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Research Article

Two new endophytic *Microascus* species from *Capparis spinosa* L. in Iran

Samira Karimzadeh[1], Naser Safaie[1*], Shideh Mojerlou[2] and Leila Ebrahimi[3]

1. Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran.
2. Department of Horticulture and Plant Protection, Faculty of Agriculture, Shahrood University of Technology.
3. Department of Entomology and Plant Pathology, Faculty of Agricultural Technology, College of Agriculture & Natural Resources, University of Tehran, Tehran, Iran.

Abstract: To identify the endophytic fungi of the caper plant, samples were collected from the healthy leaves, stems, and roots from Bushehr and Qom provinces during the summer and early autumn of 2022. Isolation and purification of fungal isolates were performed on potato dextrose agar and 2% water agar culture media, respectively. A total of 102 fungal isolates were obtained. Morphological characteristics and molecular data of the ITS region and *TUB* gene were used to identify the fungal species. Based on the results, *Microascus cirrosus* and *M. terreus* were identified as new endophytic fungi of caper for the first time in the world, and *M. terreus* was introduced as a new record for the funga of Iran.

Keywords: caper, *Microascaceae*, phylogeny, symbiosis, Iran

Introduction

In natural and agricultural ecosystems, plants are associated with plenty of microbial communities in the rhizosphere, endosphere, and phyllosphere, as well as pollen and seed (Nair and Padmavathy, 2014; Liu *et al*., 2017). Endophytes are bacteria or fungi that colonize healthy plant tissue inter- or intracellularly without causing apparent symptoms (Wilson, 1995). Fungal endophytes include a wide variety of plant-pathogenic and saprophytic fungi that have a long incubation period before the appearance of disease symptoms, which is necessary to establish a symbiosis (Hassan, 2007). Endophytic fungi include a very diverse group of fungi in terms of ecological niche and taxonomic position and have been isolated from different plants in different ecosystems (Maki, 2006; Harveson *et al*., 2011)

Based on fossil evidence, endophytes were associated with plants about 400 million years ago (Krings *et al*., 2007). During evolution, the habitat of plants changed from water to land, and they had to face atmospheric conditions characterized by high carbon dioxide, poor soil nutrients, fluctuations in temperature, and difficulty in access to water. In such conditions, fungi help plants to withstand unusual conditions and survive in the soil (Bonfante and Selosse, 2010).

In general, endophytes play important physiological and ecological roles in the lives of their hosts (Tintjer and Rudgers, 2006). The interaction between plants and endophytes is

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*Corresponding author: nsafaie@modares.ac.ir
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important for nutrient acquisition, growth, development, and increased tolerance to various environmental stresses (Schulz and Boyle, 2005). In this way, the host plant provides nutrients to the endophytes, promoting growth rate, limiting pathogen damage and enhancing their host plants' abiotic stress tolerance (Arnold and Herre, 2003). Research showed that most plants coexist with endophytic or mycorrhizal fungi in natural ecosystems (Petrini, 1986).

Caper *Capparis spinosa* L. is an important medicinal plant of the Capparaceae family (Alzahrani *et al*., 2021). It includes 350 species of tropical or subtropical origin, mostly distributed in the Mediterranean regions (Chedraoui *et al*., 2017). This plant is native to the Mediterranean, West and Central Asia tropical regions. It is scattered in different parts of Iran, including the slopes of Alborz, Shiraz, Yazd, and Hormozgan (Emad *et al*., 2013). Due to its deep root system and extensive vegetation, it is placed on the soil as a dense and wide canopy, which prevents soil erosion. This mechanism allows it to grow in dry, stony areas, waterlogged, sandy, and loamy soils with low nutrients (Chedraoui *et al*., 2017). This plant usually grows at low altitudes; some can even be found at altitudes more than 1000 meters above sea level (Chalak *et al*., 2007). The extensive root system leads to a high water-consumption efficiency and a significant ability to seek and absorb water from the environment, which is very effective in conserving water during scattered rains and provides favorable conditions for animals and soil microbiota (Zuo *et al*., 2012; Gan *et al*., 2013).

