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Original Paper

Diversity of the culturable endophytic fungi producing L-asparaginase in arid Sinai, Egypt

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Abstract
Twenty-five endophytic fungal species were recovered and identified from a twenty-three plant species collected from Saint Katherine Protectorate, South Sinai Egypt, during the period of 2017-2019` with a total count of 4,466 CFU. *Alternaria alternata* recorded the highest count of 1382 CFU out of 4466 CFU and the highest frequency of occurrence with 22 cases of isolation out of 23 plant species. In relation to host plant, *Euphorbia obovata* hosted the highest count (366 CFU), while *Artemisia herba-alba* hosted the highest fungal species richness value with 9 species out of 25. Isolated taxa were screened for L-asparaginase production where 7 strains belonging to 4 different species showed a positive result with an enzymatic activity ranging from 44.5 ± 1.66 to 152.58 ± 0.63 U ml^{-1}. *Lasiodiplodia theobromae* isolated from *Teucrium polium* recorded the highest activity with 152.58 U ml^{-1}. The present study suggests that *L. theobromae* has the potential to be used as an alternative and reliable source of *L*-asparaginase for potent anticancer agents.

Keywords
endophytic fungi; *L*-asparaginase; *Lasiodiplodia theobromae*; *Teucrium polium*; Saint Katherine Protectorate

Introduction
Endobiontic fungi are microfungi that host plant tissue intercellularly and/or intracellularly without any apparent pathological symptoms (Wilson, 1995; Das and Varma, 2009). To be able to sustain steady symbiosis, plant-associated fungi develop chemical substances that enhance the development of plants and help them to acclimatize to harsh environments (Gouda et al., 2018). As a treasure mine of bioactive metabolites, endophytic fungi are considered a sustainable source of various natural products such as antioxidants, quinones, saponins, alkaloids, steroids, phenolic acids, terpenoids, and tannins that exhibit antimicrobial and anticancer properties (Verma et al., 2009; Abo Nahas, 2019).

Recently, the term "bioprospecting" refers to the discovery and commercialization of natural products from natural-biological sources (Beattie et al., 2011; Abdel-Azeem et al., 2012; Salem and Abdel-Azeem, 2014). Fungi have been included in the interest of biotechnology because their immense importance to the ecosystem components, especially to human (Strobel and Daisy, 2003). In the last two decades scientific community worldwide has focused on the fungal plant-associated fungi as a sustainable and ecofriendly source for production of bioactive metabolites that may be effective candidates for the first-class drugs for treatment of human diseases (Bacon and White, 1994; Zhang et al., 1999; Strobel and Daisy, 2003; Guo et al., 2008; Kumaran, 2008; Duan et al., 2009; Shweta et al., 2010). It was indicated that endophytic fungi produce about 51% of new bioactive substances in comparison with 38% from terricolous fungi (Schulz et al., 2002). Unique environments, diversity,

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ethnobotanical history and endemicity should be considered during selection and sampling processes of endophytic fungi-hosting plants (Abdel-Azeem et al., 2019a).

Leukemic cells require large amounts of asparagine for proliferation and growth. *L*-asparaginase controls the tumor by lowering the serum's asparagine (Egler et al., 2016). El-Nagga et al. (2014) recommended *L*-asparaginase as a potential drug which can treat acute lymphoblastic leukemia as an alternative to the classical chemotherapy protocols. *L*-asparaginase (EC 3.5.1.1) belongs to a group of homologous amidohydrolases family, which catalysis the hydrolysis of the amino acid *L*-asparagine to *L*-aspartate and ammonia (Dias et al., 2016). *L*-asparaginase discovered firstly from guinea pig serum (Clementi, 1922) and its tumor activity were reported afterwards by recognizing the anti-leukemic activity of the guinea pig serum on treated mice (Broome, 1963). First microbial source of *L*-asparaginase was *E. coli* but *L*-asparaginase is broadly distributed among plants, animals and microorganisms (Kumar et al., 2013a). Prokaryotic sources of *L*-asparaginase showed many side effects like hypersensitivity which usually lead to anaphylaxis and hypersensitivity (Duval et al., 2002). Based on aforementioned reasons, fungi are considered as an alternative and effective source of *L*-asparaginase (Kumar and Sobha, 2012). More than 600,000 species of plant associated micro-fungi are theorized to exist worldwide (Schmit and Mueller, 2007). In Egypt, endophytic fungi are a potential fungal resources still unexplored (Abdel-Azeem, 2010).

The mountainous region of southern Sinai exhibits greater biodiversity than the rest of Egypt, and 4,350 km^2 of this area was declared a Protectorate in 1996 (Zalat et al., 2008). In South Sinai, Bedouin's traditional folk medicine depends upon approximately 170 plant species which inhabiting the area (Fayed and Shaltout, 2004). No solid study on endophytic fungi producing *L*-asparaginase exists in Egypt. In this study, we isolated endophytic fungi from 23 plant species growing on the mountainous region in Saint Katherine Protectorate, in addition to analyzing the biodiversity and seasonal variation in the numbers of these fungi and screening their potentiality to produce *L*-asparaginase.

