

Diversity of inulinase-producing fungi associated with two Asteraceous plants, *Pulicaria crispa* (Forssk.) and *Pluchea dioscoridis* (L.) growing in an extreme arid environment

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ABSTRACT

Inulinases are potentially valuable enzymes catalyze the hydrolysis of plant's inulin into high fructose syrups as sweetening ingredients for food industry and ethanol production. The high demands for inulinase enzymes have promoted interest in microbial inulinases as the most suitable approach for biosynthesis of fructose syrups from inulin. Arid land ecosystem represents a valuable bioresource for soil microbial diversity with unique biochemical and physiological properties. In the present study, we explored the fungi diversity associated with the rhizosphere and rhizoplane of two desert medicinal plants namely *Pluchea dioscoridis* and *Pulicaria crispa* growing in the South-Eastern desert of Aswan, Egypt. A total of 180 fungal isolates were screened based on their ability to grow on potato dextrose agar medium supplemented with 1% inulin. The isolated fungal colonies were morphologically identified according to cultural characteristics and spore-bearing structure. In addition, the inulinase activity of the isolated fungi was examined

spectrophotometrically. Among these, *Aspergillus terreus* var. *terreus* 233, *Botrytis cinerea*, *Aspergillus aegyptiacus*, *Cochliobolus australiensis* 447 and *Cochliobolus australiensis* exhibited high inulinase activity ranging from 5.05 to 7.26 U/ml. This study provides a promising source of microbial inulinase, which can be scaled up for industrial applications.

Keywords: Microbial inulinase; Arid land; *Pluchea dioscoridis*; *Pulicaria crispa*.

1. INTRODUCTION

Fungi are a diverse group of microorganisms comprising seven known phyla, including Ascomycota, Basidiomycota, Microsporidia, Glomeromycota, Blastocladiomycota, Chytridiomycota and Neocallimastigomycota [1]. Fungi communities are essential soil components as both decomposers and plant symbionts, playing critical roles in the ecological and biogeochemical processes in natural environments [2-5]. Fungi communities can undergo

transitory variations in its structure due to the selective pressure exerted by the surrounding environment [6, 7]. For example, it is well known that production and diffusion of root exudates are affected by plant health and development, and these exudates exercise a selective microbial stimulation/inhibition that varies according to the unique rhizosphere nutrient pool [3, 5, 7, 8]. In this context, plant roots represent a favorable habitat for diverse fungi populations, including those that colonize the soil zone around the root (Rhizosphere) as well as the root surface (Rhizoplane) [8-10]. Rhizosphere and rhizoplane areas are always affluent in various fungal populations with vigorous activities in close association with host plant [11]. Therefore, the co-evolution of plant roots and soil fungi plays a significant role in soil physical and biological processes that increase biodiversity and soil fertility in agricultural and natural systems [3, 4, 7].

Inulin is a widespread naturally occurring storage polysaccharides found in the roots of several plants belonging to Gramineae and Asteraceae families [12, 13]. Inulin composed of fructose unit chains with various length, linked by β -2,1-D-fructosyl-fructose bonds, and generally terminated by a single glucose unit connected by the α -D-glucopyranosyl bond [13, 14]. Inulinases (2,1- β -D-fructan fructanohydrolases) are potentially useful enzymes catalyze the conversion of plant inulin into high fructose syrups as sweetening ingredients for the food industry and ethanol production [13-15]. Although inulinases were first isolated from plants, it is very difficult to separate plant inulinase in sufficient quantities for industrial and biochemical applications [12]. In addition, the acid hydrolysis of inulin to fructose displays several drawbacks, which resulted in difructose anhydrides as a final result with no sweetening properties [12, 16]. These drawbacks have forced interest in microbial inulinases as the most suitable method for biosynthesis of fructose syrups from inulin [13, 14, 17]. There are several fungal species that can produce inulinase enzymes, among which, *Aspergillus*, *Penicillium*, and *Khyveromyces* species are the most common fungi that used for inulinase industrial production [18]. The increasing potential of inulinase applications promoted the screening of new inulinase-producing fungi with high thermostable character. In the present study, we isolated, identified rhizosphere

and rhizoplane fungi from two desert medicinal plants including *Pluchea dioscoridis* and *Pulicaria crispa* growing naturally in the South-Eastern desert of Aswan governorate, Egypt. The isolated fungi were screened based on their ability to grow on potato dextrose agar (PDA) medium supplemented with 1% inulin as the only carbon source, followed by quantitative analysis of their inulinase enzymatic activity using spectrophotometric analysis. The obtained results demonstrated the potential of the desert-adapted plants like a rich bioresource for various fungal strains with high inulinase activity, which can be used for industrial purpose.

2. MATERIALS AND METHODS

2.1. Study area

This study was performed at Aswan University, Aswan governorate, Egypt (24° 5' 15" N 32° 53' 56" E). The rhizosphere and rhizoplane samples from *P. dioscoridis* and *P. crispa* were collected from three different locations including Aswan University campus (Location I, sites 1-6), Aswan airport road (location II, sites 7-8) and Aswan dam road (Location III, sites 9-10). The climatic conditions in this region ranged from very hot dry summer (30 to 50°C) to moderately cold dry winter (10 to 25°C) [3].

2.2. Preparation of rhizosphere and rhizoplane samples

In each study site, three replicates were established with one-meter distance from each other, at 20-35 cm depth from the surface. *P. dioscoridis* and *P. crispa* roots were carefully uprooted, and roots with adhering soil particles were placed in sterilized plastic bags and transported to the laboratory. The rhizosphere samples were collected according to Timonin [19], and Moubasher and Abdel-Hafez [20]. One gram of roots was lightly scraped to collect the firmly adhering soil particles to the root surface by using sterilized hairbrush and spatula. The collected rhizosphere-soil samples were subjected to serial dilution (10^{-4}) using sterile distilled water (SDW). One ml of the rhizosphere soil suspension was transferred to PDA plates amended with chloramphenicol (500 mg l⁻¹). The

plates were incubated in an incubation chamber at $28 \pm 1^\circ\text{C}$ for 7 days. The number of fungal colonies (cfu g^{-1} soil) formed on PDA dilution plates in each sample was calculated.

