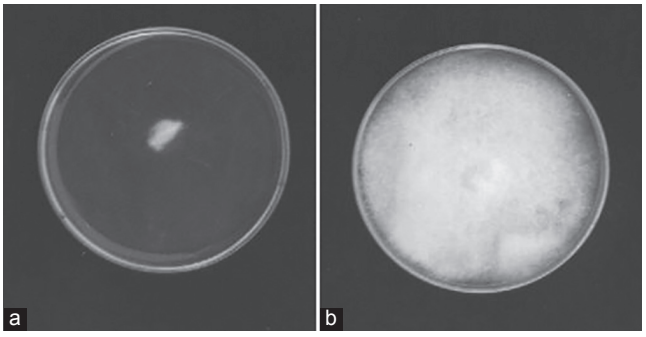
In addition, the isolated fungi was reviewed and compared with the same species deposited at Assiut University Mycological Center (AUMC).

Results and discussion

The screening survey, which was developed at El-Wady El-Assiuty, resulted in the isolation and identification of 37 species and one species variety belonging to 21 genera from the soil, 71 species and four species varieties belonging to 33 genera from the phyllosphere on glucose–Czapek's agar at 28 ± 1°C, and 56 species apart from the isolation and identification of *Papulaspora immersa* from the phyllosphere of wheat plants (*Triticum aestivum* L.) and an unidentified ascomyceteous fungal species from the phyllosphere of pomegranate (*Punica granatum* L.). Finally, three species varieties belonging to 26 genera from the phylloplane on glucose–Czapek's agar at 28 ± 1°C besides the isolation and identification of *Arthrinium sacchari* from the phylloplane of Guava (*Psidium guajava* L.) were identified. From the Assiut petroleum farm 56 species and three species varieties belonging to 29 genera were isolated and identified from the soil, besides the isolation and identification of *Beltrania querna*, 69 species and three species varieties belonging to 30 genera from the phyllosphere on glucose–Czapek's agar at 28 ± 1°C, and 43 species and one species variety belonging to 21 genera from the phylloplane on glucose–Czapek's agar at 28 ± 1°C.

The four species that were isolated and identified for the first time are as follow:

Figure 1



Seven-day-old *Arthrinium sacchari* (Speg.) M.B. Ellis colonies on (a) Czapeck's agar and (b) PDA, showing markedly better growth on PDA. PDA, potato dextrose agar.

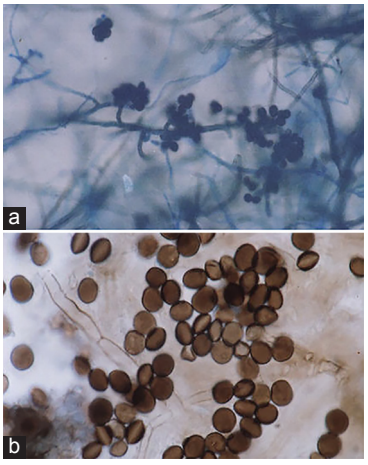
Arthrinium sacchari (Speg.) M.B. Ellis

Colonies grow well on potato dextrose agar, attaining a diameter of 9 cm after incubation at 28 ± 1°C for 7 days; limited growth (about 1 cm) was observed on glucose–Czapek's agar after 7 days. Colonies are compact or widely diffuse, black or dark blackish brown (Figs. 1 and 2). Conidiophores are 1–1.5 μm thick and septa are numerous and brown. Conidia are one-celled, smooth, almost round in face view, lenticular, solitary, lateral or terminal, frequently flattened, and with a hyaline rim, which is either brown or dark brown, measuring 6–8 μm in face view and 3–4 μm in thickness. *Arthrinium sacchari* was isolated from the phylloplane of Guava (*Psidium guajava* L.) cultivated in El-Wady El-Assiuty. According to the literature review, several compounds with antitumor activity were isolated from the culture extract of *Arthrinium sacchari*, including cytochalasin E [39], decarboxyhydroxycitrinone, libertellenone F, libertellenone E, myrocin D, libertellenone C, and myrocin A [40].