Materials and Methods

Study area

The Protectorate of Saint Katherine covers about 4,350 km^2 of Southern Sinai (Abdel-Azeem, 2009). Saint Katherine is located at an elevation of 1,500 to 2,624 meters above sea level (m a.s.l.) which includes the main mountains in the area. The area is situated between 28°30' to 28°35' North and 33°55' to 34°30' East (Abdel-Azeem et al., 2019a). The area is composed of igneous and metamorphic rocks; chiefly granites are intensely dissected and rugged. The area is characterized by an extremely arid climate with long, hot and rainless summers and cool winters. The mean annual precipitation in the area is 45 mm but the high mountains receive more precipitation (100 mm y^{-1}) as rain and snow. In some parts of this place, floods resulting from connective rains have sometimes been observed during winter and spring. The maximum air temperature ranged from 20.2 °C to 32.7 °C and the minimum temperature ranged from 1.9 °C to 20.2 °C with the lowest temperature in December and January and the highest temperature in July and August (Abdel-Azeem et al., 2019a).

Within the Protectorate, more than 400 species of higher plants have been recorded, of which 19 species are endemic, 10 are extremely endangered and 53 are endangered (Guenther, 2005). Approximately 170 plant species that inhabit south Sinai are used traditionally in folk medicine (Fayed and Shaltout, 2004; Abdel-Azeem et al., 2012; 2019a).

Plant species sampling

Some dominant plant species (Table 1) belonging to thirteen families were collected from different 11 sites (for more data check supplementary material). Aerial parts of the plants were collected in sterilized polyethylene bags and transferred to the laboratory, where they were subsequently plated

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out. Samples were collected under permission of the Saint Katherine Protectorate for scientific purposes and no endangered species were involved in the study.

Table 1 - List of collected plant species and their Families dominant in the study area

Family	Species
Asteraceae	*Achillea fragrantissima* Sch. Bip.
	Artemisia judaica Täckh.
	Artemisia herba-alba Asso.
	Chiliadenus montanus (Vahl). Brullo
	Tanacetum sinaicum Delil ex DC.
Boraginaceae	*Alkanna orientalis* Boiss
Euphorbiaceae	*Euphorbia obovate* Decne
Lamiaceae	*Ballota undulata* Benth.
	Origanum syriacum Boiss.
	Phlomis aurea Decne.
	Rosmarinus officinalis L.
	Teucrium polium Decne.
	Thymus decussatus Benth
Oleaceae	*Olea europaea* L.
Peganaceae	*Peganum harmala* L.
Plantaginaceae	*Plantago sinaica* Decne.
Rhamnaceae	*Rhamnus frangula* L.
Rosaceae	*Crataegus sinaica* Boiss.
Rubiaceae	*Galium odoratum* Scop.
	G. sinaicum Boiss.
Scrophulariaceae	*Verbascum sinaiticum* Benth.
Solanaceae	*Hyoscyamus muticus* L.
Zygophyllaceae	*Fagonia mollis* Delile

Isolation and phenotypic identification of plant-associated fungi

A total number of 2,760 of plates were used for recovering endophytic fungi (120 plate/plant) during this study. Surface sterilization technique adopted by Abdel-Azeem and Salem (2012) was applied: pant samples washed under running tap water for 10 min, then stem tissues cut down to pieces each of a 2 cm in length, while leaves tissues cut into (2 x 2 cm) 4 cm^2 segments and surface sterilized by sodium hypochlorite and 70% ethanol and plated out on isolation media. For primary isolation, Potato Dextrose Agar (PDA) and Malt Extract Agar medium (MEA) were supplemented with Rose Bengal (1:1,500) and chloramphenicol (50 ppm) to suppress bacterial growth (Abdel-Azeem et al., 2016).

Isolated taxa were identified morphologically to species level on standard media based on the phenotypic characters and relevant identification keys [Raper and Thom (1949), Pitt (1980) for *Penicillium* spp.; Raper and Fennell (1965), Klich (2002) for *Aspergillus* spp.; Booth (1971), Leslie and Summerell (2006) for *Fusarium* spp.; Ellis (1971, 1976) for Dematiaceous hyphomycetes; Guarro et al. (2012) for ascomycetes; Von Arx (1981), Domsch et al. (2007) for miscellaneous fungi; Cannon (1986), Von Arx (1986), Asgari and Zare (2011), Doveri (2013) for *Alternaria* spp.].

Authors of the fungal taxa names were abbreviated according to Kirk and Ansell (1992). All name corrections, authors and taxonomic assignments of the recorded species in the present study checked against the Index Fungorum Partnership (www.indexfungorum.org).