The rhizoplane samples were collected from plant roots by washing one-gram of roots with SDW and cut into approximately 1 cm pieces. Root pieces were allowed to surface-dry under sterile conditions prior to placement into PDA plate. Five pieces of root were placed on PDA plates amended with chloramphenicol (500 mg l^{-1}) and incubated at $28 \pm 1^\circ\text{C}$ for 7 days [21]. The number of fungal colonies (cfu g^{-1} root) formed on PDA dilution plates in each sample was calculated.

2.3. Identification of fungi

All the isolated fungal colonies were morphologically identified according to cultural characteristics (color, texture, and pigmentation), and spores and spore-bearing structure using standard identification manuals [22-28].

2.4. Screening of inulinase-producing fungi

All isolated strains from the rhizosphere and rhizoplane samples were screened for their ability to grow on Czapek's agar medium supplemented with inulin as the only carbon source and were incubated at $28 \pm 1^\circ\text{C}$ for 7 days. A total of 180 isolates were able to grow on inulin-Czapek's agar medium, and these strains were further used for inulinase assay.

2.5. Inulinase assay

A loop of the selected fungal isolates was inoculated separately in 5 ml fermentative medium consisted of inulin 10.0, NaNO_3 3.0, K_2PO_4 1.0, MgSO_4 0.5, KCl 0.5 and FeSO_4 0.001 g/l. The inoculated-cultures were incubated at 30°C for 12 days. The fermented broth was centrifuged at 10000 rpm, for 20 minutes at 4°C , and the supernatant was filtered and used as the crude enzyme extract. Inulinase activity was carried out spectrophotometrically according to Singh et al. [29]. The reaction mixture consisted of one ml crude enzyme extract and 0.8 ml of 2% (w/v) inulin dissolved in 0.05 M sodium acetate buffer (pH 5.5). The reaction

mixture was incubated at 37°C for 60 min, then 2 ml dinitrosalicylic acid (DNS) reagent was added to the reaction. The mixture was boiled for 10 min, immediately cooled on ice and absorbance was measured at 540 nm. One enzyme unit was defined as the amount of enzyme that produces 1 μmol of fructose from inulin per min under standard assay conditions.

3. RESULTS AND DISCUSSION

Since the early 90s, the plant-soil fungi associations have been the subject of intensive research, which enable us to gain an insight into the critical role of soil fungal symbionts in plant ecology and physiology [30]. However, little is known about the plant-rhizosphere and rhizoplane fungi associations in the desert ecosystems where water availability is limited. In our recent studies, we were able to illustrate the significant roles of *Trichoderma longibrachiatum* isolated from desert soil in Egypt that confer beneficial agronomic traits to onion (*Allium cepa*) [3], and the ecophysiological role of *Thermomyces* endophyte CpE-mediated heat stress tolerance in cucumber, which will facilitate the cultivation of heat-tolerant cucumber (*Cucumis sativus*) [4]. This study extended the previous work to isolate prospective rhizosphere and rhizoplane fungi from two desert medicinal plants *P. dioscoridis* and *P. crispa* and their potential for inulinase enzyme production.

3.1. Fungal occurrence and diversity

A total of 180 fungal isolates were isolated from the rhizosphere and rhizoplane of *P. dioscoridis*, and *P. crispa* based on their ability to grow on PDA medium supplemented with 1% inulin as the only carbon source (Figs. 1-2, Supplementary Fig. S1). The morphological identification of all fungal isolates was confirmed based on the anamorph and teleomorph characters using a light microscope (Fig. 3). A total of 62 fungal isolates were obtained from rhizoplane of *Pluchea dioscoridis*, while 28 fungal isolates were isolated from rhizosphere of *P. dioscoridis* (Fig. 4A). In addition, five isolates were overlapped between *P. dioscoridis* rhizosphere and rhizoplane (Fig. 4A).

