Survey of all mycobiota associated with rhizosphere and rhizoplane of different cultivated plants in new reclaimed soil, upper Egypt, and examination of the most common fungal isolates to produce mycotoxins

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This survey was designed to study the diversity and occurrence of rhizosphere and rhizoplane fungi in the protectorate of Assiut in Egypt, followed by testing the ability of the most common isolated fungal strains to produce mycotoxins. Not many mycological studies have been carried out to describe the fungal flora of this area, which will be of great significance for the endemic mycobiota. Rhizosphere and rhizoplane samples were collected from the protectorate of Assiut, which represents one of the largest distinctive regions of newly reclaimed soil at the Assiut Governorate. The identification of the isolated fungi during our investigation was carried out using the morphological and microscopic features according to many references and confirmed by the Assiut University Mycology Center (AUMC). The most common four fungal species were examined for their capability to produce mycotoxins; in addition, chemical confirmatory tests for mycotoxins were examined.

Keywords:
fungi, identification, isolation, mycotoxins, rhizoplane, rhizosphere

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Introduction

Many papers have reported the pathogenicity of fungi that inhabit rhizosphere and rhizoplane of many crops [1,2]. Very few investigations have been carried out on the mycobiota of the newly reclaimed localities at the Assiut Governorate, especially the protectorate of Assiut, which represents one of the largest newly reclaimed areas at the Assiut Governorate cultivated with different important crops. The terms rhizosphere and rhizoplane are now used widely by microbial ecologists and pathologists. Several studies have been carried out to characterize the flora of root surface and soil adhering to the roots of some plants [3–5]. In Egypt, the root surface fungi have received some attention in cultivated and desert plants [6–9]. El-Hissy *et al*. [8] studied the composition of rhizosphere fungi in root and stem segments of *Helianthus annuus*, *Chrysanthemum coronarium*, *Nigella sativa*, *Datura innoxia*, and *Hyoscyamus muticus*, which is presumably affected selectively by root metabolites. Abdel-Hafez [4] recovered 55 species and one variety belonging to 24 genera as rhizosphere fungi from four fern plants growing in Saudi Arabia, and found that *Aspergillus*, *Penicillium*, *Fusarium*, and *Mucor* were the most common genera of rhizosphere fungi. Moubasher *et al*. [10] reported that *Fusarium solani* and *Fusarium oxysporum* were the most common fungi isolated from the rhizoplane of five plants. Mazen *et al*. [9] reported that the total count of fungi of rhizosphere and rhizoplane fungi of five plants did not show regular seasonal periodicity and this was because of the abnormally high counts of some fungi in some months. Abdel-Hafez *et al*. [11] reported that the most widespread rhizosphere fungi of wheat plant were *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Alternaria alternata*, *F. oxysporum*, *Humicola grisea*, *Scopulariopsis brevicaulis*, and *Paecilomyces variotii*, whereas the most common rhizoplane fungi of wheat plant were *A. niger*, *A. fumigatus*, *A. alternata*, *F. oxysporum*, and *F. solani*. Abdel-Hafez *et al*. [12] studied the seasonal fluctuations of rhizosphere and rhizoplane fungi of sugarcane and they found that *A. flavus*, *A. niger*, *Cochliobolus spicifer*, *Gibberella fujikuroi*, *Nectria haematococca*, and *Penicillium chrysogenum* were the most common species. Abdel-Hafez *et al*. [13] reported that the most common rhizosphere species of wheat plant were *A. alternata*, *A. fumigatus*, *Aspergillus tamarii*, *Cochliobolus lunatus*, *F. oxysporum*, *Gliocladium roseum*, and *H. grisea*.

Not many mycological studies have been carried out to describe the fungal flora of newly reclaimed soil of the protectorate of Assiut in Egypt. The present work aimed to survey all fungal isolates associated with rhizosphere and rhizoplane of different cultivated plants throughout the year in this area; also, the ability of the most common fungal isolates to produce mycotoxins was investigated.

Materials and Methods

Selected area

The protectorate of Assiut lies 25 km southeast of Assiut and represents one of the largest and distinctive regions of newly reclaimed soil at the Assiut Governorate. The main dominant plants cultivated in this area are listed in Table 1.