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Qualitative and quantitaive screening of fungal L-asparaginase

All of the recovered plant-associated fungi were screened for their capability to produce *L*-asparaginase according to Gulati et al. (1997) with some modifications. Taxa were grown on malt extract agar (MEA) at 28 °C for 15 days to enhance sporulation. Modified Czapek's Dox broth (MCDB) composed of Glucose, 2.0, *L*-asparagine 10.0; KH_2PO_4 1.52, KCL 0.52, $MgSO_4$ $7H_2O$ 0.52, traces of $Cu(NO_3)_2$ $3H_2O$, $ZnSO_4$ $7H_20$, $FeSO_4$ $7H_2O$ (g l^{-1}) (Gulati et al., 1997) was supplemented with bromothymol blue as indicator and pH adjusted to 6.0, followed by sterilization for 20 min at 120 °C. Five millimeters fungal mycelial plug was inoculated to 50 ml sterile falcon tube containing MCDB and were incubated at 28 °C for 5 days. Color change from yellow to blue was recorded as an indication for the release of ammonium due to *L*-asparaginase activity of positive taxa.

Nine strains belongig to five species (showing blue color) were grown at MCDB without bromothymol blue and incubated under static condition for 5 days at 28 °C. All the cultures were filtered using Whatman filter papers No.1. One ml of culture filtrate was mixed with 0.1 ml of 40 mM asparagine, 0.4 ml dH_2O and 0.5 ml of 50 mM Tris-buffer (pH 7.0). Mixture was incubated at 37° C for 30 min. Reaction was stopped by adding 1.5 M trichloroacetic acid, followed by centrifugation at 10,000 rpm for 5 min at 4 °C. After having discarged the pellet, Pellets obtained discarded, 3.7 ml of dH_2O and 1 ml of Nessler's reagent were vortexed with 0.1 ml of the supernatant and incubated at room temperature for 10 min. Blank solution was prepared containing all the reagents except fungal filtrate. Absorbance was measured at 450 nm using UV spectrophotometer (JEANWAY 6305, UK). All readings were noted in triplicates and the amount of ammonia liberated from the crude sample was calculated from the standard curve of ammonia previously created (Dias et al., 2016).

Enzyme activity was calculated using the following equation:

$$\text{Enzyme activity (units ml}^{-1}) = \frac{(\mu\text{mol of ammonia liberated}) * (\text{initial volume of mixture in ml})}{(\text{Vol. of enzyme mixture in final rxn in ml}) * (\text{Incubation time}) * (\text{Vol. of enzyme used})}$$

Fungal taxa selected for production of *L*-asparaginase were molecularly identified. The CTAB extraction procedure (Arenz and Blanchette, 2011) was applied to extract genomic DNA. PCR amplification of the internal transcribed spacer (ITS) region of the genomic rDNA was carried out by using the primer combination of ITS1F/ITS2 (White et al., 1990) according to Abdel-Azeem et al. (2019b). By using Geneious 9.0 (Kearse et al., 2012) software a consensus sequence was assembled and compared to those deposited in GenBank using BLASTn for identification.

Data Analysis

The data presented in tables and figures were checked for normality and outlier detection. Fungal diversity was studied using Shannon diversity and Margalef richness indices according to Muthukrishnan (2012). Three variables (isolation media, seasonal variation and plant species) were analysed using multivariate test and analysis of variance (ANOVA) using the SPSS ver. 23 for Mac OS. To determine differences among groups ANOVA was followed by Duncan's multiple range test (DMRTs) at $p < 0.05$.

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Results

Overview and biodiversity of the recorded plants associated fungi

In this study twenty five fungal species were isolated; the isolated species were belonging to 16 genera, 10 families, 8 orders, 5 classes and three phyla. Taxa were distributed as 22 ascosporic (anamorphic and teleomorphic), two zygosporic species and one basidiosporic taxon (for more data check supplementary materials). Taxa with uncertain taxonomic position (*Incertae sedis*) were distributed among orders and families (for more data check supplementary materials).

The prevailing genera were *Aspergillus* (4 species; 16% of the total species number), *Fusarium* and *Alternaria* (3 species; 12%) *Penicillium* and *Chaetomium* (2 species; 8%) and the remaining taxa were represented only by one species each.

Family Aspergillaceae contained the highest number of species (7 species) belonged to 3 genera among all families. The species genus ratio (S/G) per family showed that family Necteriaceae recorded 3 species within one genus by recording a ratio of 3 followed by Aspergillaceae (2.3), Chaetomiaceae (2), Pleosporaceae (1.7), while the other families recorded a ratio of 1 (for more data check supplementary materials).

Data concerning frequency classes showed that *Alternaria alternata* and *Aspergillus niger* recorded the highest frequency of occurrence while, *Aspergillus flavus* and *Penicillium chrysogenum* showed medium occurrence.The other species showed low to rare frequency of occurrence (Table 2).

A total count of 4,466 CFU was recorded during this study from the collected 23 plant species. Among the isolated taxa, *A. alternata* recorded the highest total count with 1,382 CFU followed by *A. niger* (1225) and *P. chrysogenum* with 757 CFU respectively. Other taxa showed total count ranging from 8 to 300 CFU/plant (Table 2).