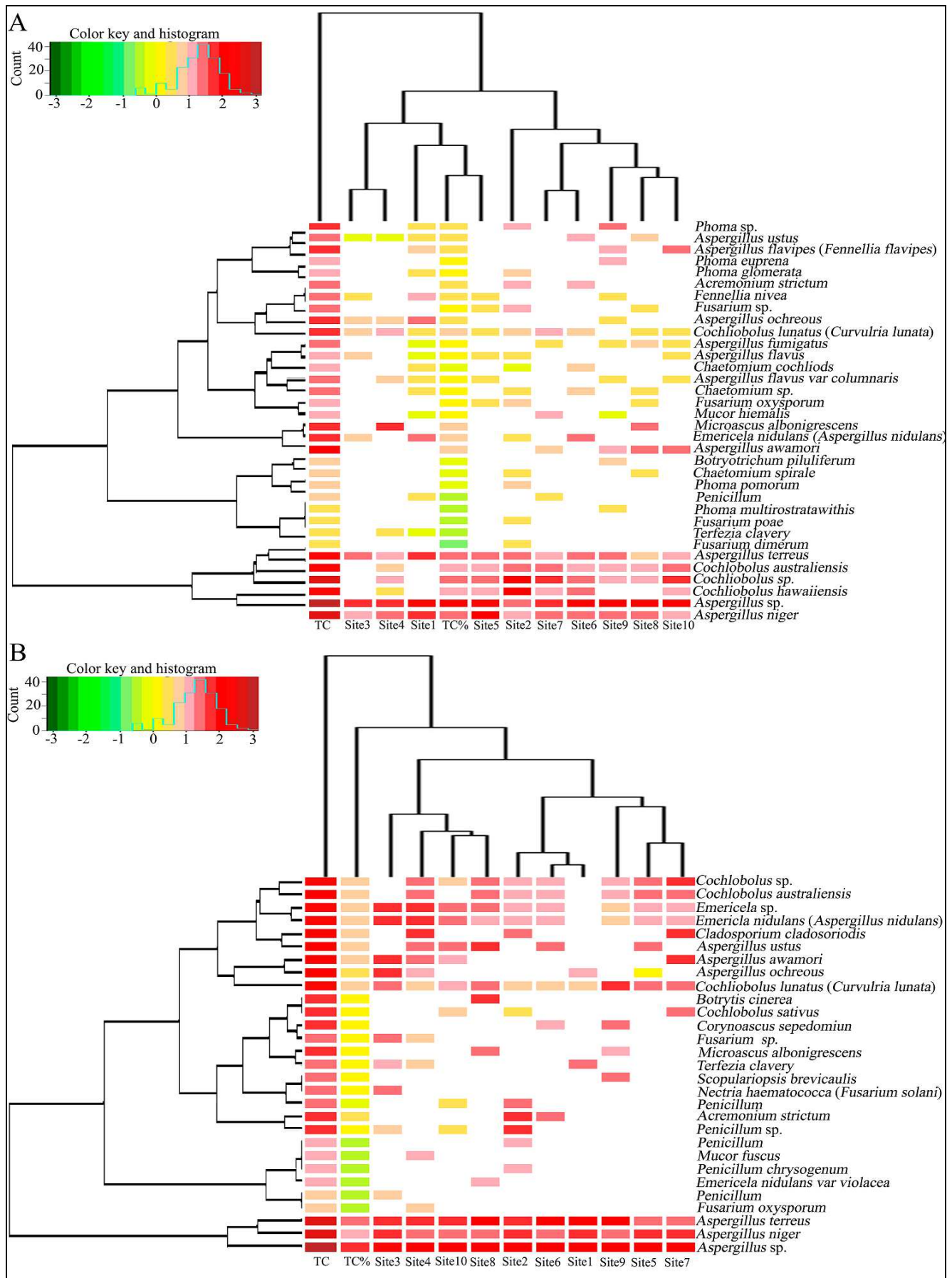


Figure 1. Heatmap clustering of the total number of fungi species isolated from *Pluchea dioscoridis*-rhizoplane (A) and *P. dioscoridis*-rhizosphere (B) at different sites (1-10). To construct heatmap color scale, the fungal counts were normalized using logarithm of base 10. TC, total count.

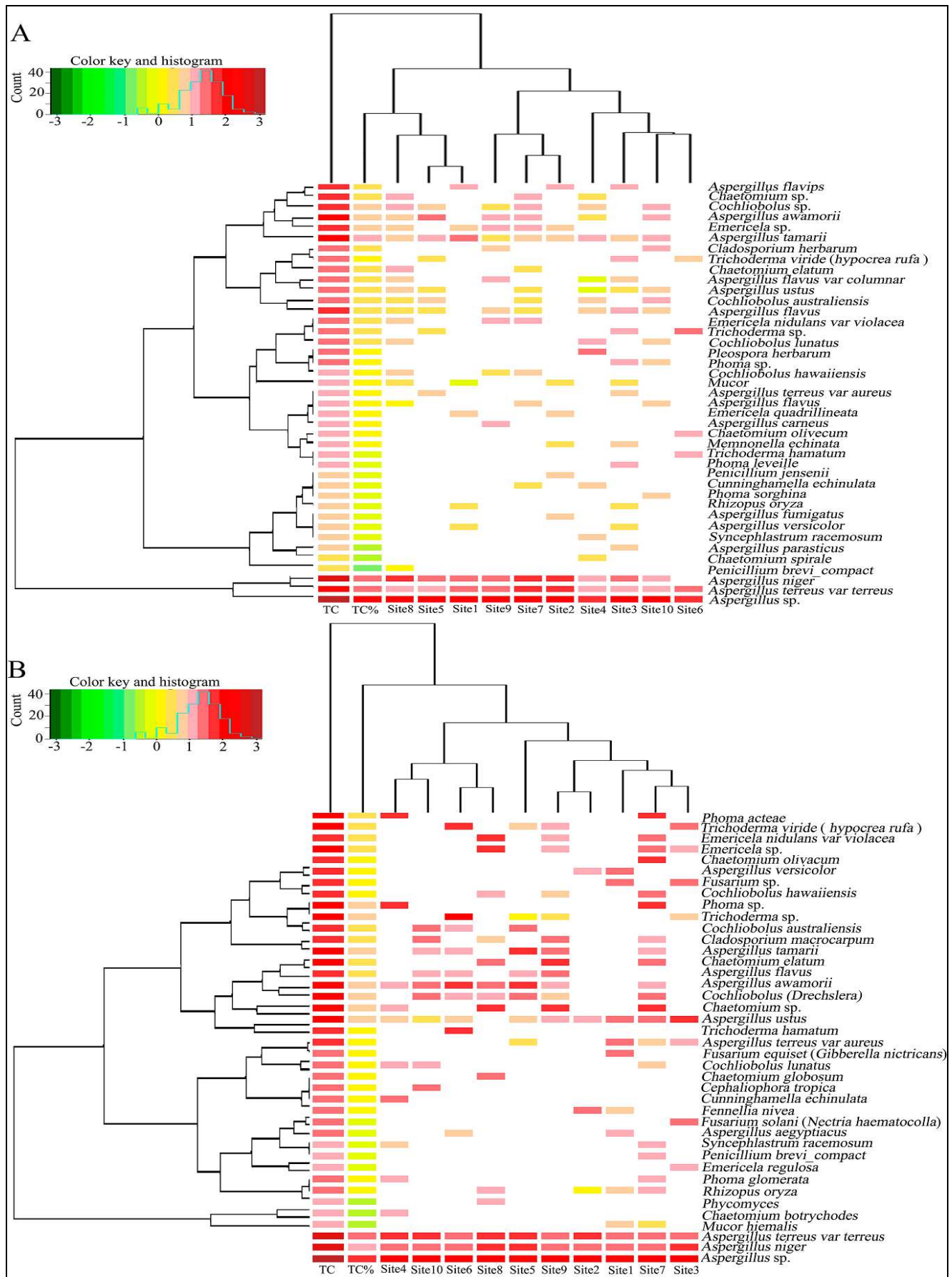
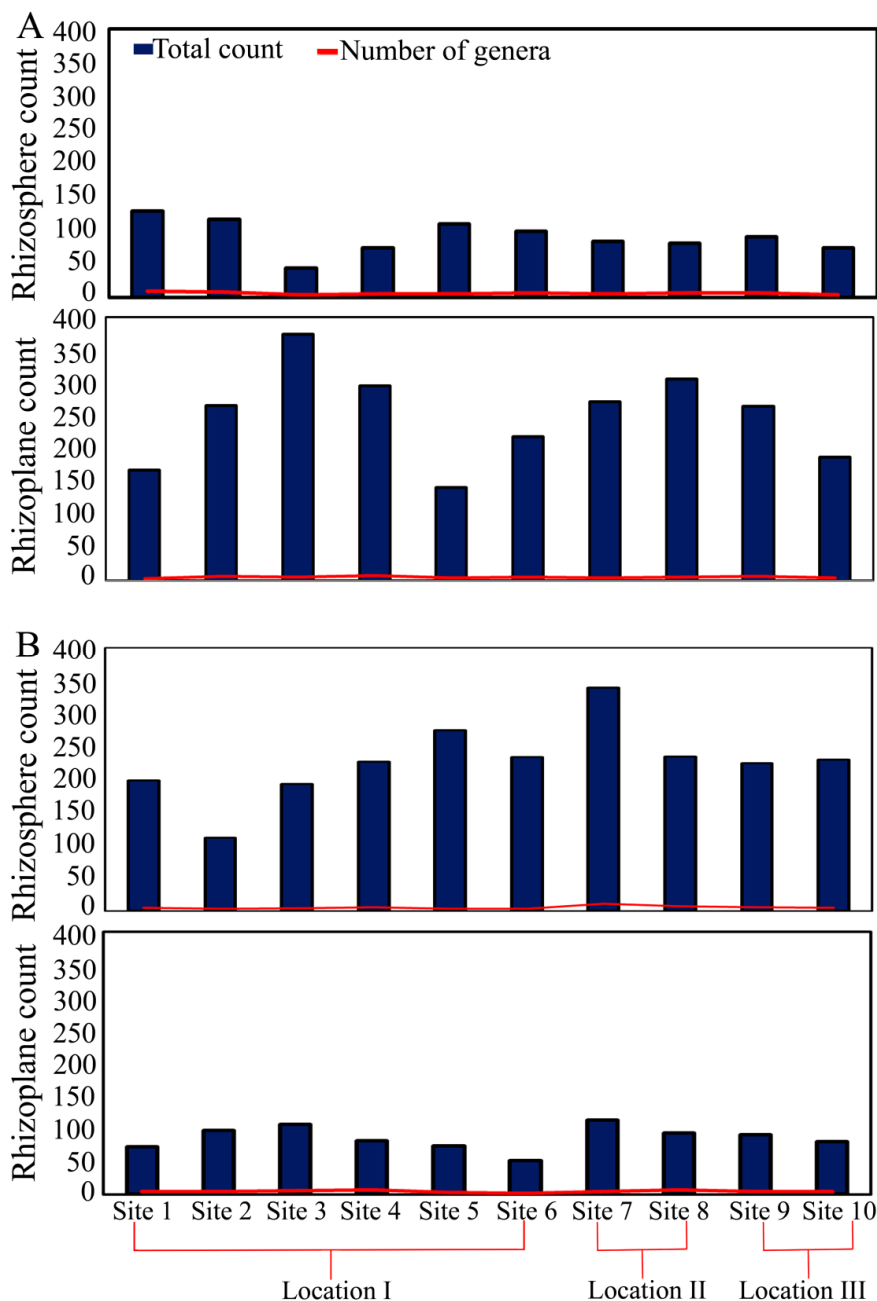


