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Alternaria arborescens and *Alternaria angustiovoidea*, two new additions to soil fungi of Egypt

Mohamed Ahmed Abdel-Sater[1,2], Mady Ahmed Ismail[1,2], Nemmat Abdel-Gawad Hussein[1,2], Rania Mohamed Sayed[2*]
[1]*Department of Botany and Microbiology, Faculty of Science, Assiut University, Assiut 71511, Egypt.*
[2]*Assiut University Mycological Centre, Assiut University, Assiut 71511, Egypt.*
[*]*Corresponding author email address: beltrania2005@yahoo.com*

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Abstract. In the current study, eight new recorded isolates related to the genus *Alternaria*, section Alternata were isolated from soil, sorghum, and wheat grains in Assiut Governorate, Egypt. In addition to DNA sequence analysis, morphological characteristics revealed their identity as *Alternaria arborescens* (2 isolates) and *A. angustiovoidea* (6 isolates). To the best of our knowledge, this is the first record of these species in Egypt. The two genetically identified strains of both species were deposited in the culture collection of Assiut University Mycological Centre with accession numbers of *A. arborescens* AUMC 14106 and *A. angustiovoidea* AUMC 14107. Sequences of the two strains' internal transcribed spacer (ITS) gene were uploaded to Gen Bank with accession numbers *A. arborescens* MN240306 and *A. angustiovoidea* MN242398.

Keywords: Mycobiota, *Alternaria*, soil, sorghum, wheat, phenotypic.

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1. Introduction

The genus *Alternaria* has many species and is considered an essential plant pathogen to many plants in the families Solanaceae, Cucurbitaceae, and Brassicaceae [1]. The taxonomy of that genus has been based on conidial characters, which include shape, color, septation, and sporulation patterns [2]. Several studies concerned with the descriptions and revision of *Alternaria* and related genera [3,4] resulted in a growing number of new species. Simmons [3] classified the genus *Alternaria* into two broad sections based on the conidium body length.

Section I includes species with conidium body length long (above 100 µm) and section II for species with conidium body length short (up to 50 µm), and medium (50-100 µm); these sections divided into groups based on conidium morphology and sporulation pattern. Based on these classifications, Simmons [3] could identify 276 *Alternaria* species. At the same time, Woudenberg et al. [5] revealed that *Alternaria* section *Alternaria* consists of 11 species and one species complex. Past two decades, phylogenetic investigations were used to support morphological identification [2,4].

Eight isolates of *Alternaria* were identified as *A. arborescens* (2 isolates), and *A. angustiovoidea* (6 isolates) were isolated from soil, sorghum grains, and wheat grains. Two new records of *Alternaria* were added to fungi of Egypt as *A. arborescens* and *A. angustiovoidea,* which never been isolated previously in Egypt. *A. arborescens* AUMC 14106, and *A. angustiovoidea* AUMC 14107 were selected for further morphological descriptions, and molecular analysis.

2.Materials and methods

2.1. Strains isolation

Two isolates of *A. arborescens* and six of *A. angustiovoidea* were isolated using acidified weak potato-dextrose agar (AWPDA) [6], dichloran chloramphenicol peptone agar (DCPA) [7], dichloran glycerol agar (DG18) [8]; and potato carrot agar with manganese (PCA-Mn) [9]. These isolates were isolated from garden soil samples collected from the Assiut University

campus and sorghum and wheat grains collected from local markets in Assiut Governorate, Egypt. The dilution plate method was used for the isolation of fungi from the soil and the direct plating method from grains. All plates were incubated for 10 days at 25 °C. The developing colonies were isolated in pure cultures and maintained using PDA slants [10] and the cotton balls method [11].

2.2. Morphological studies of *Alternaria* strains

The isolates were grown at a light/dark cycle of 8/16 h on PCA, OA, and MEA media [10] for microscopic identification. Three replicate-plates of all media were incubated at 22 °C. Growth rates and colony characteristics (diameter, appearance, and sporulation on the cut-agar surface) were recorded after 7 days of incubation. Colony colors and reverse were identified, according to Kornerup and Wanscher [12].