Comparing the total colony count of recovered fungi per plant, *Euphorbia obovata* came first by recorded the highest count with 366 CFU, followed by *Thymus decussates*, *Tanacetum sinaicum*, *Verbascum sinaiticum*, *Galium odoratum* with 341, 295, 279, 272 CFU/plant respectively. The other plant species showed a total count ranging between 116 to 216 CFU (Table 3).

Regarding the hosting plant' species richness, *Artemisia herba-alba* showed the highest richness value by hosting 9 species, followed by *Achillea fragrantissima, Ballota undulata, Rosmarintus officinalis* with 8 fungal species, *Thymus decussates* recorded 7 species and other plant species showed richness value ranging from 1 up to 6 (for more data check supplementary materials).

Seasonally, spring recorded the highest number of taxa with 21, followed by summer, autumn and winter with 20, 15 and 12 respectively.

Regarding the diversity of the isolated endophytic fungi in relation to hosting plant species, *Artemisia herba-alba* recorded the highest values of Margalef richness index and Shannon diversity index with values of 3.64 and 2.20, respectively, followed by *Achillea fragrantissima*, *Ballota undulata ,Rosmarintus officinalis*, *Tanacetum sinaicum* with values of 3.37 for Margalef index and 2.08 for Shannon index; other plant species exhibited a range of 2.49 to 1.44 for Margalef index and 1.95 to 0.69 for Shannon index (for more data check supplementary materials).

The diversity of the isolated fungal species analyzed statistically using Multi-way ANOVA analysis showed high significance values in relation to plant species, with F-ratio ranging from 105.15 to 4.84, while the seasonal variation showed a variation of significance from high to no significance values with F-ratio ranging from 31.31 to 0.46; thetype of isolation media showed a range of significance from high to no significance results with F-ratio from 26.3 to 0.04 (Table 5).

Regarding the biodiversity of recorded taxa over seasons, *A. alternata* recorded the highest number of cases of isolation over the four seasons with 73, followed by *A. niger* with 67 cases of isolation (Table 4).

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Table 2 - The total count (TC), number of cases of isolation (NCI out of 23 plants species), frequency (F) and frequency classes (FC) of recovered taxa during this study

Taxa	TC	NCI	%F	FC
Alternaria alternata (Fr.) Keissl.	1382	22	95.7	H
Aspergillus niger Tiegh	1225	20	87.0	H
Penicillium chrysogenum Thom	757	16	69.6	M
Aspergillus flavus Link	300	12	52.2	M
Penicillium brevicompactum Dierckx	174	8	34.8	L
Alternaria atra (Preuss) Woudenb. & Crous	100	5	21.7	R
Cladosporium cladosporioides (Fresen.) G.A. de Vries	68	7	30.4	L
Rhizopus stolonifer (Ehrenb.) Vuill.	67	4	17.4	R
Chaetomium globosum Kunze	60	3	13.0	R
Aspergillus terreus Thom	44	6	26.1	L
Trichoderma viride Pers	35	6	26.1	L
Talaromyces stipitatus C.R. Benj.	33	1	4.3	R
Sarocladium strictum (W. Gams) Summerb.	29	1	4.3	R
Fusarium oxysporum sensu Smith & Swingle	28	1	4.3	R
Syncephalastrum racemosum Cohn ex J. Schröt.	25	1	4.3	R
Cochliobolus bicolor A.R. Paul & Parbery	23	3	13.0	R
Exserohilum monoceras (Drechler) KJ. Leonard & Suggs	20	1	4.3	R
Lasiodiplodia theobromae (Pat.) Griffon & Maubl.	15	1	4.3	R
Fusarium solani (Mart.) Sacc.	15	1	4.3	R
Aspergillus nidulans (Eidam) G. Winter	14	1	4.3	R
Chaetomium madrasense Natarajan	13	1	4.3	R
Alternaria solani (Ellis & G. Martin) L.R. Jones & Grout	11	1	4.3	R
Nigrospora oryzae (Berk. & Broome) Petch	11	1	4.3	R
Phanerodontia chrysosporium (Burds.) Hjortstam & Ryvarden	9	1	4.3	R
Fusarium cylindricum (Mont.) Sacc.	8	1	4.3	R

Frequency class: H, the species observed is "High" when recovered from 18 or more plants; M, species observed is "Medium" when recovered from (17-12) plants; L, the species observed is "Low" when recovered from (9-3) plants; R, species frequency is "Rare" when recovered from (5-0) plants.

Table 3 - The total count of endophytic fungi recorded per plant species

Plant species	Total count	Plant species	Total count
Euphorbia obovata	366	*Alkanna orientallis*	162
Thymus decussates	341	*Fagonia mollis*	162
Tanacetum sinaicum	295	*Plantago sinaica*	162
Verbascum sinaiticum	279	*Artemisia herba-alba*	161
Galium odoratum	272	*Gallium siriacum*	156
Rosmarintus officinalis	216	*Crataegus sinaica*	141
Tecrium polium	214	*Rhammus frangula*	139
Peganum harmala	189	*Hyoscyamus muticus*	136
Phlomis aurea	187	*Ballota undulate*	132
Olea europaea	184	*Origanum syriacum*	121
Artemisia Judaica	169	*Chiliadenus montanus*	116
Achillea fragrantissima	166		
Mean	194.17	Minimum	116.00
Standard Error	14.56	Maximum	366.00
Standard Deviation	69.81	Sum	4466.00

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As an overview on the dominating recorded fungal endophytic taxa over the nine collection sites, *Alternaria alternata* showed the highest frequency over 7 different sites, while *Aspergillus terreus* showed a high frequency over 4 different sites (for more data check supplementary materials).