Regarding using caper as a biological control agent, studies showed that its endophytes could control human diseases. For instance, three fungi, *Eladia* sp., *Cladosporium sphaerospermum*, and *Alternaria* sp., were identified, of which *Eladia* sp. 3-C, had the ability to control *Pseudomonas aeruginosa* and *Alternaria* sp. 6-F could control *Staphylococcus aureus* bacteria (El-Said Osman *et al*., 2020). In the study of Rajabi *et al.*, during sampling in 10 areas of Iran, 72 isolates were obtained, of which *Chaetomium globosum* Kunze, *Diaporthe foeniculina* (Sacc.) Udayanga & Castl., *Mucor circinelloides* Tiegh and *Stemphylium vesicarium* (Wallr.) E.G. Simmons was reported for the first time in Iran from caper, and as endophytic fungi for the first time in the world (Rajabi *et al*., 2021), and it is interesting to note that two fungal isolates *Alternaria alternata* M28 and *Paecilomyces maximus* M7 were capable of producing Rutin, one of the flavonoids and antioxidants (Rajabi *et al*., 2023).

Due to the lack of comprehensive study on the endophytic fungi of this valuable medicinal plant and the importance of endophytic symbiosis in plants resistance to biotic and abiotic stresses, the present study aimed to identify endophytic fungi of caper plant in natural habitats in Iran.

Materials and Methods

Sampling and fungal isolation

To isolate endophytic fungi, samples were randomly collected from the healthy parts of the leaf, stem, and root from 16 mature caper plants in flowering and/or sometimes fruiting stage from Bushehr and Qom provinces during the summer and early autumn of 2022. The collected samples were placed in separate paper bags and transferred to the laboratory of the Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran.

Isolation was done within 48 hours after sampling. For this purpose, the plant samples were first thoroughly washed under running tap water to remove dust and epiphyte fungi. Then, the surface of the leaf, stem, and root samples was cleaned with cotton dipped in 96% ethanol. Fifteen pieces of 5 × 5 mm were selected from each organ .Finally, disinfestation was carried out according to the method of Kusari *et al*. (2009) with a few changes. Briefly, plant segments were disinfected twice with 70% ethanol for 30 s to 2 min (based on the tissue thickness, e.g., leaf pieces for 30 s, stem pieces for 60 s, and root pieces for 2 min), and finally washed three times for 1 m by sterilized distilled water and dried on sterilized filter paper. Disinfested leaf, stem, and root pieces were transferred to potato dextrose agar (PDA). The hyphal tip or single spore

method using 2% water agar and PDA culture medium was carried out for purification of fungal isolates (Singleton *et al*. 1990).

Morphological identification

For morphological identification, the purified fungal isolates were cultured on PDA, oatmeal agar (OA), and potato carrot agar (PCA) at 25 °C for 7 d up to 4 weeks (Jagielski *et al.*, 2016).

For the macroscopic characteristics, after the test time passed, the diameter of the colonies and colony colors were assessed using Rayner's color charts (1970). Microscopic slide mounts were prepared using lactophenol and lactophenol-cotton blue solutions, and 50 of each fungal characteristic, including ascomata, ascus, ascospores, conidiogenous cells (annellides) and the morphology of conidia, were studied (Abdollahi Aghdam and Fotouhifar, 2017) using Olympus BH2 light microscope. Photomicrographs were taken using an Olympus DP72 digital camera attached to an Olympus BX51 microscope with differential interference contrast. Representative isolates of each identified species were deposited in the Microbial Collection of the Agricultural Biotechnology Research Institute of Iran (Karaj).

Molecular identification

DNA extraction, PCR, and sequencing

DNA extraction from mycelia grown on PDA at 25 °C for 7-10 days was performed using the method provided by Safaie *et al*. (2005). The ITS region was amplified using ITS1 and ITS4 primers (White *et al.*, 1990), and for accurate identification of the species, the *beta-tubulin* gene (*TUB*) was amplified by Bt2a and Bt2b primers (Glass and Donaldson 1995).