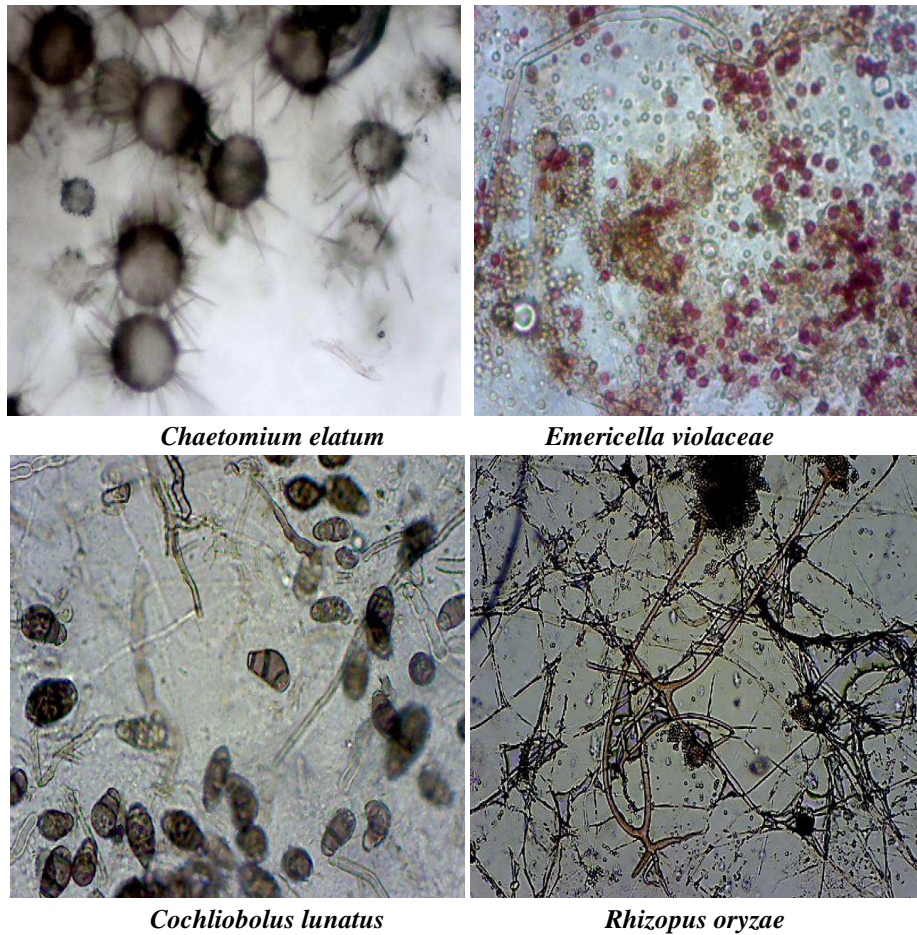
Figure 2. Heatmap clustering of the total number of fungi species isolated from *Pulicaria crisperhizoplane* (A) and *P. crisperhizosphere* (B) at different sites (1-10). To construct heatmap color scale, the fungal counts were normalized using logarithm of base 10. TC, total count.