On the 5th day of incubation at 22 °C, a rectangular block of agar (5.0 × 20.0) mm was removed from the margin of the colony at the right angle to the radius, then re-incubated [3,13]. On the 7th day, colony development, sporulation patterns, and conidial chains were observed. Slide preparations were examined using a drop of lactic acid to evaluate microscopic features (conidiophores and conidial characteristics). The isolates were described and identified based on macro and micro-morphological characteristics following the keys and descriptions [3].

2.3. Growth of the isolates and DNA extraction

The isolates were grown on PDA plates and incubated at 25 °C for 7 days. For DNA extraction, a small piece of fungal mycelia of the tested isolates was collected and transferred to 2 mL-Eppendorf tubes. Fungal DNA was extracted and isolated using the SolGent purification bead in the SolGent Company (Daejeon, South Korea).

2.4. PCR and rDNA sequencing

The PCR reaction was performed using SolGent EF-Taq. In the PCR tubes, 1µl of DNA template, 1 µl 2.5 mM dNTP mix, 0.2 unit of Taq polymerase, 5 µl of 10x complete buffer, and 40 µl of sterile dH_2O, 10 pmol of ITS1 (5′ TCC GTA GGT GAA CCT TGC GG 3′) as forwarding primer and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) as the reverse primer was added for DNA amplification [14]. Then the PCR amplification was carried out using the following sequence: one round of amplification consisting of denaturation at 95 °C for 15 min, followed by 30 cycles of denaturation at 95 °C for 20 secs, annealing at 50 °C for 40 sec and extension at 72 °C for 1 min, with a final extension step of 72 °C for 5 min. The PCR products were then purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) before sequencing. The purified PCR products were confirmed on 1% agarose gel by electrophoresis using a size marker. The bands were eluted and sequenced in the forward and reverse directions [15].

2.5. Phylogenetic analysis

Sequence data for the closest matching *Alternaria* species, including the representative type strains, were downloaded from GenBank. Sequences produced in this study and those from GenBank were aligned using Clustal_X [16] and optimized manually. Phylogenetic analysis was conducted using the Maximum Likelihood method and the General Time Reversible model [17]. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed [18]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. Initial tree(s) for the heuristic search was obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+*G*, parameter = 0.1000)]. The rate variation model allowed some sites to be evolutionarily invariable [(+*I*), 49.71% sites]. All positions containing gaps and missing data were eliminated (complete deletion option). There were 512 positions in the final dataset. Evolutionary analyses were conducted in MEGA X software version 10.1.6 [19].

3. Results and discussion

3.1. Morphological characteristics of *Alternaria arborescens* E.G. Simmons (AUMC 14106)

Colonies were attaining a diameter of 40-45 mm, 35-40 mm, and 50-55 mm PCA, OA, and MEA respectively after 7 days at 25 °C. Colonies on PCA flat, olive-grey to dark olive-grey (2F1-2), centrally greyish; margin entire, paler than the colony center. Sporulation sparse, few conidial chains were observed after 12 days; reverse the same color as the colony (olive grey). Colonies on OA lanuginose, dark olive-grey (2F2) with greyish mycelia on the surface; margin entire, paler than the colony center; reverse greyish-black to black. On MEA, colonies mole, taupe (4E1) in the center, grey to medium grey (4B-C1) towards the margin; margin entire, raised than the colony surface, pale grey; reverse olive to coal (3E3 – 3F1) (Figure 1).

