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Molecular Identification of Alternaria spp. Isolated from Wheat Seeds in Salah AL-Deen Governorate in Iraq

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ABSTRACT

Three local isolates of Alternaria spp. were collected from wheat seed in Salah AL-Deen. These were morphologically identified confirmed as *Alternaria alternata*, *A. tenuissima* and *A. saponaria* on the basis of internal transcribed spacer (ITS) sequence similarity by using two primers ITS-1 ITS-4 synthesized on the basis of conserved regions of the eukaryotic rRNA gene. The studied local isolates were compared with the global isolates preserved in the Genbank (NCBI). The wheat seed samples studied in this study were contaminated with different percentage of fungi. Alternaria spp. is post-harvested contaminated fungi. Therefore, the Silos will not receive the yield unless it meets the conditions for good storage. The molecular study confirmed the morphological diagnosis of Alternaria spp. Thus, the selected species showed genetic relationship by using DNA sequencing. Further, the bs showed a base pair which indicated that studied isolates belonged to Alternaria spp.

Key words: *Alternaria*, wheat, ITS sequence, Genbank, wheat

INTRODUCTION

The genus *Alternaria* spp. is a widespread fungi in different environments, atmosphere, seeds, dust, soil indoor environments (Jun-Fu Li *et al.,* 2023), considered as a common contaminant for various agricultural commodities (Woudenberg *et al.*, 2015; Da Cruz Cabral *et al.*, 2017; Poursafar *et al.*, 2019). Further, it is one of the groups of saprophytic fungi, but, some species have a pathogenic potential on plants. It is returned to the *Deuteromycetes* but some species represented the conidial phase of *Ascomycetes* genus as the *Leptosphaeria* genus thus about 299 species are in the genus *Alternaria* (Burchett and Burchett, 2017). This genus is widely distributed in soil on aerial plant surfaces. Species of this genus are common field pathogenic that may affect grain yields in the field or can cause post-harvested losses of plant products (Tariq *et al*, 2020). The species *A. alternata* is abundant in the airspora, especially during the ripening and harvesting of grain crops. Ripening ears of wheat are colonized by *A. alternata* soon after emergence (Filiz and Çakir, 2017). It is recorded as the most common sub-epidermal fungus of wheat grain, *A. alternata* alone or with other fungi such as *A. triticina* can cause a clear black or brown discolouration of wheat kernels, called black spot disuse (Filiz and Çakir, 2017; Hoffmann *et al.*, 2021). This can result in decreased quality and yield of grain. This genus can produce about 14 different secondary metabolites in the form of mycotoxins or phytotoxins (Lawrence *et al.* 2016; Poursafar *et al.*. 2019; Jaata, 2021). Some of these are beneficial can be used in the application of biotechnology as myco-herbicides or bio-control agents (Woudenberg *et al.*, 2015; Lawrence *et al.*, 2016; Jun-Fu Li *et al.,* 2023). On the other hand, the most of them have a toxic effect to human and animals (Jaata, 2021). The genus *Alternaria nees* was firstly described in *A. tenuis* by Nees Von Essenbeck. This was the first stage in the study of *Alternaria* (Poursafar *et al.*, 2019) and the last stage was observed by Grum-Grzhimaylo *et al.* (2016) based on molecular phylogenetic methods to the taxonomy of *Alternaria* which had many efforts to classify the genus based only on the morphological characteristic (Jun-Fu Li *et al.*, 2023).

MATERIALS AND METHODS

Each isolate was grown on PDA after 5-7 days at 25±2°C. DNA was extracted directly from the aerial mycelium by the tube with L shape from fungus. DNA to be used as template for (PCR) reaction were stored at 4°C for use in regular work and at -20°C for long term storage. Genomic DNA was extracted using the Fungal/ Bacterial DNA Isolation kit (ZYMO, USA) following the manufactures recommendations for fungal DNA. The PCR amplification was performed in a total volume of 25 µl containing 1.5 µl DNA, 5 µl Taq PCR Pre Mix (Intron, Korea). 1 µl of each primer (10 pmol), then distilled water was added into tube to a total volume of 25 µl. The detais of the primers are mentioned in Table 1. The thermal cycling conditions were done as: Denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 52°C for 45 s and 72°C for 45 s with final incubation at 72°C for 7 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem). The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302 nm) after red stain staining (Intron Korea). To analyse the molecular data accurately and to confirm the molecular diagnosis of the studied fungal species and to determine closest sequence matches of these species with the recorded isolates in GenBank they were sequenced by International Biotechnology Company Bioneer, Korea. ITS sequence analysis was performed using the analysis program (BLAST). Sequences of each isolate were deposited in GenBank. A comparison of 5.8 rDNA gene sequence of the test strain against nucleotide collection (nr/nt) database was done using BLAST program.