The 25 µl PCR mixture contained 9 µl of sterile deionized water, 12 µl 2X PCR Mastermix (Pishgam Company), one pmol of each primer, and 2 µl of 30 ng/µl DNA. PCR was carried out in a Thermal cycler device (Epgradiant, Eppendorf) under the following conditions: 95 °C for 3 min; 35 cycles of 94 °C for 40 s, 58 °C and 55 °C for 45 s (for ITS and *TUB*, respectively) and 72 °C for 60 s; 72 °C for 10 min. The PCR products were sequenced by Pishgam Company (www.pishgambc.com).

Phylogenetic analysis

After sequencing, to ensure the accuracy of the obtained data, sequences were compared with relevant sequences in Genbank (NBCI) using the BLAST search tool (Altschul *et al*., 1990).

A megablast search of ITS sequences showed that isolate GRCA10-7 has an identity of 100.00% with *Microascus terreus* (strain CBS 665.71, GenBank: KX923938), and *TUB* sequences BLAST search displayed 99.79% identity with *M. terreus* (CBS 807.73, GenBank: KX924373). For isolate CA04BS-2, the closest hits using the ITS sequence were *Microascus cirrosus* (CBS 277.34, GenBank: LM652401) identities at 99.47%.

The combined ITS and *TUB* sequences alignments of the strains of this study and taken from the GenBank were compared. All sequences were aligned using Muscle software of MEGA 7.0, and the resulting alignment was manually edited. *Yunnania carbonaria* and *Yunnania smithii* were added as outgroup taxa (Woudenberg *et al*., 2017).

Analysis was performed based on Bayesian inference (BI). The estimates of invariant sites (GTR + G + I) were used in phylogeny. The Bayesian analyses were performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) and a random starting tree, running the chains for 2000000 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The Markov chain Monte Carlo (MCMC) method within a Bayesian framework was used to estimate the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) using the 50% majority rule. The convergence of model parameters and topology was assessed based on the average standard deviation of split frequencies and potential scale reduction factor values. The output file of the phylogenetic program was visualized using Dendroscope V.3.2.8 (Huson and Scornavacca, 2012), and both ITS and *TUB* Bayesian trees were drawn in CorelDRAW software version 17. All sequences in the present study were submitted to the GenBank (NCBI) database, and their accession numbers are shown in Table 1.

Table 1 Used sequences of ITS and *TUB* (Woudenberg *et al*., 2017 and this study) in phylogenetic analysis.