Supplementary Figure S1. Total count of fungal isolates isolated from *Pluchea dioscoridis*-(A) and *Pulicaria crispa*- (B) rhizoplane and rhizosphere at studied sites.

Similarly, the total number of fungal isolates isolated from *Pulicaria crispa*-rhizoplane and rhizosphere was 46 and 44, respectively, whereas nine fungal isolates were overlapped (Fig. 4B). The high number of fungi species obtained from the rhizoplane and rhizosphere of desert medicinal plants indicates the significant role of root exudates in soil fungal diversity and dynamics, which are important for plant adaption to the arid land ecosystem [4]. Our data was in accordance with the early reports that showed a high diversity of fungal species

were detected in the rhizosphere and rhizoplane of *Hyoscyamus muticus* and *Hordelymus europaeus* relative to other soil samples [31, 32]. In addition, *Aspergillus niger*, *A. terreus* and *Aspergillus* sp. were the most dominant fungi in the all sample sites (Figs. 1-2). *Aspergillus* spp. were highly abundant in the all sample sites represented by 13 identified species and three varieties, followed by *Chaetomium*, *Emericella*, and *Phoma* that were represented by six identified species (Figs. 1-2).



Chaetomium elatum

Emericella violaceae

Cochliobolus lunatus

Rhizopus oryzae

Figure 3. Morphology of some isolated fungi.

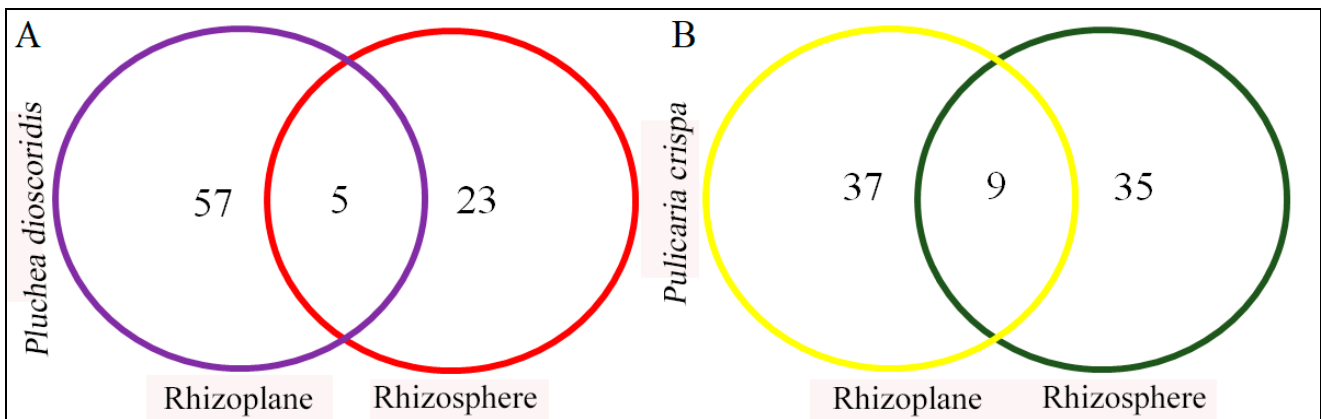


Figure 4. Venn diagram of the total number of fungal isolates isolated from *Pluchea dioscoridis*- (A) and *Pulicaria crispa*- (B) rhizoplane and rhizosphere.

Our data was in accordance with the study by Lima et al. [33] demonstrating that *Aspergillus* spp. were highly abundant in the rhizosphere and rhizoplane of *P. crispa* and *P. dioscoridis*. *Aspergillus* spp. have a wide range of optimum growth temperature ranging from 25 to 40°C and minimum growth temperature around 10°C compared with other fungi [34]. The

dynamic range of *Aspergillus* growth temperature is an important physiological character, which enables *Aspergillus* spp. to survive under different environmental conditions. Among all recovered species during this work, there were twenty three identified species and one variety belonging to 14 terrestrial fungal genera were recovered from *Pluchea dios-*

coridis in rhizosphere samples. In addition, 31 identified species and 2 varieties appertaining to 17 genera were recovered from *Pulicaria crispa*. While, 27 species and two varieties belonging to 13 genera were isolated and identified from *Pluchea dioscoridis*, whereas thirty one and three varieties appertaining to 15 genera were recovered from *Pulicaria crispa* in rhizoplane samples. Out of all recorded species in this investigation, 12 identified species were isolated from rhizoplane only of the two plants were; *Aspergillus carneus*, *A. flavus* var. *columnaris*, *A. fumigatus*, *A. parasiticus*, *Botryotrichum piluliferum*, *Chaetomium cochliodes*, *Phoma euprena*, *Phoma* sp., *P. pomorum*, *Emericella quadrilineata*, *Memoniella echinata* and *Pleospora herbarum*. In Pakistan, Qureshi et al. [35] isolated *Fusarium solani*, *Cochliobolus australiensis*, *Macrophomina phaseolina* and *Rhizoctonia solani* from rhizoplane of 65 plants belonging to 58 genera and 19 families. The following 14 fungal species; *Aspergillus egyptiacus*, *Botrytis cinerea*, *Cephalophora tropica*, *Cladosporium cladosporioides*, *C. macrocarpum*, *Chaetomium bostrychodes*, *C. globosum*, *Cochliobolus sativus*, *Emericella rugulosa*, *Gibberella intricans*, *Mucor fuscus*, *Nectaria haematocoa*, *Phoma acteae* and *Phycomyces* sp. were isolated only from rhizosphere of the two tested plants. Most of the fungal genera that were recorded in this investigation were repeatedly reported for rhizosphere of different wild and cultivated plants in the South-Eastern desert of Egypt and different parts of the world by [29, 32, 36-39]. In the rhizoplane of *Pluchea dioscoridis*, there were four species found to be dominant are; *Aspergillus ustus*, *Aspergillus flavus* var. *columnaris*, *Aspergillus flavus* and *Aspergillus fumigatus*, *Phoma viride*, *Fusarium* sp. and *Chaetomium cochliodes* were showed an intermediate frequency while remaining isolated were found in low frequencies whereas, in rhizoplane of *Pulcaria crispa*, *A. tamarii*, *A. awamori*, *A. flavus* and *Aspergillus ustus*, *Cochliobolus australiensis* and *Emericella violacea* were found to be dominant. *Mucor hiemalis* and *Rhizopus oryzae* were showed an intermediate frequency while, remaining isolates were found in low frequency, these genera were represented by few species that did not exceed five species for each genus. *Acremonium*, *Botryotrichum*, *Fusarium* and *Microascus* which were recovered