Distribution of endophytic fungi hosting plants collected from different elevation wadis showed that low elevation wadis (Abu Sayla and Itlah) recorded the highest mean of content per plant species with 2.14 species/plant followed by mid elevation wadis (Shyraj, Farsh Sefafa, Arbaein and Fara`) with 1.78 fungal species/plant and high elevation wadis (Sefsafa, Ahmar and Abas) with 1.6 fungal species/plant (for more data check supplementary materials).

The combined effect of plant species, isolation medium and seasonal variation show a range of significance from high to no significance with F-ratio ranging from 16.42 to 0, 21.13 to 1.29 respectively, while the combined effect of seasonal variation and media types showed a variation of significance values from medium to no significance with F-ratio ranging from 0.04 to 4.31 (Table 5).

Regarding the effect of three variables combined, Table 5 showed a high significance with some fungal endophytic species with F-ratio ranging from 12.1 to 1.75 and showed a range of significance from low to no significance with the other endophytic fungal species with F-ratio ranging from 0.04 to 1.41.

Qualitative and quantitative screening of L-asparaginase

L-asparaginase producing taxa shifted the pH from acidic (yellow) to the alkaline (blue) conditions (Fig. 1a). Out of the screened twenty-five taxa one strain of *L. theobromae* and two strains for each of *F. oxysporum, C. cladosporioides, A. flavus* showed positive *L*-asparaginase activities (Fig. 1b).

Quantitatively, activity of *L*-asparaginase have been assayed spectrophotometrically for the positive recorded taxa and results showed a range of activity between 44.5 ± 1.66 to 152.58 ± 0.63 U ml^{-1} (Fig. 2). *L*-asparaginase activity results analyzed statistically using ANOVA one-way analysis of variance and showed F-ratio of 1382.2 and $p < 0.001$ (Fig. 1). *Lasiodiplodia theobromae* recorded the highest activity results with 152.58 ± 0.63 U ml^{-1} (Figs. 1c and 2).

All the positive *L*-asparaginase species were sequenced and deposited at the NCBI under accession numbers of: MH752509 *A. flavus* SCUF2020, MH752510 *F. oxysporum* SCUF2021, MH752511 *C. cladosporioides* SCUF2022 and MK537303 *L. theobromae* SCUF-TP2016.

Discussion

Screening of bioactive natural products secreted by endophytic fungi got attention by Egyptian scientists lately (Abdel-Azeem, 2010; Abdel-Azeem and Salem 2012; Abdel-Azeem et al., 2012; Salem and Abdel-Azeem 2014; Abdel-Azeem et al., 2016; 2018; 2019a) as they directed their researches on endemic and medicinal plants grow over areas of great biodiversity e.g. Saint Katherine Protectorate. From the collected twenty-three plant species a 25 fungal species with a total count of 4,466 CFU were isolated.

During the presented study, twenty-five fungal species have been isolated with a 4,466 colonies from twenty-three collected plant species. Abdel-Azeem et al. (2016) recovered a total count of 1490 CFU from 27 plants in Saint Katherine Protectorate with a mean value of 178.64 CFU, while each plant showed a mean value of 194.17 CFU.

All of the twenty-five taxa were recorded previously as plant-associated fungi with no new records from Saint Katherine Protectorate (Abdel-Azeem, 2009; Abdel-Azeem et al., 2016; 2018; 2019a; Abo Nahas, 2019).

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Table 4 - Number of cases of isolation of plant-asscoiated fungi over the four seasons

Fungal species	**NCI**				**Total NCI**
	Spring	**Summer**	**Autumn**	**Winter**	
Alternaria alternata	16	18	17	22	73
Aspergillus niger	15	17	16	19	67
Penicillium chrysogenum	12	13	9	13	47
Aspergillus flavus	8	9	8	10	35
Aspergillus terreus	4	5	4	4	17
Penicillium brevicompactum	5	4	3	4	16
Alternaria atra	4	3	3	3	13
Cladosporium cladosporioides	4	3	1	4	12
Trichoderma viride	3	1	2	3	9
Rhizopus stolonifer	1	3	2	3	9
Cochliobolus bicolor	2	1	1	2	6
Chaetomium globosum	2	2	0	1	5
Fusarium oxysporum	1	1	1	1	4
Fusarium solani	1	1	1	1	4
Talaromyces stipitatus	1	1	1	1	4
Exserohilum monoceras	1	1	1	0	3
Lasiodiplodia theobromae	1	1	1	0	3
Nigrospora oryzae	1	1	1	0	3
Syncephalastrum racemosum	1	1	1	0	3
Sarocladium strictum	1	1	0	0	2
Alternaria solani	1	1	0	0	2
Aspergillus nidulans	1	1	0	0	2
Chaetomium madrasense	1	0	0	0	1
Phanerodontia chrysosporium	1	0	0	0	1
Fusarium cylindricum	0	1	0	0	1
Yeast	0	0	0	0	0
No. of Taxa/Season	21	20	15	12	

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Table 5 - The multivariate analysis (ANOVA) for the effect of plant species, isolation media and seasonal variation on taxa composition.