Rhizosphere samples

Plants in Table 1 from the above-mentioned area were uprooted and gently shaken to remove superfluous soil, placed in sterilized plastic bags, and transferred to the laboratory to determine rhizosphere fungi. Samples were collected every month during the growing season of the plants.

Rhizoplane samples

From the same above area, plants (Table 1) were collected every month as well as uprooted, dislocated from the adhering soil, and directly transferred into clean and sterilized plastic bags. Then, they were kept in a refrigerator for further fungal analysis.

Isolation and identification of fungi

Rhizosphere fungi

Isolation and identification of fungi were carried out according to Timonin [14] and used in this laboratory by Moubasher and Abdel-Hafez [15] as follows:

(1) Blocks of soil containing plants roots were cut out and gently crushed, with as little tearing of roots as possible. The roots were removed and gently shaken to remove superfluous soil. Two grams of roots were placed with adhering soil particles in a weighed flask that contained 100 ml of sterile water. After thorough shaking, suitable dilutions were prepared.
(2) To determine the weight of rhizosphere soil, the roots were removed from the original dilution flasks and washed. The washing was collected in the original flask and then evaporated on a water bath; the soil residues were dried to a constant weight in an oven at 105–116°C. The flask containing the dry soil was weighed and the dilution factors were calculated, allowance being made for the amount of soil removed while preparing the dilutions.
(3) One milliliter of the rhizosphere soil suspension was transferred to a sterile Petri dish and cover with melted but cooled agar medium. For every sample of rhizosphere, five plates were used, poured with glucose – Czapek's agar. The plates were incubated at 28 ± 1°C for 7 days, during which the developing fungi were examined microscopically for identification.

Table 1 List of common plants cultivated in the protectorate of Assiut

Ocimum basillicum L. (Basil)
Zea mays L. (Maize)
Helianthus annuus L. (Sun-flower)
Hordeum vulgare L. (Barley)
Artemisia herba-alba Del. (Wormwood)
Vicia faba L. (Broad bean)
Datura stramonium L. (jimsonweed)
Rosmarinus officinalis L. (Rosemary)
Trifolium alexandrinum L. (Egyptian clover Fenugreek)
Trigonella foenum-graecum L. (Bird's Foot)
Zygophyllum coccineum L. (Syrian bean caper)

Rhizoplane fungi

The roots of plants were subjected to a series of washing with sterile-distilled water. They were dried thoroughly between sterile filter papers, cut into equal segments (each about 1 cm), and five of them (per dish) were placed on the surface of the agar medium [11]. For every sample of rhizoplane, 20 plates were incubated at 28 ± 1°C for 7 days, during which the developing colonies were identified.

Identification of fungal genera and species

Identification of the isolated fungi during our investigation was carried out using the morphological and microscopic features according to:

Ames [16] for *Chaetomium* spp., Booth [17] for *Fusarium* spp., Domsch *et al.* [18] for soil fungi in general, Ellis [19] for *Dematiaceous hyphomycetes*, Moubasher [20] for fungi in general, Pitt [21] for *Penicillium* spp., Raper and Fennell [22] for *Aspergillus* spp., and Rifai [23] for *Trichoderma* spp. Also, identification of the isolated fungi was reviewed and compared with the same species stored at the Assiut University Mycological Center (AUMC).

Screening for mycotoxins production

Fungal isolates

The four most common fungal species were examined for their ability to produce mycotoxins.

Cultivation

Each isolate was inoculated into 250 ml Erlenmeyer flasks. Each flask contained 50 ml of glucose – Czapek's liquid medium supplemented with 0.2% yeast extract and 1% peptone. The flasks were sterilized at 1.5 atmospheres for 20 min and inoculated after cooling

with 2 ml of the inoculum's suspension. The cultures were incubated at 28±1°C as a stationary cultivation for 8 days in case of *Chaetomium globosum* and *C. spicifer* isolates. *F. oxysporum* and *F. solani* cultures were incubated at 28±1°C for 8 days and then at 15°C for another 8 days as static cultures.