Species	Strain	Host/ Substrate	Location	GenBank no.	
				ITS	*TUB*
Microascus alveolaris	CBS 268.49	*Avena sativa*	USA	KX923823	KX924257
M. alveolaris	CBS 270.49	Hordeum vulgare	USA	KX923825	KX924259
M. atrogriseus	CBS 410.76	Burnt soil	Netherlands	KX923831	KX924266
M. atrogriseus	DTO 191-C2	Indoor horse arena	Netherlands	KX923833	KX924268
M. brunneosporus	CBS 138276	Human	USA	KX923834	KX924269
M. cinereus	CBS 664.71	Human	USA	KX923835	KX924270
M. cinereus	CBS 138709	Human	USA	KX923837	KX924272
M. cirrosus	CBS 217.31	*Prunus* sp., leaf	Italy	KX923838	KX924273
M. cirrosus	**ABRIICC 10403**	***Capparis spinosa***	**This study**	**OR681555**	-
M. cleistocarpus	CBS 134638	Discarded cloth	China	KX923851	KX924286
M. croci	CBS 158.44	*Crocus* sp.	Netherlands	KX923852	KX924287
M. croci	DTO 220-I5	Indoor	Indonesia	KX923855	KX924290
M. expansus	CBS 138127	Human, sputum	USA	KX923859	KX924294
M. intricatus	CBS 138128	Human BAL fluid	USA	KX923872	KX924307
M. intricatus	DTO 223-A6	Indoor	Micronesia	KX923873	KX924308
M. longicollis	CBS 752.97	*Anacardium occidentale*	Brazil	KX923874	KX924309
M. melanosporus	CBS 854.68	Compost soil	Germany	KX923877	KX924312
M. melanosporus	DTO 255-C3	-	Germany	KX923904	KX924339
M. micronesiensis	CBS 141523	Indoor	Micronesia	KX923905	KX924340
M. micronesiensis	DTO 223-A5	Indoor	Micronesia	KX923906	KX924341
M. paisii	DTO 109-G6	Indoor	Denmark	KX923912	KX924347
M. paisii	DTO 255-B8	Polystyrene	Germany	KX923921	KX924356
M. pseudopaisii	CBS 141581	Air, basement	Netherlands	KX923923	KX924358
M. pseudopaisii	DTO 116-A4	Air, basement	Netherlands	KX923924	KX924359
M. pyramidus	CBS 212.65	Desert soil	USA	KX923925	KX924360
M. pyramidus	CBS 663.71	Soil	USA	KX923927	KX924361
M. senegalensis	CBS 277.74	Mangrove soil	Senega	KX923929	KX924363
M. senegalensis	DTO 342-H4	-	-	KX923937	KX924371
M. terreus	CBS 665.71	Soil	USA	KX923938	KX924372
M. terreus	**ABRIICC 10404**	***C. spinosa***	**This study**	**OR681556**	**OR689840**
M. trigonosporus	CBS 198.61	-	-	KX923944	KX924378
M. trigonosporus	DTO 220-I8	Indoor	Micronesia	KX923947	KX924382
Scopulariopsis asperula	CBS 390.52	*Calliphora vomitoria*	France	KX923962	KX924392
S. asperula	CBS 289.38	Human	Italy	KX923960	KX924394
S.africana	CBS 118736	Mud, salt pan	South Africa	KX923954	KX924388
S. albida	CBS 415.51	-	Germany	KX923955	KX924389
S. brevicaulis	CBS 340.39	Bone	South Africa	KX923972	KX924405
S. brevicaulis	CBS 118472	-	UK	KX923984	KX924417
S. candida	CBS 353.36	Indoor air	-	KX924011	KX924446
S. candida	DTO 139-E8	Indoor	Germany	KX924019	KX924453
Y.carbonaria	CBS 205.61	Soil	Panama	KX923820	KX924254
Y. smithii	CBS 855.68	Garden soil	Germany	KX923822	KX924256

The sequences studied in this research are shown in bold.

Results

Sampling and fungal isolates

102 fungal isolates were obtained from leaves, stems, and roots, of which 14 belonged to the genus Microascus based on macroscopic and microscopic characteristics. The species *M. terreus* was more common (53.85%) than *M. cirrosus*, with 46.15% abundance. Of the isolates obtained from the two regions examined, 46.15% were from Bushehr Province, and 53.85% were from Qom. Furthermore, the majority (61.5%) of the total

number of isolates came from root tissue; the second was leaf tissue (23.1%), and the third was stem tissue (15.4%). In the root tissue, which has the largest number of isolates of this genus, *M. terreus* accounts for 53.8% and *M. cirrosus* for 7.7% (Fig. 1). Based on the results of morphological features and molecular data, *M. terreus* is introduced as a new record for the funga of Iran, and *M. cirrosus* is reported as an endophytic fungus from caper for the first time in the world.

Phylogenetic analysis

The amplified partial length of the ITS1-5.8S-ITS2 region and *TUB* gene were 598 bp and 612 bp, respectively. In the phylogenetic analysis based on the ITS1-5.8S-ITS2 and *TUB* sequences, CA04BS-2 was placed next to *M. cirrosus* (CBS 217.31) with a posterior probability of 1.00 and isolate GRCA10-7 was placed next to *M. terreus* (CBS 665.71) with a posterior probability of 1.00 (Fig. 2).

Taxonomy

Microascus cirrosus Curzi, Boll. R. Staz. Patalog. Veget. Roma, N. S. 10(3): 308 (1930).

Examined isolates: Iran, Bushehr province, Bushehr City, 2022/07/23, Samira Karimzadeh, (CA03BL-4, CA04BS-2, and CA02BR-6).