Beltrania querna Harkn

Colonies are effuse, brown to black, and attain a diameter of 9 cm after 7 days of incubation at 28 ± 1°C on potato dextrose agar; stroma usually present setae that are simple, dark, smooth or verrucose, and thick-walled, arising from flat, radially lobed basal cells that are up to 400 μm long (Figs. 3 and 4). Conidiophores are straight or flexuous, pale olive to brown, smooth, septate, up to 200 × 2–7 μm conidiogenous cells terminal, polyblastic sympodial, clavate, denticulate, separating cells when present 8–12 × 4–7 μm. Conidia are solitary, biconic, appendiculate, the free end usually being spicate, without septa, smooth, and pale olive to dark reddish brown, with a hyaline band. Biconic

Figure 2



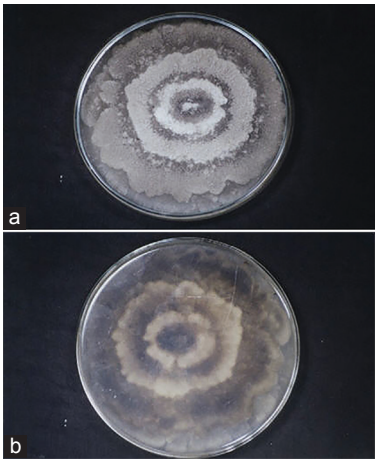
Arthrinium sacchari (Speg.) M.B. Ellis: (a) hyphae, conidiophores and conidia (×400); (b) dark lenticular conidia each with a hyaline rim (×1000).

conidia are mostly asymmetrical, with the proximal end being U-shaped, measuring 15–30 × 7–14 μm, with a 2–5 mm appendage. *Beltrania querna* was isolated from soil collected from Assiut petroleum farm. The following isocyclic compounds were isolated from *Belternaria* spp., with known antifungal activity [41]: (–)-β-eudesmol; β-eudesmol (–)-chrysanthemol rhombidiol; eudesm-4(14)-ene-9β,11-diol (–)-pterocarpol (–)-5β-hydroxy-β-eudesmol rhombitriol; 5α, 8α-dihydroxy-β-eudesmol (–)-longilobol.

Papulaspora immersa hotson

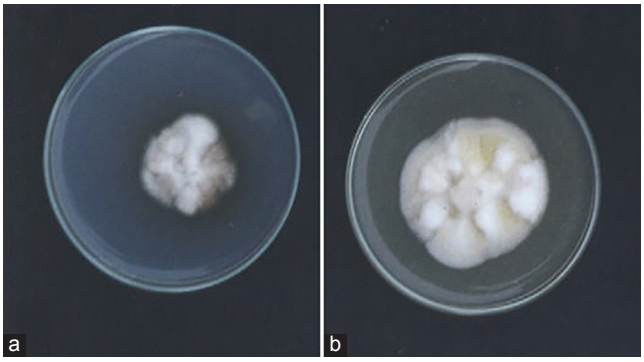
Colonies reach 6.5 cm diameter after 7 days of incubation at 28 ± 1°C on PDA and 4 cm on glucose–Czapek's agar. Papulaspores originate from intercalary cells, are pale brownish yellow, irregular in outline, 88–150 (–260) μm in diameter, often submerged in the agar central cells, and are comparatively large, angular, and darker than the peripheral cells. Reverse brownish yellow (Figs. 5 and 6). *Papulaspora immersa* was isolated from the phyllosphere of wheat (*Triticum aestivum* L.) plants cultivated in El-Wady El-Assiuty. Twelve substances of medical importance different from anticancer, antimicrobial, or antituberculosis agents were reported to be produced by *Papulaspora* spp. [42–47].

Figure 3



Beltrania querna Harkn., fast growing dark-colored colony on PDA: (a) colony surface, (b) colony reverse. PDA, potato dextrose agar.

Figure 5

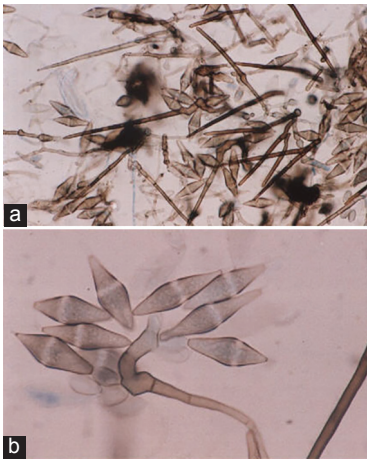


Papulaspora immersa Hotson; 7-day-old colonies on (a) Czapek's agar and (b) PDA. PDA, potato dextrose agar.