from *Pluchea dioscoridis* in moderate and low incidence, were completely missed in *Pulicaria crispa*. Whereas, *Cladosporium*, *Cunninghamella*, *Memnoniella*, *Pleospora*, *Rhizopus*, *Syncephalastrum* and *Trichoderma* which were represented by two or one identified species and present in moderate to low incidence, were recovered from *Pulicaria crispa* and completely absent in *Pluchea dioscoridis*. All fungal genera and species which were recovered during this work were previously recovered from soil, rhizosphere, rhizoplane and endophytic fungi at different parts of the world and on different habitats by many investigators [35, 38, 40-45].

3.2. Inulinase activity of fungal isolates associated with Asteraceous plants

A total of 180 fungal isolates were able to grow on PDA medium supplemented with 1% inulin. Out of which, 23 fungal isolates (12.77%) exhibited high inulinase activity ranging from 5.05 to 7.26 U/ml (Figs. 5-6). Whereas 67 fungal isolates (37.22%) displayed moderate inulinase activity, ranging from 3.01 to 4.99 U/ml (Figs. 5-6). *Aspergillus terreus* var. *terreus* 233 isolated from *P. dioscoridis*-rhizoplane, and *Botrytis cinerea* isolated from *P. dioscoridis*-rhizosphere exhibited the highest inulinase activity ranging from 6.28 to 6.41 U/ml (Fig. 5A-B). On the other hand, *Chaetomium cochliods* isolated from *P. dioscoridis*-rhizoplane, and *Emericla nidulans* var. *nidulans* isolated from *P. dioscoridis*-rhizosphere displayed the lowest inulinase activity, ranging from 0.17 to 0.34 U/ml (Fig. 5A-B). Similarly, *Aspergillus aegyptiacus* isolated from *P. crispa*-rhizoplane, and *Cochliobolus australiensis* 447 isolated from *P. crispa*-rhizosphere exhibited highest inulinase activity ranging from 7.10 to 7.26 U/ml (Fig. 6A-B). However, *Mucor hiemalis* isolated from *P. crispa* rhizoplane and rhizosphere displayed the lowest inulinase activity of 0.85 U/ml (Fig. 5A-B). In general, our results indicated that the isolated fungal species exhibited significant differences in inulinase activities, and several *Aspergillus* spp. isolated in this study showed high inulinase activity in comparison with other fungi species. The high inulinase activity of *Aspergillus* spp. seems to be a physiological characteristic of this species to enable them to extract nutrition under severe environmental condi-

tions. Similar studies have also indicated the high inulinase activity derived from *Aspergillus* spp. [46, 47]. The histogram analysis of inulinase activity of fungal isolates isolated from *P. dioscoridis*-

rhizosphere and rhizoplane exhibited a right-skewed/asymmetrical distribution due to the high number of fungi showed low inulinase activity (Fig. 7).