Colonies growth on OA and PCA attained a diameter of 46 mm and 35 mm after 14 days at 25 °C, respectively. The colony on the OA was buff initially, gradually turning umber, and it was sienna color on PCA. In both colonies, it started to darken from the center, and black granular ascomata formed.

Ascomata globose to sub-globose, black, 110–220 (av. 165) µm diam., with cylindrical necks with 58–61 (av. 59.5) × 25–34 (av. 29.5) µm diam. Peridium black, composed of thick-walled textura angularis. Asci ovate to globose, 8-spored, 9–11 (av. 10) × 8–10 (av. 9) µm. Ascospores concavo-convex or sometimes plano-convex to oval, pale brown, 5–6 (av. 5.5) × 3–4 (av. 3.5) µm. On vegetative hyphae, annellides singly or in groups of two or three, 10–15 (av. 12.5) × 2.5–3.5 (av. 3) µm with abruptly narrowed apices. Conidia arranged in chains, sub-globose or oval to ovoid shaped, with a truncate base and light brown, that their size was 4–5.5 (av. 4.25) × 3–4 (av. 3.25) µm (Fig. 3).

Microascus terreus (Kamyschko) Jagielski, Sand. -Den. & Gené, in Jagielski, Sandoval-Denis, Yu, Yao, Bakuła, Kalita, Skóra, Krzyściak, de Hoog, Guarro & Gené, *Fungal Biology* 120(4): 597 (2016).

Examined isolates: Iran, Qom province, Qom City, **2022**/10/12, Samira Karimzadeh, (GRCA6-12, GRCA7-11 and GRCA10-7).

Colonies growth on OA and PCA attained a diameter of 27–29 mm after 14 days (51-53 mm after 21 days) at 25 °C. Colonies were buff to Isabelline with immersed and regular margin, that on PCA turned a little ochreous, and granular black ascomata at the center began to form and became abundant.

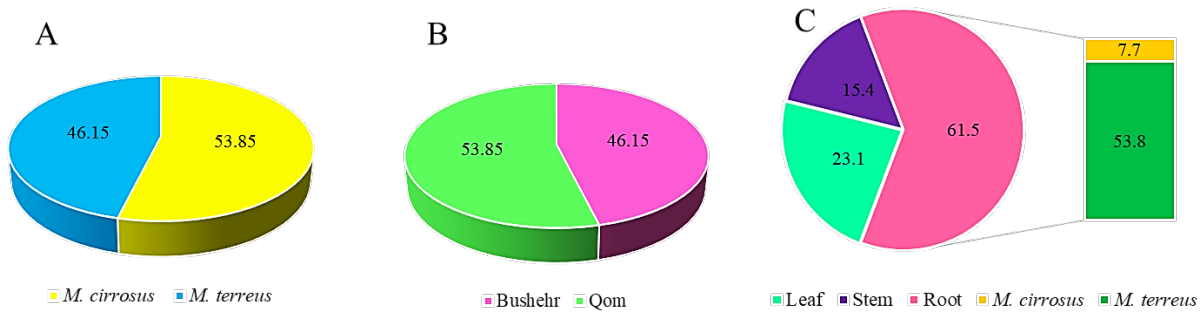

Figure 1 A) Abundance percentage based on fungal species, B) Sampling areas, and C) Plant tissue.