Unidentified ascomyceteous fungal species

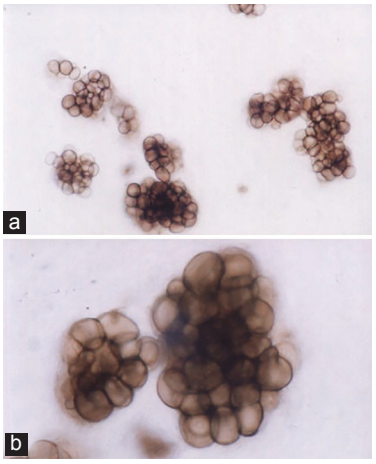
Colonies on PDA and glucose–Czapek's agar attained diameters of 2.5 and 2 cm, respectively, after 7 days of incubation at 28 ± 1°C. Mycelium at first appeared light brown, becoming dark brown with age, then brownish black, and finally reverse black; tiny drops of exudates appeared after 3 weeks of incubation; cultures produced ascomata after 3–4 weeks at 28 ± 1°C. Ascomata were

Figure 4



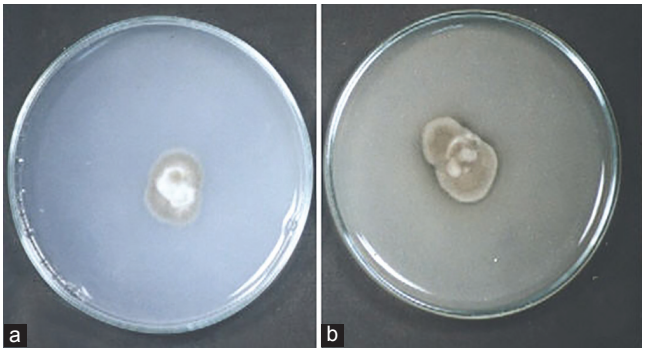
Beltrania querna Harkn.: (a) dark-colored conidiophores, setae, and conidia (×400); (b) enlarged part of conidiophore showing the terminal, polybastic sympodial conidiogenous cell, separating cells, and the biconic, appendiculate conidia (×1000).

Figure 6



Papulaspora immersa Hotson. (a) Dark irregular papulaspores showing central cells (×400) and (b) peripheral cells (×1000).

Figure 7



Unidentified ascomyceteous species: growth of the fungus on (a) Czapek's agar and (b) PDA, showing dark-colored mycelium.

dark, solitary, firmly adhering to the substratum, globose, and measured 360 mm in diameter. Asci were numerous, globose to subglobose with delicate, thin, transparent walls, and eight-spored, measuring 14–16 mm. Ascospores were ellipsoid to fusiform each, with one transverse septum, appearing light to dark brown under the microscope, measuring 10.5–12 mm in length×5.5–6.5 mm in width; no anamorph was observed (Figs. 7 and 8). This culture was isolated from the phyllosphere of pomegranate (*Punica granatum* L.) cultivated in El-Wady El-Assiuty.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References

1 Falloon RE. Fungi pathogenic to ryegrass seedlings. Plant Soil 1985; 86:79–86.

2 Dewan MM, Sivasithamparom K. Occurrence of species of *Aspergillus* and *Penicillium* in roots of wheat and rye grass and their effect on root rot caused by *Gaeumannomyces graminis* var. *tritici*. Aust J Bot 1988; 36:701–710.

3 Tsay JG. *Aspergillus* seedling blight of soybean and factors affecting its development. Plant Prot Bull 1990; 32:183–194.

4 Kiziewicz B, Zdrojkowska E, Gajo B, Godlewska A, Muszyńska E, Mazalska B. Occurrence of fungi and fungus-like organisms in the Horodnianka River in the vicinity of Białystok. Poland Wiad Parazytol 2011; 57:159–164.