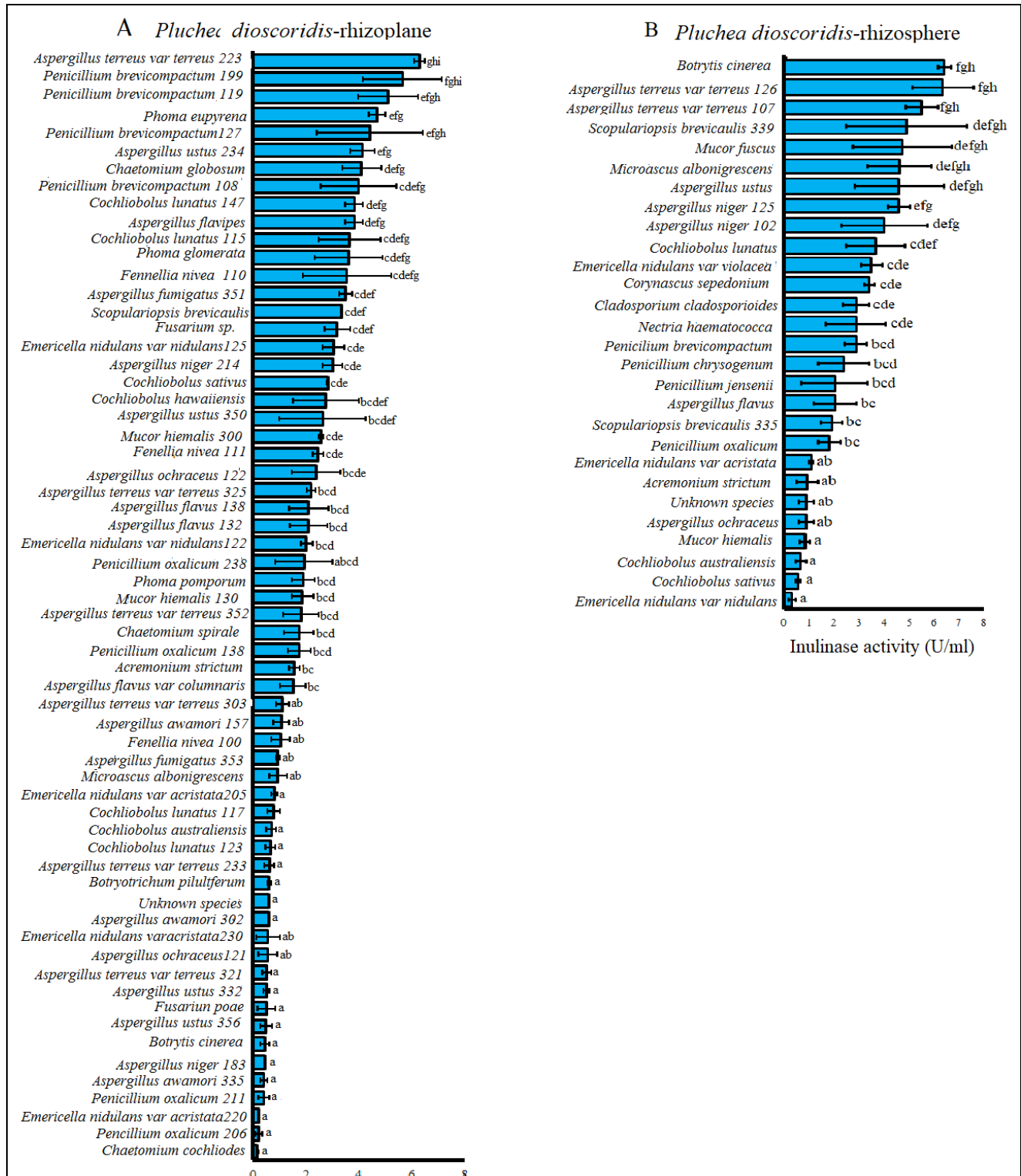


Figure 5. Inulinase activity (U/ml) of fungi species isolated from *Pluchea dioscoridis*-rhizoplane (A) and rhizosphere (B).

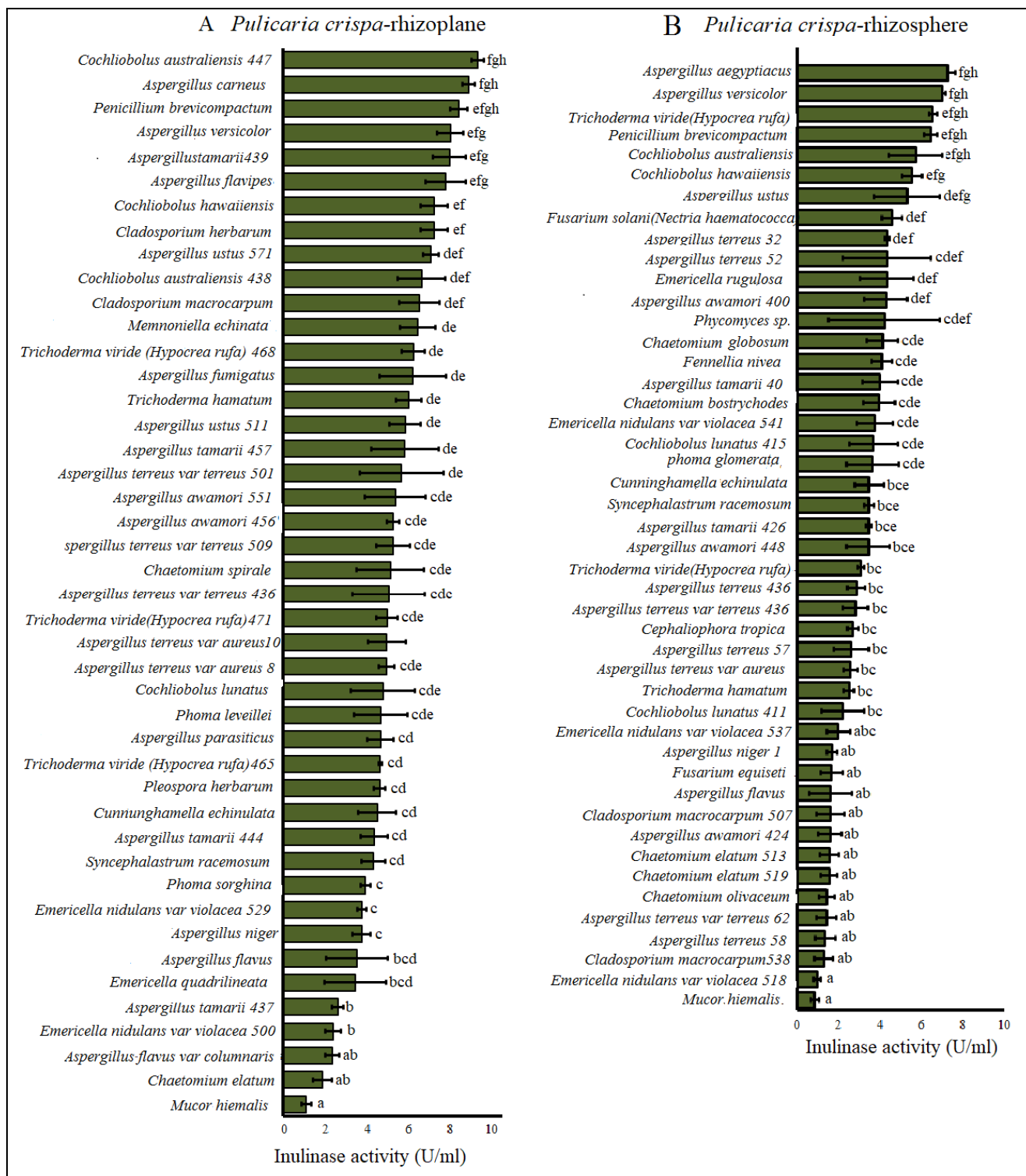


Figure 6. Inulinase activity (U/ml) of fungi species isolated from *Pulicaria crisperhizoplane* (A) 0020 and rhizosphere (B).