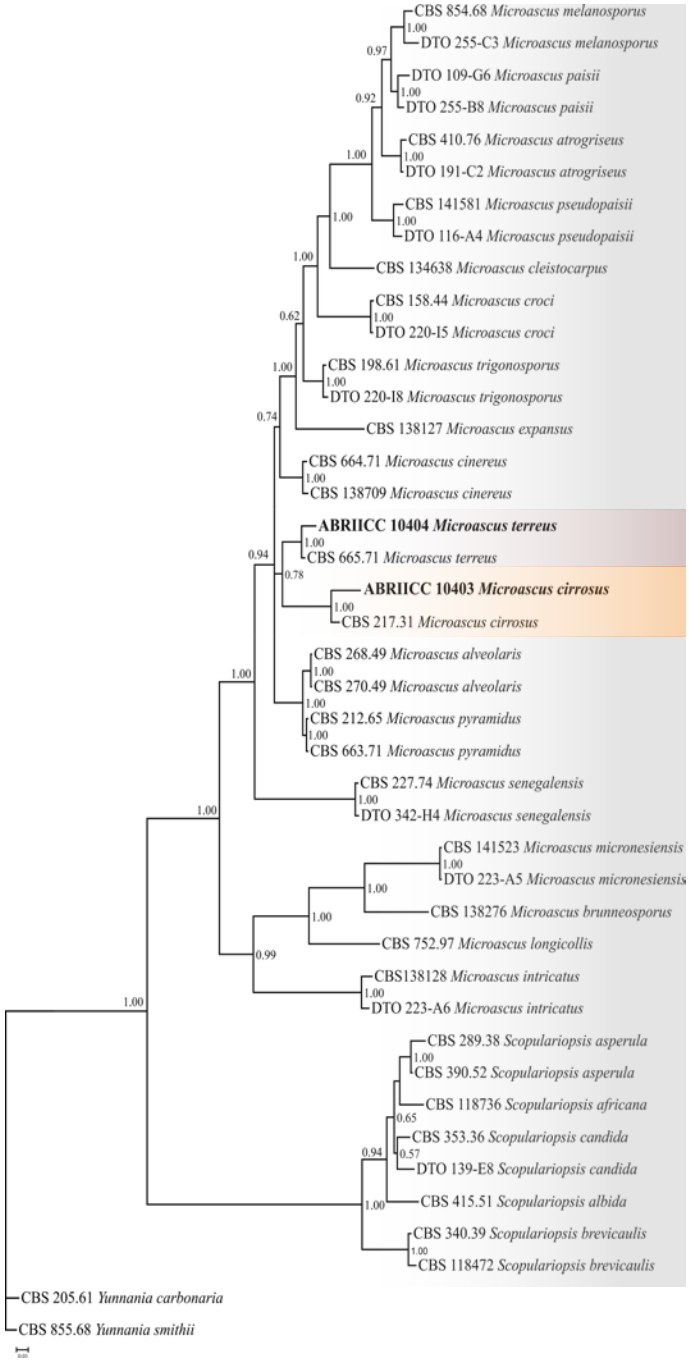

Figure 2 Gene tree inferred by Bayesian 50% majority rule consensus tree inferred using ITS region and *TUB* gene under the GTR + G + I model using 42 nucleotide sequences. *Yunnania carbonaria* (CBS 205.61) and *Yunnania smithii* (CBS 855.68) were used as outgroups. Bayesian posterior probabilities (BPP) are given more than 50% for appropriate clades. New sequences are in bold font.

Ascomata globose to subglobose, black 177–271 (av. 224) µm diam., with a short ostiola neck, glabrous and black, with 42.5–44 (av. 43.25) × 31–33.5 (av. 32.25) µm diam. Peridium dark brown or black with a textura angularis. Asci ellipsoides to globose, 8 spored, 9–11 (av. 10) × 7.5–9 (av. 8.25)

µm. Ascospores triangular with concave sides and yellowish brown, 5–6 (av. 5.5) × 3.5–4 (av. 3.75) µm. Annellides single, lateral and sessile on vegetative hyphae, 7–11 (av. 9) × 2–3.5 (av. 2.75) µm. Conidia arranged in chains, sub-globose or oval to ovoid shaped, basally truncate, and light brown in color, 3.5–5 (av. 4.25) × 3–3.5 (av. 3.25) µm diam (Fig. 4).