Extraction of the crude toxins

After incubation, the content of each flask (medium+mycelium) was homogenized in a high-speed blender (16 000 rpm) with 100 ml chloroform. The chloroform extract was decanted off and re-extracted by another 100 ml chloroform. The chloroform extracts were combined, washed with an equal volume of distilled water, dried over anhydrous sodium sulfate, filtered and then concentrated under vacuum, and the dry material was transferred to a dram vial with a small amount of chloroform, which was evaporated to near dryness. The content of each flask, after decanting the chloroform extract, was extracted again by 100 ml of 90% aqueous methanol. The aqueous methanol extract was decanted off and re-extracted by another 100 ml methanol. The aqueous methanol extracts were combined, concentrated under vacuum, which were extracted again by acetonitrile (three times), concentrated, transferred to a dram vial, and evaporated to near dryness [24].

Thin layer chromatographic determination of mycotoxins

Thin layer chromatography plates G60 F254 were used for the qualitative analysis of mycotoxins.

Solvent systems

To separate the different mycotoxins, the solvent systems of the following compositions were used, all of reagent grade:

(1) Benzene : methanol : acetic acid (90 : 2: 15, v/v/v) for strigmatocystin [25].
(2) Dichloromethane : methanol (95 : 5, v/v) for trichothecenes [26].
(3) Benzene : acetone (95 : 5, v/v) for zearalenone [26].

Application and development

The samples to be analyzed were applied as 0.01 ml solutions in chloroform or methanol or a mixture of both using micropipettes. The spots were dried during application with a flow of cold air. The plates were developed in developing tanks 15 × 30 × 30 cm in diameter (Zeiss, Jena, Germany) saturated with solvent vapor. Each substance was chromatographed in two series in all the solvent systems. When the front of the systems reached a height about 15 cm above the origin, the development was interrupted, the chromatogram was dried in air, and then detection was carried out.

Determination and reagents

The developed plates were detected before and after spraying with the different reagents under short wave (254 nm) and long wave (354 nm) ultraviolet irradiation (UV IS, Desage, Heidelberg, Germany), mycotoxins were identified by comparison with appropriate reference standards after each of the following treatments:

Strigmatocystin

The compound shows dull brick red fluorescence under short wave UV light. Fluorescence changes to yellow on spraying with aluminum chloride solution (20 g $AlCl_3 \cdot 6H_2O$) in 100 ml ethanol with the plate heated at 100°C for 5 min [27].

Trichothecenes

4-*p*-Nitrobenzyl pyridine reagent: solution of reagent in chloroform : carbon tetrachlorides (2 : 3) was used as 1% (for detection) or as 3% (for quantification). The plates were heated at 105°C for 30 min after spraying and then resprayed by tetraethylene pentamine as a 10% solution in the same solvent [28].

(1) Sulfuric acid reagent: a solution of 20% sulfuric acid in methanol was used. The plates were heated at 110°C for 10 min.
(2) *P*-anisaldehyde reagent: consisted of a mixture of 0.5 ml of *p*-anisaldehyde+85 ml of methanol +10 ml of glacial acetic acid +5 ml of concentrated sulfuric acid. The plates were heated at 130°C for 15 min after spraying [29].

Zearalenone

Zearalenone fluoresces blue-green under long wave light and more greenish under short wave UV light. It is ferric chloride and 2,4-dinitrophenylhydrazine positive and develops green spots with 50% sulfuric acid in methanol that rapidly turns to yellow [30]. Standard samples of the different mycotoxins used in this study were purchased from Sigma Chemical Company (USA).

Chemical confirmatory tests for mycotoxins

Strigmatocystin

The identity and quantity of strigmatocystin in the extracts were determined using the method described by Schroeder and Kelton [31].

Trichothecenes

The presence of trichothecenes was confirmed by the formation of different color reactions reported by Gorst and Peter [32] using celinum sulfate, Ehrlich reagent, vanillin, and 2,4-dinitrophenylhydrazine.

Results

In this survey, 24 genera, 54 species, and three species varieties were isolated and identified from rhizosphere and 23 genera, 45 species, and two species varieties were isolated and identified from rhizoplane of the different cultivated plants in the protectorate of Assiut on glucose–Czapek's agar medium at 28 ± 1°C. *Aspergillus* was the most dominant genus with respect to its occurrence with 11 species; in addition, one species variety belonging to *Aspergillus* was identified. *Aspergillus japonicus* was the most common species. *A. terreus* and *A. flavus* were the second and third common species. *Aspergillus ustus*, *Aspergillus aegyptiacus*, and *Aspergillus versicolor* were also isolated at high frequencies. However, *Aspergillus sydowii* and *Aspergillus ochraceus* were isolated at moderate frequencies as can be seen in Table 2. *Fusarium* (eight species) was the second common genus with respect to the occurrence and the most common species were *F. oxysporum* and *F. solani*, whereas *Fusarium equiseti* was isolated at a moderate frequency. *Chaetomium* (two species) and *Emericella* (three species and two