Fungal species	Variables						
	Plants	Media	Plants* Media	Season	Plants* Season	Media* Season	Plants* Media* Season
	F-ratio with degree of significance						
	F-ratio	F-ratio	F-ratio	F-ratio	F-ratio	F-ratio	F-ratio
A. alternata	30.41***	13.46***	4.41***	5.33**	11.52***	0.99$^{n.s}$	1.17$^{n.s}$
A. atra	36.22***	0.18$^{n.s}$	0.21$^{n.s}$	2.98*	4.31***	2.13$^{n.s}$	1.68***
A. solani	14.12***	5.72*	5.72***	5.41***	5.41***	1.98$^{n.s}$	1.98***
A. flavus	41.47***	3.99*	3.75***	19.02***	9.63***	0.93$^{n.s}$	0.81$^{n.s}$
A. nidulans	20.79***	0$^{n.s}$	0$^{n.s}$	14***	14***	1.13$^{n.s}$	1.13$^{n.s}$
A. niger	59.4***	26.3***	4.36***	7.35***	8.5***	3.19*	1.41*
A. terreus	11.69***	0$^{n.s}$	0.83$^{n.s}$	2.76*	4.22***	0.21$^{n.s}$	0.45$^{n.s}$
C. globosum	37.31***	12.73***	4.15***	31.31***	21.13***	3.48*	2.35***
C. madrasense	14.97***	2.22$^{n.s}$	2.22***	14.97***	14.97***	2.22$^{n.s}$	2.22***
C. cladosporioides	17***	0.07$^{n.s}$	10.81***	9.56***	5.67***	1.17$^{n.s}$	2.01***
C. bicolor	15.6***	2.5$^{n.s}$	1.76*	0.46$^{n.s}$	1.99***	2.64*	1.75***
E. monoceras	27.18***	0.27$^{n.s}$	0.27$^{n.s}$	7.43***	7.43***	0.45$^{n.s}$	0.45$^{n.s}$
F. cylindricum	8.15***	2.04$^{n.s}$	2.04**	8.15***	8.15***	2.04$^{n.s}$	2.04***
F. oxysporum	29.19***	0.15$^{n.s}$	0.15$^{n.s}$	1.29$^{n.s}$	1.29$^{n.s}$	2.23$^{n.s}$	2.23***
F. solani	17.12***	0.68$^{n.s}$	0.68$^{n.s}$	1.5$^{n.s}$	1.5**	0.89$^{n.s}$	0.89$^{n.s}$
L. theobromae	13.02***	2.83$^{n.s}$	2.83***	5.61***	5.61***	0.37$^{n.s}$	0.37$^{n.s}$
N. oryzae	11.29***	2.33$^{n.s}$	2.33***	2.33$^{n.s}$	2.33***	4.32**	4.32***
P. brevicompactum	105.15***	2.58$^{n.s}$	5.34***	1.76$^{n.s}$	5.74***	2.74*	12.1***
P. chrysogenum	55.42***	17.04***	16.42***	0.73$^{n.s}$	12.05***	0.56$^{n.s}$	1.86***
P. chrysosporium	12.06***	0.15$^{n.s}$	0.15$^{n.s}$	12.06***	12.06***	0.15$^{n.s}$	0.15$^{n.s}$
R. stolonifer	36.98***	0.21$^{n.s}$	4.65***	11.1***	8.77***	2.89*	1.47**
S. strictum	32.52***	0.04$^{n.s}$	0.04$^{n.s}$	15.2***	15.2***	0.04$^{n.s}$	0.04$^{n.s}$
S. racemosum	26.68***	0.04$^{n.s}$	0.04$^{n.s}$	3.12*	3.12***	0.16$^{n.s}$	0.16$^{n.s}$
T. stiptatus	33.29***	3.7$^{n.s}$	3.7***	2.39$^{n.s}$	2.39***	0.52$^{n.s}$	0.52$^{n.s}$
T. viride	4.84***	1.1$^{n.s}$	0.21$^{n.s}$	1.46$^{n.s}$	2.89***	0.08$^{n.s}$	0.13$^{n.s}$