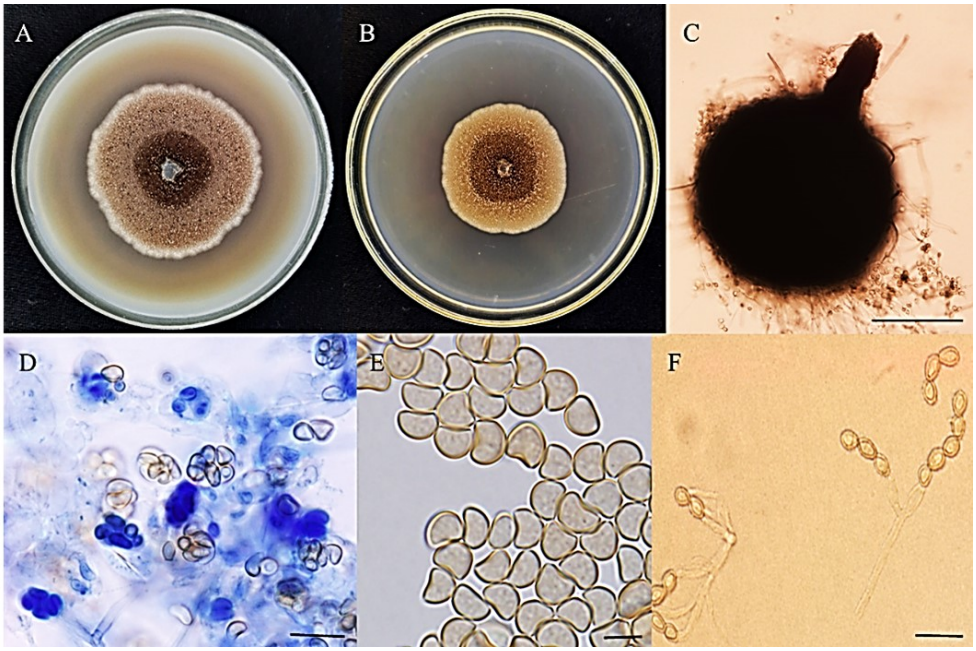

Figure 3 *Microascus cirrosus*, isolate CA04BS-2: A and B) Colony on OA and PCA after 14 days at 25 °C, C) Ascomata, D) Asci, E) Ascospores and F) Annellides and conidia. Scale bars: C = 100 µm; D-F = 10 µm.

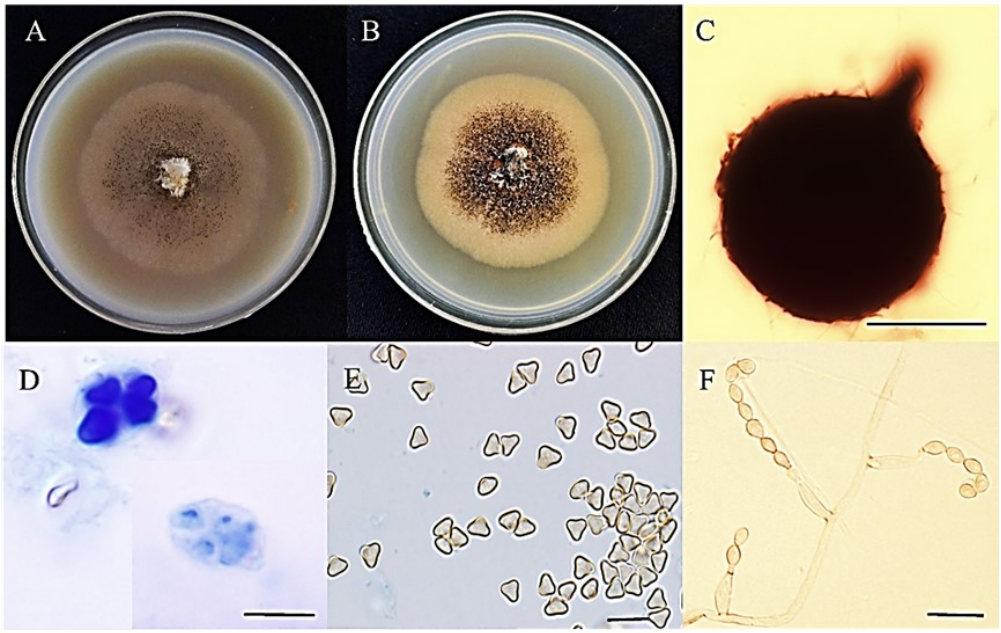

Figure 4 *Microascus terreus*, isolate GRCA10-7: A and B. colony on OA and PCA after 21 days at 25 °C, C. Ascomata, D. Asci, E. Ascospores, and F. Annellides and conidia. **Scale bars**: C = 100 µm; D-F = 10 µm.