Table 2 Occurrence of fungi isolated from the protectorate of Assiut

Genera and species	Protectorate of Assiut		Genera and species	Protectorate of Assiut	
	Rhizosphere	Rhizoplane		Rhizosphere	Rhizoplane
Acremonium roseolum	–	R	*Fusarium lateritium*	–	R
Acremonium strictum	M	L	*Fusarium moniliforme*	R	–
Alternaria alternata	L	M	*F. moniliforme* var. *subglutinanas*	–	L
Alternaria chlamydospora	–	R	*Fusarium nivale*	R	R
Aspergillus aegyptiacus	H	M	*Fusarium oxysporum*	H	H
Aspergillus carbonarius	–	R	*Fusarium semitectum*	R	L
Aspergillus flavipes	L	R	*Fusarium solani*	H	M
Aspergillus flavus	H	H	*Humicola grisea*	R	–
A. flavus var. *columnaris*	R	–	*Macrophomina phaseolina*	L	M
Aspergillus japonicus	H	H	*Mucor fuscus*	R	R
Amelleus melleus	L	–	*Mucor circinelloides*	R	R
Aspergillus ochraceus	M	R	*Myrothecium roridum*	R	R
Aspergillus sydowii	M	–	*Myrothecium verrucaria*	M	M
Aspergillus tamarii	R	–	*Papulaspora immersa*	R	–
Aspergillus terricola	–	R	*Penicillium brevicompactum*	L	–
Aspergillus terreus	H	H	*Penicillium chrysogenum*	M	L
Aspergillus ustus	H	M	*Penicillium corylophilum*	–	R
Aspergillus versicolor	H	H	*Penicillium funiculosum*	R	–
Botryotrichum piluliferum	M	R	*Penicillium purpurogenum*	M	R
Chaetomium globosum	H	H	*Penicillium roqueforti*	R	–
C. spirale	R	–	*Pestalotia spp.*	R	M
Circinella muscae	R	–	*Phoma herbarum*	M	M
Cladosporium cladosporioides	R	M	*Phoma leveillei*	–	R
Cochliobolus hawaiiensis	R	R	*Rhizoctonia solani*	–	L
Cochliobolus lunatus	R	–	*Rhizopus stolonifer*	H	H
Cochliobolus spicifer	H	H	*Setosphaeria rostrata*	R	R
Cunninghamella echinulata	M	M	*Stachybotrys chartarum*	H	M
Cunninghamella elegans	R	R	*Stemphylium botryosum*	–	R
Doratomyces microsporus	R	–	*Trichoderma hamatum*	–	M
Emericella nidulans	H	H	*Trichoderma harzianum*	–	R
E. nidulans var. acristata	M	L	*Trichoderma koningii*	–	R
E. nidulans var. lata	R	–	*Ulocladium botrytis*	R	–
Emericella quadrilineata	M	–	*Ulocladium chartarum*	L	R
Emericella rugulosa	R	–	*Ulocladium chlamydosporum*	L	L
Fusarium culmorum	R	M	Total number of genera	24	23
Fusarium equiseti	M	L	Total number of species	54	45
Fusarium graminearum	R	–			

H, high occurrence (>5 months throughout the year); L, low occurrence (2 months); M, moderate occurrence (between 3 and 5 months); R, rare occurrence (1 month).