*** $p < 0.0001$, ** $p < 0.01$, * $p < 0.01$, n.s not significant

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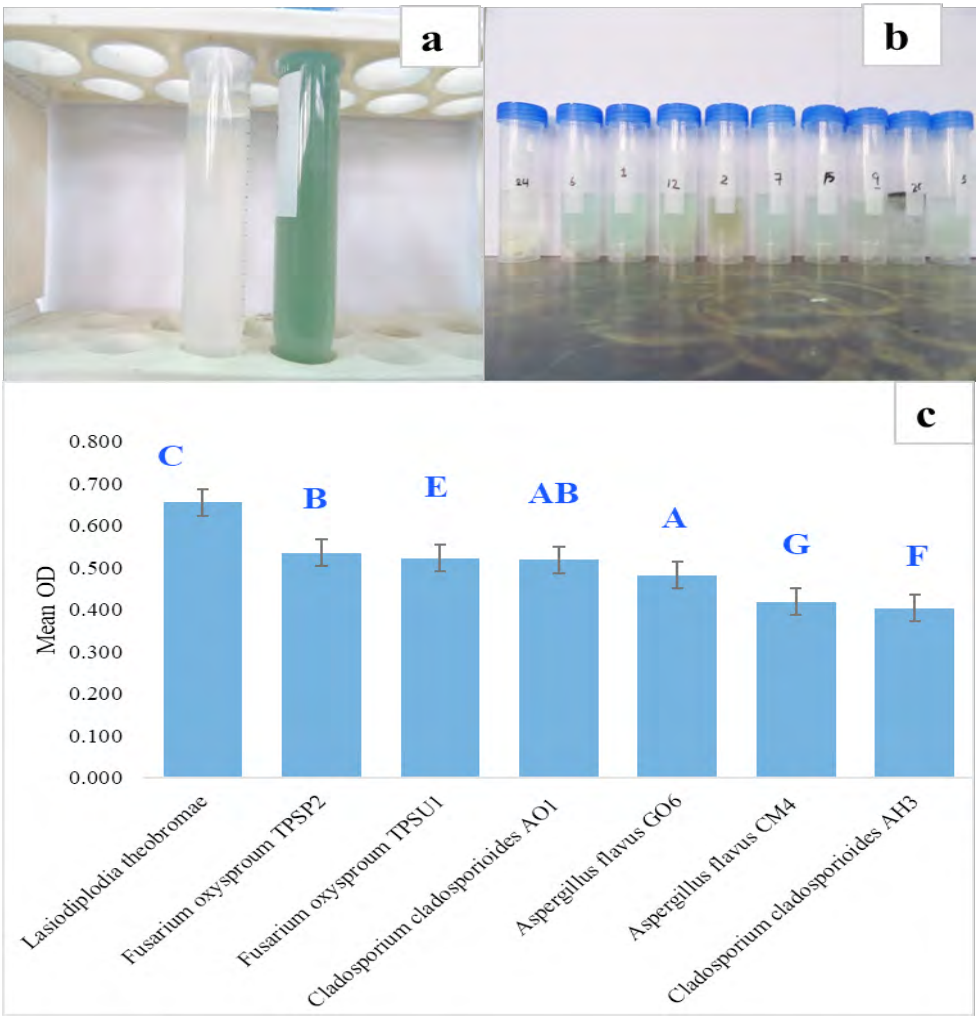

Figure 1 - Positive and negative colour changes using bromothymol blue indicator (a). Positive *L*-asparaginase morphotypes (b). Ranges of OD values with Duncan grouping (c)

Regarding the occurrence levels of the isolated endophytic species; *A. alternata* and *A. niger* showed high occurrence, *A. flavus* and *P. chrysogenum* showed medium occurrence, and the other species showed low to rare occurrence. Our results are in agreement with Salem and Abdel-Azeem (2014) and Abdel-Azeem et al. (2016, 2018, 2019a). They reported that *A. alternata* and *A. niger* were recovered with 25% of frequency and 250 cases of isolation and with 2.8% of frequency and 28 cases of isolation respectively. As an overview for the biodiversity of endophytic fungi among the collected plant species; *Artemisia herba-alba* showed the highest richness value of nine species, followed with, *Achillea fragrantissima, Ballota undulata, Rosmarintus officinalis, Tanacetum sinaicum.* Our data was compatible with Abdel-Azeem (2009) and Abdel-Azeem and Salem (2012).

On studying the diversity of the recorded endophytic fungi among the collected plant species using Shannon and Margalef indeces, *Artemisia herba-alba* recorded the highest diversity indeces, while the other plant species showed a varied diversity indeces. These diversity levels are similar to those reported by Li et al. (2016) who examined the plant-associated fungi isolated from *Zanxthoylum bungeanum.*