5 Türkoğlu A, Castellano M. New records of truffle fungi (Basidiomycetes) from Turkey. Turk J Bot 2013; 37:970–976.

6 Durrell LW, Shields, LM. Fungi isolated in culture from soil of Nevada test site. Mycologia 1960; 52:636–644.

7 Hudson HJ. *Fungal saprophytism (studies in Biology)*. London: Edward Arnold Limited; 1972.

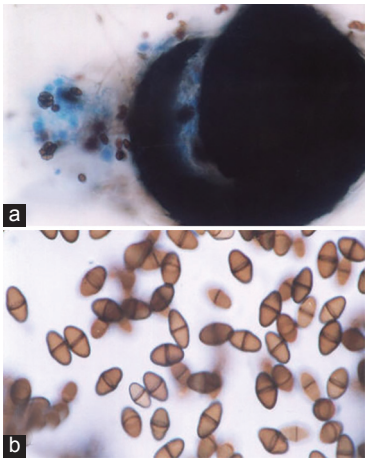
8 Fathi SM, El-Husseini TM, Abu-Zinada AH. Seasonal variations of soil mycoflora and their activities in Riyadh region, Saudi Arabia. Bull Fac Sci Riyadh Univ 1975; 7:17–29.

9 Ali MI. On the fungal flora of Saudi Arabia. 1-Wadi Hanifa. Bull Fac Sci Riyadh Univ 1977; 8:7–20.

10 Moubasher AH, Mazen MB, Abdel-Hafez AI. Some ecological studies on Jordanian soil fungi. I-Records of Mesophilic Fungi Naturalia Monospeliensis. Serie Bot 1977; 27:5–23.

11 Abdel-Hafez SI. Phyllosphere and phylloplane fungi of wheat cultivated in Saudi Arabia. Mycopathologia 1981; 75:33–38.

Figure 8



Unidentified ascomyceteous species. (a) Dark blackish ascoma and eight-spored globose-to-subglobose asci (´400). (b) Dark brown ellipsoidal ascospores, each with one transverse septum (´1000).

12 Halwagy R, Moustafa AF, Kamel SM. Ecology of soil mycoflora in the desert of Kuwait. J Arid Environ 1982; 5:109–125.

13 Moubasher AH, El-Dohlob SM. Seasonal fluctuations of Egyptian soil fungi. Trans Brit Mycol Sco 1970; 54:45–51.

14 Moubasher AH, Moustafa AF. A survey of Egyptian soil fungi with special reference to *Aspergillus, Penicillium* and *Penicillium* related genera. Trans Br Mycol Soc 1970; 54:35–44.

15 Abdel-Fattah HM, Moubasher AH, Abdel-Hafez SII. Fungus flora of root and leaf surface of broad bean cultivated in Oases, Egypt. Naturalia Monspeliensia Ser Bot 1977; 27:167–177.

16 Moubasher AH, Abdel-Hafez SII. Further study on seasonal fluctuation of Egyptian soil fungi. Mycopathologia 1978; 63:11–19.

17 Abdel-Hafez SII, Mohawed SM, El-Said AHM. Seasonal fluctuations of soil fungi of Wadi Qena at eastern desert of Egypt. Acta Mycologica 1989; XXV:113–125.

18 Abdel-Hafez AII, Mazen MB, Galal AA. Glycophilic and cellulose-decomposing fungi from soils of Sinai Peninsula, Egypt. Arab-Gulf J Sci Res 1990a; 8:153–168.

19 Abdel-Hafez SII, Mazen MB, Shaban GM. Seasonal fluctuations of rhizosphere and rhizoplane fungi of Egyptian wheat plant. Bull Fac Sci Assiut Univ 1990b; 19:173–184.

20 Abdel-Hafez SII, Moharram AM, Abdel-Sater MA. Monthly variations in the mycobiota of wheat fields in El-Kharga Oasis, Western desert, Egypt. Bull Fac Sci Assiut Univ 2000; 29:195–211.

21 Last FT. Seasonal incidence of *Sporobolomyces* on cereal leaves. Trans Br Mycol Soc 1955; 38:221–239.

22 Ruinen J. Occurrence of *Beijerinckia* species in the phyllosphere. Nature London 1956; 177:220–221.

23 Last FT. In: Preece TF, Dickinson CH, eds. Introduction. *Ecology of leaf surface microorganisms*. London: Academic Press; 1971.

24 Barragán A, Bernardi M, Rossi W. New records of Laboulbenia (Fungi, Ascomycota) from Ecuador and other countries. J Plant Taxon Geogr 2013; 68:25–34.

25 Sharma PD. Experimental studies of some micro-fungi from decaying shoots of *Setaria glauca*. Trans Brit Mycol Soc 1974; 63:397–400.

26 Eicker A. Non-parasitic mycoflora of the phylloplane and litter of *Panicum coloatum*. Trans Brit Mycol Soc 1976; 67:275–281.