Discussion

Today, it is proven that plants host many types of microbial symbionts (Müller and Döring, 2009). With all available evidence, symbionts such as endophytes have been recognized for their ability to protect their host against stresses and to be used as biological control agents. Therefore, the isolation and characterization of endophytic microorganisms from plants that have not yet been studied can enable the discovery of new species (Strobel and Daisy, 2003).

In this study, an investigation of 14 endophytic fungal isolates recovered from healthy leaves, stems, and roots of *C. spinosa* collected from Bushehr and Qom in Iran showed that the isolates belonged to the *Microascus* genus. Detailed morphological features and phylogeny analyses based on ITS and *TUB* sequences identified the isolates as two different species, namely *M. cirrosus* and *M. terreus*. Characteristics of *M. cirrosus* isolates were similar to the description of this species by Mirzaee *et al*. (2010). It has been reported from *Prunus* sp. In Italy (Sandoval-Denis *et al*., 2016), and from leaf spot in pistachio for the first time in Iran (Mirzaee *et al*., 2010). Characteristics of *M. terreus* isolates were also similar to the description of this species from soil (Ukraine) provided by Jagielski *et al*. (2016). It has been reported from *Helianthus annuus* L. seed soil (USA) and saline desert soil (Kuwait) (Woudenberg *et al*., 2017). *Microascus alveolaris* is morphologically similar to this species but differs by ascospore size, which is 4–6 (av. 5) × 3–5 (av. 4) µm, and shorter annelids (6–7 (av. 6.5) µm) (Sandoval-Denis *et al*., 2016). Based on the results of the phylogeny of our study, although *M. cirrosus* is the sister group of *M. terreus*, they are different in terms of morphology, especially in the shape of ascospores, where ascospores are triangular in *M. terreus*, and concavo-convex to oval in *M. cirrosus*. Also, the annelids shape was single annellide in *M. terreus*, but singly or in groups of two or three in *M. cirrosus*. Both species are reported as endophytes of the caper plant for the first time in the world, and *M. terreus* is a new record for the funga of Iran.

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دو گونه اندوفیت جدید *Microascus* از *Capparis spinosa* L. در ایران

سمیرا کریم‌زاده[1]، ناصر صفایی[1*]، شیده موجرلو[2] و لیلا ابراهیمی[3]

۱- گروه بیماری‌شناسی گیاهی، دانشکده کشاورزی، دانشگاه تربیت مدرس، تهران، ایران.
۲- گروه باغبانی و گیاه‌پزشکی، دانشکده کشاورزی، دانشگاه صنعتی شاهرود، شاهرود، ایران.
۳- گروه حشره‌شناسی و بیماری‌های گیاهی، دانشکده فناوری کشاورزی، دانشکدگان کشاورزی و منابع طبیعی، دانشگاه تهران، تهران، ایران.
پست الکترونیکی نویسنده مسئول مکاتبه: nsafaie@modares.ac.ir
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چکیده: برای شناسایی قارچ‌های اندوفیت گیاه کپر، از برگ‌ها، ساقه‌ها و ریشه‌های سالم استان‌های بوشهر و قم در تابستان و اوایل پاییز ۱۴۰۱ نمونه‌برداری شد. جداسازی و خالص‌سازی جدایه‌های قارچی به‌ترتیب روی محیط‌کشت سیب‌زمینی دکستروز آگار و آب آگار دو درصد انجام شد. درمجموع ۱۰۲ جدایه قارچی به‌دست آمد. برای شناسایی گونه‌های قارچی از ویژگی‌های ریخت‌شناختی و داده‌های مولکولی ناحیه ITS و ژن *TUB* استفاده شد. براساس نتایج به‌دست آمده، دو گونه *Microascus cirrosus* و *M. terreus* به‌عنوان قارچ اندوفیت جدید کپر برای اولین بار در جهان شناسایی شدند و *M. terreus* به‌عنوان رکورد جدید فونگای ایران معرفی گردید.

واژگان کلیدی: *Microascus*، کپر، فیلوژنی، هم‌زیستی، ایران