species varieties) were also isolated at high frequencies and the most common species were *C. globosum* and *Emericella nidulans*, whereas *E. nidulans* var. acristata and *Emericella quadrilineata* were isolated at moderate frequencies. *Penicillium* (five species), *Rhizopus* (one species), *Stachybotrys* (one species), and the fungi with dark sterile mycelia were also isolated at a high frequency. The most common species were *P. chrysogenum*, *Penicillium purpurogenum*, and *Rhizopus stolonifer*. Among rhizoplane mycobiota, 45 species belonging to 23 genera in addition to the fungi with sterile mycelia were isolated and identified during this study as shown in Table 2. *Aspergillus* was also the most dominant genus. It was recovered at a high frequency with nine species and the most common species was *A. japonicus*; also, *A. flavus*, *A. terreus*, and *A. versicolor* were isolated at high frequencies. *A. ustus* and *A. aegyptiacus* were isolated at moderate frequencies. *Fusarium* (seven species and one variety) was the second common genus and the most common species was *F. oxysporum*. However, *F. solani* and *Fusarium culmorum* were isolated at moderate frequencies. *C. spicifer*, *R. stolonifer*, *E. nidulans*, and *C. globosum* were isolated at high frequencies, whereas the remaining genera and species were isolated at other frequencies of occurrence as can be seen in Table 2.

Mycotoxins' potential to produce the selected fungal isolates

The most common eight isolates representing four species (two isolates each) were examined for mycotoxin production using glucose–Czapek's liquid medium supplemented with 0.2% yeast extract and 1% peptone as a static culture. The results showed that the extracts of the two tested isolates of each of *C. spicifer*, *F. oxysporum*, and *F. solani* in addition to one isolate of *C. globosum* were of high or moderate toxicity to brine shrimp larvae, whereas only one isolate of *C. globosum* was non toxic to the test larvae (Table 3).

C. globosum produced strigmatocystin at 420 ± 39 μg/50 ml medium, whereas the other *Chaetomium* isolate could not produce detectable amounts of this or other mycotoxins.

However, the two tested isolates of *C. spicifer* produced an unidentified toxic factor on the basis of thin layer chromatography analysis that could not be identified owing to the lack of authentic toxin references. Diacetoxyscirpenol and zearalenone were produced by all tested isolates of two *Fusarium* spp. under investigation at concentrations that ranged from 260–420 to 220–510 μg/50 ml medium, respectively. T-2 toxin was detected in the extract of only one isolate of *F. solani* at 28±32 μg/50 ml medium (Table 3).

Discussion

In this survey, 24 genera, 54 species, and three species varieties were isolated and identified from rhizosphere; in addition, 23 genera, 45 species, and two species varieties were isolated and identified from the rhizoplane of the different cultivated plants in the protectorate of Assiut. Most of these species and genera were isolated from the rhizosphere and rhizoplane of some Egyptian plants [4,6,7,11,13]. Several fungi were common only in rhizospheres such as *A. ochraceus*, *Botryotrichum piluliferum*, *E. quadrilineata*, *F. solani*, *P. purpurogenum*, and *Stachybotrys chartarum*, whereas other fungi such as *Cladosporium cladosporioides*, *F. culmorum*, *Pestalotia* spp., and *Trichoderma hamatum* were common in rhizoplane. El-Hissy *et al.* [8] isolated *Stachybotrys atra* and *A. niger*; *Cladosporium herbarum*, *A. sydowii*, and *Penicillium funiculosum*; *Fusarium moniliforme* and *A. sydowii*; *A. fumigatus* and *A. terreus*; and *C. herbarum* and *A. sydowii* as most prevalent fungi in the rhizosphere of *H. annuus*, *C. coronarium*, *N. sativa*, *D. innoxia*, and *H. muticus*, respectively, at Assiut, Egypt. Also, Abdel-Hafez *et al.* [11] studied the seasonal fluctuations of rhizosphere and rhizoplane fungi of Egyptian wheat plant and found that the most common rhizosphere fungal species were *A. niger*, *A. terreus*, *A. fumigatus*, *A. flavus*, *A. ochraceus*,

Table 3 Comparative studies on production of mycotoxins by the most common fungal isolates

Fungal species	DW ± SD	Toxicity[a]	Mycotoxins detected	MC ± SD
Chaetomium globosum (no. 164)	986 ± 100	A	Strigmatocystin	420 ± 38
C. globosum (no. 215)	1021 ± 99	D	Negative	—
Cochliobolus spicifer (no. 81)	896 ± 93	A	UITF	—
C. spicifer (no. 314)	864 ± 88	B	UITF	—
Fusarium oxysporum (no. 114)	785 ± 82	A	Diacetoxyscirpenol Zearalenone	260 ± 25220 ± 24
F. oxysporum (no. 93)	827 ± 85	A	Diacetoxyscirpenol Zearalenone	310 ± 30270 ± 32
Fusarium solani (no. 70)	731 ± 75	A	Diacetoxyscirpenol Zearalenone	420 ± 50510 ± 55
F. solani (no. 139)	659 ± 70	A	DiacetoxyscirpenolT-2 toxin Zearalenone	380 ± 45280 ± 32440 ± 55