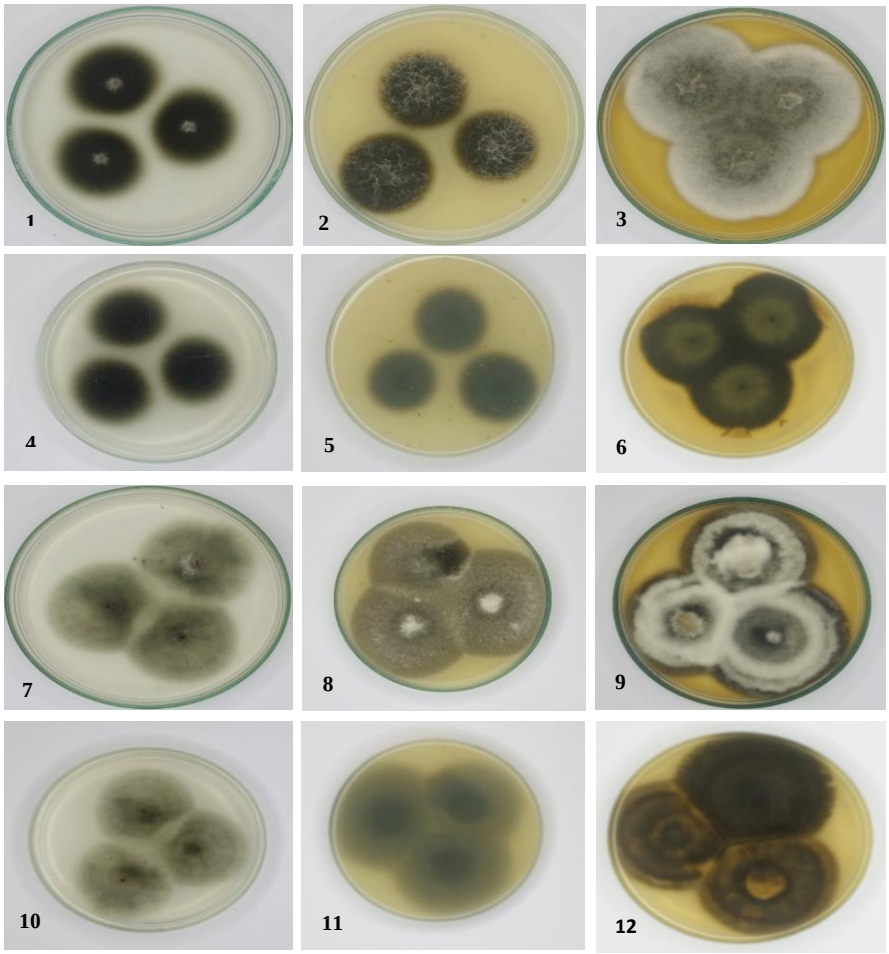

Figure 1. 7-day-old colonies at 25 °C of *A. arborescens* AUMC 14106: 1-3, obverse on PCA, OA, and MEA; 4-6 reverse on PCA, OA, and MEA. 7-9 *A. angustiovoidea* AUMC 14107: obverse on PCA, OA, and MEA and 10-12 reverse on PCA, OA, and MEA.

First conidiophores are extended up to 500 µm and width 4-5 µm bearing a terminal cluster of branches and conidial chains, dull or aggregated; conidia, punctate to verrucose, 17-40 × 7-13 µm with 1-4 transverse septa and 1-2 longitudinal or oblique septa, light tan to brown, arranged in conidial chains of 1-5 conidia. Secondary conidiophores formed at the apical portion of the terminal conidia, short 4-6 × 3-4 µm, geniculate or straightforward (Figure 2). Morphologically, the strain AUMC 14106 has similar morphological characters as the type strain of *A. arborescens* [20]; they are characterized by long primary conidiophores bearing terminal and subterminal branches of conidial chains, which gives the arborescent appearance. *Alternaria*, but the inconsistencies in morphology and molecular data makes further research necessary.

Alternaria arborescens was recorded for the first time as a pathogen, causing tomato stem-canker in California [21], and the first description was introduced [20]. It was first identified as *A. alternata* f. sp. *lycopersici*; later, it has described as *A. arborescens* based on molecular studies [22]. The species produces AL-toxin [5], and it was recorded as the causative agent of a cutaneous Alternariosis in a healthy person [23].