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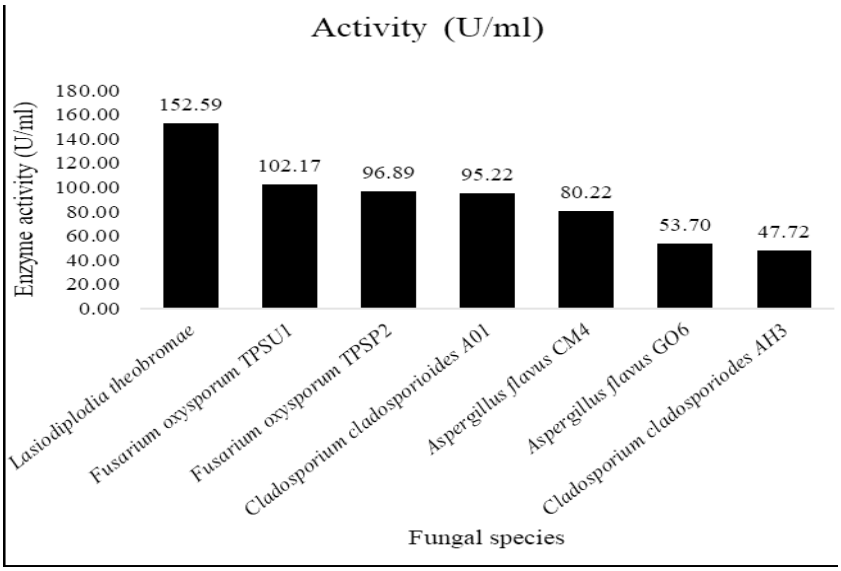

Figure 2 - *L*-asparaginase activities for the nine positive screened plant-associated fungi

On studying the effect of seasonal variation, spring recorded the highest number of taxa, followed by summer, autumn and winter. Our results is compatible with Kim et al. (2013) and Salem and Abdel-Azeem (2014) who suggested that the seasonal variation affected the biodiversity of endophytic fungi in Saint Katherine Protectorate.

Regarding the effect of elevation variation on the biodiversity of isolated endophytic taxa. Low elevation wadis recorded the highest mean of endophytic taxa per plant species followed by mid elevation wadis and high elevation wadis. Our results in agreement with Terhonen et al. (2011), Kim et al. (2013), Salem and Abdel-Azeem (2014) who suggested that the fungal diversity would increase with decreasing elevation altitude. In contrast Higgins et al. (2007) reported a high diversity of plant-associated fungi over high elevations. Furthermore, Terhonen et al. (2011) suggested that the elevation influences the biodiversity of plant associated fungi for its effets on climate.

Statistical analyses showed that plant species strongly influence the diversity of plant-associated fungi. This sociability may be referred to the chemical constituents of the plants. The ability of some of these species to live under water stress and various chemical compounds has been proven by Salem and Abdel-Azeem (2014) and Abdel-Azeem et al. (2018, 2019a) on endophytic fungi in Saint Katherine Protectorate. Furthermore, Tan and Zahou (2001) concluded that it is possible to isolate hundreds of endophytic species from a single plant, and among them, at least one generally shows host specificity. Schulz et al. (2002), Jia et al. (2016) and Khare et al. (2018) suggested that there is a close relationship between the host plant and associated fungi which already proven by Zhao et al. (2011).

Four species with seven morphotypes showed the anti-leukemic enzyme (*L*-asparaginase) production. Our result are in accordance with previous studies on the plants-associated fungi producing *L*-asparaginase carried by Kour et al. (2007) and Yadav and Sarkar (2014) on *F. oxysporum*, Ali et al. (1993) and Kumar et al. (2013) on *C. cladosporiodes*, Mishra (2006) and Rahiman et al. (2014) on *A. niger* and Nagarajan et al. (2014) on *L. theobromae.*

The positive taxa showed an enzymatic activity ranging from 44.5 U ml^{-1} to 152.58 U ml^{-1} where *L. theobromae* recorded the highest activity with 152.58 U ml^{-1} which is higher than what previously reported from *A. terrus*, *R. miehi*, *A. oryzae* and *L. theobromae* (Sarquis et al., 2004; Balbool et al., 2018).

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During this work *L. theobromae* hosted by *T. polium* showed a positive result to produce *L*-asparaginase. Plant extract of *T. polium* are used widely as antioxidants, antibacterial, antifungal and anticancer (Rajabalian, 2008; Kandouz et al., 2010; Khan et al., 2011; Movahedi et al., 2014; Emami Zeydi, 2016). It was previously suggested that both the plants-associated fungi and their host plants produce active biometabolites with similar or higher activity (Zhao et al., 2011; Jia et al. 2016; Khare et al. 2018).

Our results showed that *L. theobroma*e produces *L*-asparaginase extracellularly with a high enzymatic activity, while Narayana et al. (2008) reported that most of the *L*-asparaginase with microbial sources is intracellular. Extracellular *L*-asparaginase interacts minimally with cellular constituents and easier to extract; hence, the induction of extracellular *L*-asparaginases with high activity would be more advantageous for their expoliation as anticancer agents.

L-asparaginase was reported to be used clinically as antileukemic drug (Pieters et al., 2011; El-Nagga et al., 2014; Egler et al., 2016), and industrially as biosensor (Kumar et al., 2013b), and in the biosynsthesis of amino acids (Shrivastava et al., 2016).

In general, this study shed the light on the effect of different parameters like plant species, seasonal variations, elevation and isolation media on the diversity of endophytic fungi in Saint Katherine Protectorate. In addition, it gives an example for the ability of Egyptian fungi to be a gold mine for production of bioactive compounds in a sustainable way.

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