On the other hand, the histogram analysis of inulinase activity of fungal isolates isolated from *P. crisperhizoplane* and rhizosphere exhibited normal distribution due to the high number of fungi showed moderate inulinase activity (Fig. 7). The high inulinase activity for *Aspergillus terreus* var.

terreus 233, *Botrytis cinerea*, *Aspergillus aegyptiacus* and *Cochliobolus australiensis* 447 observed in this study was in accordance with previous reports [41, 48, 49]. For examples, Coitinho et al. [49] demonstrated the high efficiency and thermostability of inulinase enzyme purified from *Asper-*

gillus terreus using sugarcane bagasse as a substrate. *B. cinerea* xylanase activity has been reported as an essential component for their virulence effect [50]; however, this is the first report about *B. cinerea* inulinase activity, which might be a starting point

for further in-depth studies about its role in the plant-pathogen interaction. Souza-Motta et al. [41] also demonstrated the ability of filamentous fungi isolated from rhizosphere to hydrolyze inulin.

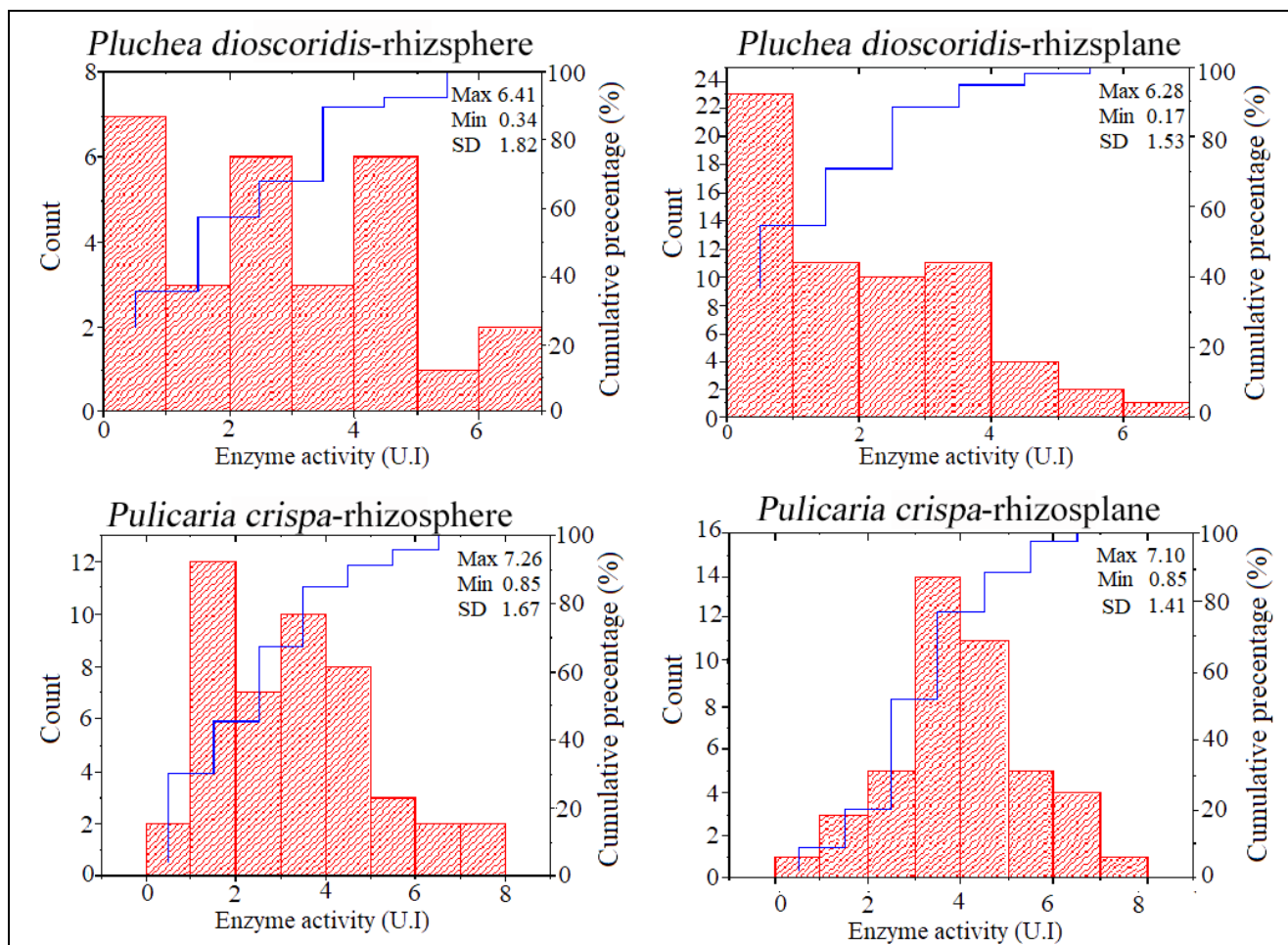


Figure 7. Histogram analysis of inulinase activity on the X-axis and total fungal isolates on the Y-axis isolated from *Pluchea dioscoridis*- and *Pulicaria crispa*-rhizoplane and -rhizosphere.

4. CONCLUSION

In conclusion, we were able to isolate and identify 180 fungal isolated from the rhizosphere and rhizoplane of two desert medicinal plants *P. dioscoridis* and *P. crispa* in the South-Eastern desert of Aswan governorate, Egypt. We also examined the inulinase activity of the isolated fungi, revealing high ability of several fungal isolates including *Aspergillus terreus* var. *terreus* 233, *Botrytis cinerea*, *Aspergillus aegyptiacus*, *Cochliobolus australiensis* 447 and *Cochliobolus australiensis*. These fungal isolates could be a potential

bio-resource for microbial inulinase production. Optimization experiments are needed for exploring the best conditions for increasing the enzyme productivity by these isolates for potential commercialization.

AUTHOR'S CONTRIBUTION

MA, SE: Project supervisors, research design, wrote and revised the manuscript; DKh, MS: experimental work, MAR: statistical analysis, Figures and wrote the first draft. All authors read and approved the final manuscript.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest regarding the publication of this article.

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