RESULTS AND DISCUSSION

The *Alternaria* spp. isolates were collected from samples of wheat seed taken from seed production store companies. Morphological and microscopic observations of the *Alternaria spp.* isolates are shown in Fig. 1. The isolates were grown on PDA at 25°C with streptomycin. The colonies were fast growing with dark coloured segmented mycelium black to olivaceous black or greyish (Dinodia *et al.*, 2022). The conidia were singly or in the form of simple or branching chains, in an acrobatic manner. The conidia were large, multi-cellular, divided by longitudinal and transverse walls, and in some species may bear an appendage called the beak, usually club-shaped or spherical. The genus *Alternaria* spp. belongs to the phylum Ascomycota, sub-phylum *Pezizomycotina*, class *Dothideomycetes*, sub class *Pleosporomycetidae*, Order *Pleosporales*, sub order *Pleosporineae*, Family *Pleosporaceae*, Genus *Alternaria* (Jun-Fu Li *et al.*, 2023).

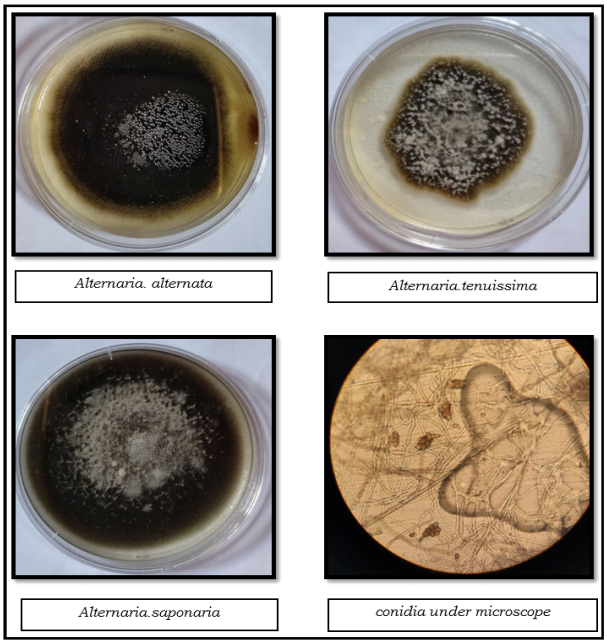

Fig. 1. Culture and microscope picture for fungi isolate. Culture picture using single spore technique and microscope picture with scotch tape technique.

Alternaria spp. was selected as a post-harvested and storage contaminated fungi that produced mycotoxins, which appeared in three species in the current study, as well as to support the identification based on taxonomic features by

Table 1. The specific primer of gene ITS

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5′- TCCGTAGGTGAACCTGCGG -3′	60.3	50	500-650 base pair
Reverse	5′ TCCTCCGCTTATTGATATGC-3′	57.8	41	

using the PCR technique. The genetic sequences of primers used in this study were obtained from the Genbank on the NCBI. The ITS region of three fungal isolates is amplified in Fig. 2. Also, the thermal cycles of the primers and the nucleotide sequences of *Alternaria* spp. were confirmed with the primers ITS-1, ITS-4 as the electrophoresis distance shown was 600 bp in Fig. 3. The use of primers mentioned in Table 1 showed their success in the process of amplifying DNA products of *Alternaria* spp. as the band showed a base pair. These results agree with many researchers who have worked on amplifying the ITS region in fungal identification as a universal genetic code (Al-Khafaji, 2017).

The results in Table 2 and Figs. 4, 5 and 6 show the comparison of test strain against known sequences of SSU and LSU rRNA databases with 5.8 S rRNA gene sequences of *Alternaria* spp. isolates. The products amplification of the ITS region of the local isolates showed success through the possibility of determining the sequences of their nucleotides bp. The molecular study confirmed the phenotypic and microscopic identification of the studied local isolates at the level of

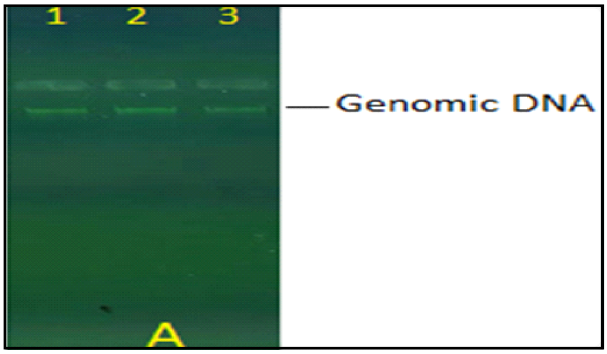

Fig. 2. Gel electrophoresis of genomic DNA extraction from three fungal isolates, 1% agarose gel at 5 vol/cm for 1 h.