3.2. Morphological characteristics of *Alternaria angustiovoidea* E.G. Simmons (AUMC 14107)

Colonies after 7 days at 25 °C attaining a diameter of 50-55 mm, 55-60 mm, and 60-65 mm on PCA, OA, and MEA, respectively. Colonies on PCA flat, zonate, grey to olive-grey (1F5-7); sporulation abundant; margin entire, paler than the colony center; reverse olive (1F4-6). On OA, colonies lanuginose, greyish-beige in the center (4C2), brownish-grey to olive-brown (4D2–4E3) towards the margin; margin entire, thin; reverse taupe, black (4F1) in the center, and olive (3E3) towards the margin. Colonies on MEA, floccose, centrally raised, greyish-orange to brownish-orange (5B3-5C3) in the center, nougat, greyish-brown, drab, mouse grey (5D3-5E3) towards the margin; margin entire, thin; reverse coffee to raw umber (5F7-8) in the center, olive-brown (4F8) towards the margin (Figure 1).

First conidiophores are simple, up to 250 × 4 µm; conidial chains unbranched with 6-8 (-10) conidia. Young conidia (with maximum one transverse septum) 5-20 × 4-7 µm; mature conidia narrow-ovoid, 40-60 × 8-14 µm, ornamented, pale to dull yellow-brown with 3-8 transverse septa and 0-1 longitudinal septum in each transverse segment; median septum darker, more constricting than the other septa; secondary conidiophores 10-25 µm long (Figure 2).

The strain AUMC 14107 is characterized mainly by the presence of aerial mycelia, and A very high percentage of its conidia are long-narrow-ovoid with numerous transverse and few longitudinal septa and constricted median septum. These morphological characteristics were closely matched with those of the type species *A. angustiovoidea*. The AASC can be distinguished from all species now recognized within the section. *A. angustiovoidea* was isolated for the first time from *Euphorbia esula* (leafy spurge) in Manitoba, Canada, from North Dakota and Iowa in the USA [24,25]. Woudenberg et al. [4] recorded *A. angustiovoidea* as a synonym for *A. alternata*.

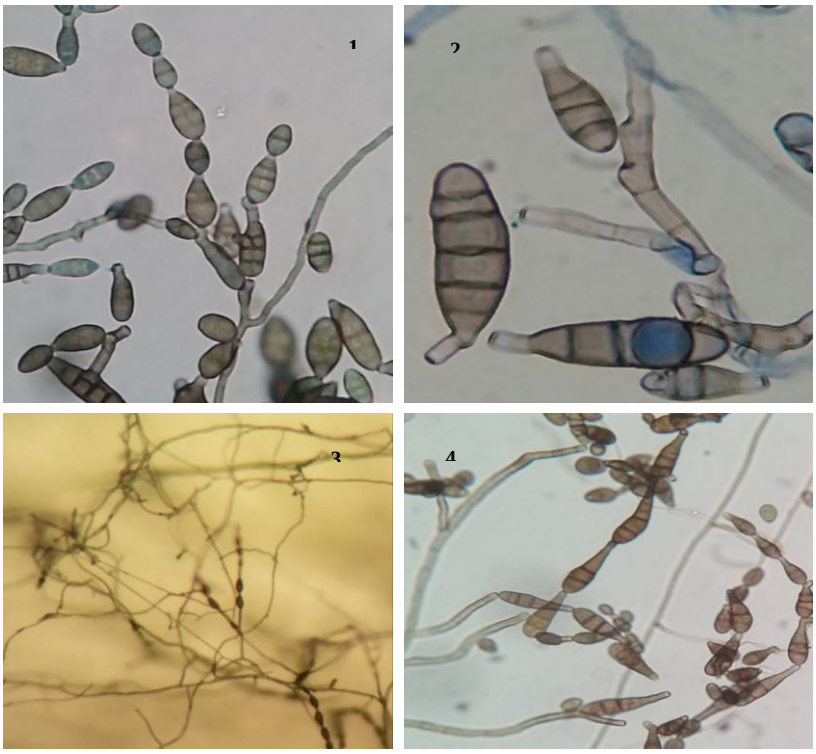

Figure 2. *A. arborescens* AUMC 14106: 1- sporulation clumps at the end of long conidiophore, 2- conidiogenous site, and conidia; *A. angustiovoidea* AUMC 14107, 3- sporulation pattern on PCA, 4 – conidia and conidiophore.