27 Mazen MB, Abdel-Hafez SI, Shaban GM. Seasonal fluctuations of phyllosphere and phylloplane fungi of Egyptian wheat. Acta Mycologica 1985; 21:109–116.

28 Nagaraja TG. Rhizosphere and phyllosphere studies in *Strychnos nux-vomic* Linn. Ad Plant Sci 1991; 4:171–173.

29 Johnson LF, Curl EA, Bono JM, Fribourg HA. *Methods for studying soil microflora plant disease relationships*. USA: Minneapolis Publi. Co.; 1959. 178.

30 Menzies JD. A dipper technique for serial dilutions of soil for microbial analysis. Soil Sci Soc Amer Proc 1957; 21:660.

31 Thom C, Raper K. *A manual of the Aspergilli*. Baltimore, MD: Williams and Wilkins Co.; 1945.

32 Martin JP. Use acid, rosebengal and streptomycin in the plate method for estimating soil fungi. Soil Sci 1950; 69:215–233.

33 Ainsworth GC. *Ainsworth and Bisby's dictionary of the fungi*. Kew Surrey, England: Commonwealth Mycological Institute; 1971.

34 Domsch KH, Gams W, Anderson T-H. *Compendium of soil fungi*. London: Academic Press; 1980. 1 and 11.

35 Ellis MB. *Dematiaceous hyphomycetes*. Kew Surrey, England: Commonweath Mycological Institute; 1971.

36 Ellis MB. *More dematiaceous hyphomycetes*. Kew Surrey, England: Commonwealth Mycological Institute; 1976.

37 Kendrick B. *Taxonomy of fungi imperfecti*. Canada: Toronto University; 1971.

38 Moubasher AH. *Soil fungi in Qatar and other Arab countries*. Doha, Qatar: The Scientific And Applied Research Centre University of Qatar; 1993. 566.

39 Gu Q, Lin Z, Wang F, Wei H, Zhu T, Wang F. Three new cytochalasins from the marine-derived fungus *Spicaria elegans* KLA03 by supplementing the cultures with l- and d-tryptophan. Chem Biodivers 2011; 8:887–894.

40 Fukai M, Ishino M, Kinoshita K, Koyama K, Miki K, Shiraishi T, *et al.* Chemical constituents of a marine fungus, *Arthrinium sacchari*. J Nat Prod 2011; 74:1645–1649.

41 Vatcharin R, Chutanat K, Souwalak P, Zubaidah H. Eudesmane sesquiterpenes from the aquatic fungus *Beltrania rhombic*a. Chem Pharm Bull 2005; 53:238–240.

42 Mårten J, Ulf E, Mattias B, Katrin M. Selective antiproliferative activity of hydroxynaphthyl-β-d-xylosides. J Med Chem 2006; 49:1932–1938.

43 Li P, Claudio ML, Angelica PI, Edward PC, James RF, Abhay RS, Douglas K. Sterols with antileishmanial activity isolated from the roots of *Pentalinon andrieuxii*. Phytochemistry 2012; 82:128–135.

44 Bastos JK, Gallo MBC, Pupo MT, Araujo BFW, Cavalcanti BC, Costa-Lotufo LV, *et al.* Chemical constituents of *Papulaspora immersa*, an endophyte from *Smallanthus sonchifolius* (Asteraceae), and their cytotoxic activity. Chem Biodivers 2010; 7:2941–2950.

45 Amal HA, Abdessamad D, Carol C, RuAngelie EE, Barbora O, Marc D, *et al.* NF kappa B inhibitors and antitrypanosomal metabolites from endophytic fungus *Penicillium* sp. isolated from *Limonium tubiflorum*. Bioorg Med Chem 2011; 19:414–421.

46 Shugeng C, Lorna C, Kaiane AH, Catalina M, Giselle T, Michael SR, Jon C. Phenolic compounds as antiangiogenic CMG2 inhibitors from costa rican endophytic fungi. Bioorg Med Chem Lett 2012; 22:5885–5888.

47 Klaiklay S, Rukachaisirikul V, Sukpondma Y, Buatong, J, Phongpaichit S, Bussaban B. Metabolites from the mangrove-derived fungus *Xylaria cubensis* PSU-MA34. Arch Pharm Res 2012; 35:1127–1131.