DW, dry weight (mg/50 ml medium); MC, mycotoxins concentration (μg/50 ml medium); UITF, unidentified toxic factor., [a]Toxicity test: A: high, for 75–100% mortality of brine shrimp larvae; B: moderate, for 50–74% mortality; C: low, for 25–49% mortality; D: none, for 0–24% mortality.

A. alternata, *Acremonium strictum*, *Mucor hiemalis*, *F. oxysporum*, *H. grisea*, and *Trichothecium roseum.* From the rhizoplane, the most prevalent species were *Alternaria grisea*, *F. oxysporum*, and *F. solani.* From the wheat cultivated in El-Karga Oasis, western desert-Egypt, Abdel-Hafez *et al*. [13] isolated *A. alternata*, *A. niger*, *A. tamarii*, *C. cladosporioides*, *C. lunatus*, *F. oxysporum*, *G. roseum*, and *P. chrysogenum* as the most common rhizosphere species.

A. alternata, *Aspergillus flavipes*, *A. strictum*, *E. nidulans* var. acristata, and *P. chrysogenum* were isolated at low frequencies in this study in rhizosphere and/or rhizoplane. *Acremonium roseolum*, *Alternaria chlamydospora*, *C. lunatus*, *Cunninghamella elegans*, *Emericella rugulosa*, *Fusarium semitectum*, *H. grisea*, *Mucor circinelloides*, and *Ulocladium botrytis* were isolated rarely in rhizosphere and/or rhizoplane. Also, most of these genera and species have been discussed in other previous works from the rhizosphere and or rhizoplane of various plants [5,7,8,11,13,33,34].

The most common fungal isolates belonging to *C. spicifer*, *C. globosum*, *F. oxysporum*, and *F. solani* (*N. haematococca*) (two isolate per each) were examined for their capability to produce mycotoxins; *C. globosum* was represented by two isolates. The extract of one isolate no. 164 had high toxicity to brine shrimp larvae and produced strigmatocystin at a concentration of 420 μg/50 ml medium, whereas the other isolate no. 215 had no toxicity to the test larvae and could not produce a detectable amount of this or other mycotoxins. Strigmatocystin is a highly toxic chemical metabolite produced by various species of *Aspergillus*, *Bipolaris*, *Penicillium*, and *Chaetomium* [35,36].

The two tested isolates of *C. spicifer* produced an unidentified toxic factor according to the biological assay. The extract of the first isolate no. 81 had high toxicity, whereas the extract of the second no. 314 had moderate toxicity to the test larvae. Panaccione *et al*. [37] reported that the genetic, biochemical, and molecular analyses indicated that the specific pathogenicity of *Cochliobolus carbonum* is because of the production of the cyclic tetrapeptide HC-toxin. Shukla *et al*. [38] found that the toxin produced by *Drechslera maydis* (called drechslerol-C) caused necrotic and chlorotic lesions on the leaves of *Cheilocostus speciosus* at concentrations ranging from 2.85×10^{-5} to 2.28×10^{-4} mol/l.

The two tested isolates of both *F. oxysporum* and *F. solani* had high toxicity to brine shrimp larvae. Diacetoxyscirpenol and zearalenone were produced by the four tested *Fusarium* isolates at concentrations ranging from 260–420 to 220–510 μg/50 ml medium, respectively. T-2 toxin was detected in the extract of only one isolate of *F. solani* (no. 139) at 280 μg/50 ml medium. The production of zearalenone by *F. oxysporum* and *F. solani* has been reported previously [24,39].

Conclusion

In this survey, 24 genera, 54 species, and three species varieties were isolated and identified from rhizosphere and 23 genera, 45 species, and two species varieties were isolated and identified from the rhizoplane of the different cultivated plants in the protectorate of Assiut. *C. spicifer*, *C. globosum*, *F. oxysporum*, and *F. solani* (*N. haematococca*) (two isolates each) were the most common isolates and hence were examined for their capability to produce mycotoxins.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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