3.3. Molecular analysis

The ITS dataset comprised 12 sequences, of which two sequences of *Alternaria* species were produced in the current study, in addition to 10 sequences of the most similar *Alternaria* species obtained from the GenBank database (Figure 3).

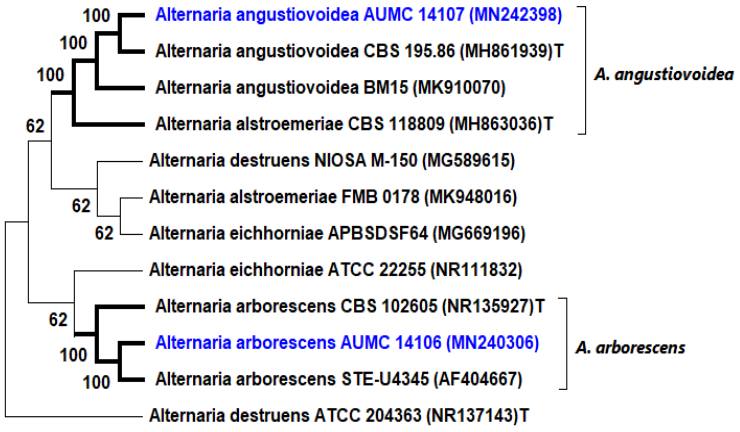

Figure 3. Maximum Likelihood phylogenetic tree of *A. arborescens* AUMC 14106 and *A. angustiovoidea* AUMC 14107 aligned with other closest matching *Alternaria* species the ITS gene sequences (GenBank accession numbers in parentheses).

Based on megablast search using the ITS sequence of *Alternaria* spp., AUMC 14106, and AUMC 14107, the closest matches in GenBank nucleotide database were related to *A. angustiovoidea* CBS 195.86 (= MH861939) and *A. destruens* ATCC 204363 (= NR137143) with 99.82% similarity for each; *A. arborescens* CBS 102605 (= NR135927); with 99.81% similarity. Also, the strain in the current study matched by 99.64% and 99.27% with *A. alstromeriae* CBS 118809 (= NR163686) and *A. eichhorniae* ATCC 22255 (= NR111832), respectively. The nucleotide alignment and phylogenetic analysis showed that strain AUMC 14106 was nested between *A. arborescens* CBS 102605 (type strain) and *A. arborescens* STE-U4345 while strain AUMC 14107 was located with *A. angustiovoidea* CBS 195.86 (type strain). Based on the phylogenetic analysis, it has been clarified that the strain AUMC 14106 was *A. arborescens,* and strain AUMC 14107 was *A. angustiovoidea*.

4. Conclusion

In this research work, eight recorded isolates related to the genus *Alternaria* were isolated from soil, sorghum, and wheat grains in Assiut Governorate, Egypt. DNA sequence analysis revealed their identity as *Alternaria arborescens* (2 isolates) and *A. angustiovoidea* (6 isolates), the first record of these species in Egypt. The two genetically identified strains of both species were deposited in the culture collection of Assiut University Mycological Centre with accession numbers of *A. arborescens* AUMC 14106 and *A. angustiovoidea* AUMC 14107. Sequences of the two strains' internal transcribed spacer (ITS) gene were uploaded to GenBank with accession numbers *A. arborescens* MN240306 and *A. angustiovoidea* MN242398.

Conflicts of interest. There is no conflict of interest.

ORCID
Mohamed Ahmed Abdel-Sater: https://orcid.org/0000-0003-4996-8787
Mady Ahmed Ismail: https://orcid.org/0000-0001-5611-390X
Nemmat Abdel-Gawad Hussein: https://orcid.org/0000-0002-8572-2437
Rania Mohamed Sayed: https://orcid.org/0000-0002-6825-3173

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