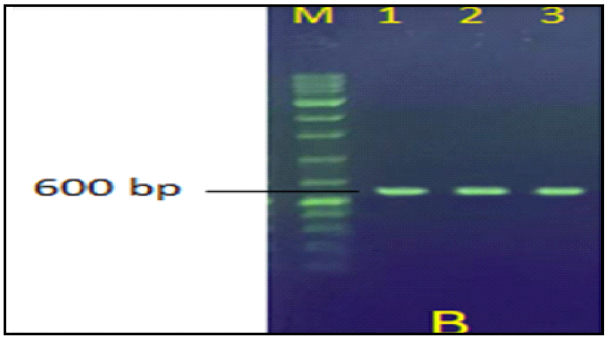

Fig. 3. PCR product of the band size 600 bp. The product was electrophoresis on 2% agarose at 5 volt/cm³ at 1X TBE buffer for 1 h. The number 1 represented the band of *Alternaria alternata;* 2. *A. tenuissima* and 3. *A. saponaria.*

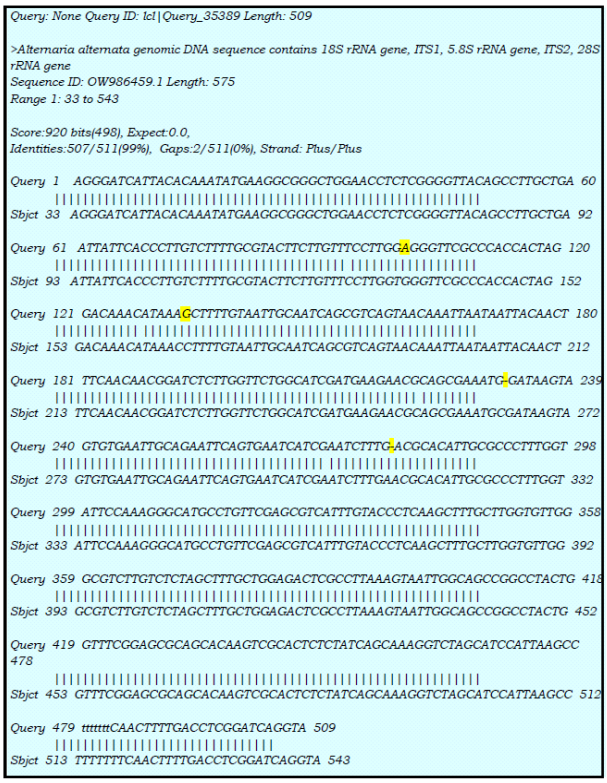

Fig. 4. Nucleotide sequences of ITS region 5.8S rRNA to the fungi *Alternaria alternata* isolate Mha-1 with the high identical isolate *Alternaria alternata OW986459.1.*

Table 2. Molecular identification of fungi isolate based on the identity percentage of nucleotide sequences at 5.8S rRNA gene with other fungi strains in Genbank

Accession number of local identified fungi	OQ789234.1	OQ789239.1	OQ789238.1
Type of isolate fungi recorded in Genbank	*Alternaria alternata* isolate Mha-1	*Alternaria tenuissima* isolate Mha-2	*Alternaria saponaria* isolate Mha-3
Ratio of similarity	99.22	98.87	98.55
Country	Belgium	Hungary	Netherland
Accession number	OW986459.1	MT134984.1	KC584215.1
Type of high identical fungi	*Alternaria alternata*	*Alternaria tenuissima* isolate N10F2	*Alternaria saponaria* strain CBS 116492

Fig. 5. Nucleotide sequences of the ITS region 5.8S rRNA to the fungi *Alternaria tenuissima* isolate Mha-2 with the high identical isolate *Alternaria tenuissima* MT134984.1.

Fig. 6. Nucleotide sequences of the ITS region 5.8S rRNA to the fungi *Alternaria saponaria* isolate Mha-3 with the high identical isolate *Alternaria saponaria* KC584215.1.

genus and species when compared with the global isolates in the Genbank (NCBI). The nucleotides sequences of three fungal isolate species in Iraq were determined and preserved in the Genbank (NCBI). *Alternaria alternata* showed 99.22% homology with the isolate with accession number OW986459.1 in Belgium; *A. tenuissima* showed 98.87% homology with the isolate at accession number MT134984.1 in Hungary and *A. saponaria* showed 98.55% with the isolate at accession number KC584215.1 in Netherland. This percentage was referred as the presence of molecular differences between number of nucleotide base pair at about 2-4 bp. This variability could be due to mutation, somatic hybridization and heterokaryosis (Ogada *et al.*, 2021), or may be returned to biodiversity and geographical distances between the isolates leading to adaption of all isolates to the biological and ecological conditions (Turzhanova *et al.*, 2020).

CONCLUSION

The wheat seed samples studied in Salah Al-Deen Governorate were all contaminated with fungi in different percentage by *Alternaria* spp., one of the post harvest contaminated fungi. Therefore, periodic and continuous examination of stored wheat varieties to determine the amount of contamination in fungi load is very important. The silos should not receive the grains unless it meets the conditions for good storage. The molecular study confirmed the morphological diagnosis of *Alternaria* spp. by their genetic relationship by using DNA sequencing. The use of specific primers tested in the study showed its success in the process of DNA amplification of *Alternaria* spp. as the bands showed a base pair which indicated that the isolates under study belonged to *Alternaria* spp., as the ITS region is a universal genetic code to diagnose fungi.

REFERENCES

Al-Khafaji, Nada Ahmed Fairouz (2017). Molecular characteristics of isolates of the fungus *A. niger* that produce toxins and pollute grains and the possibility of controlling them in the laboratory. Master thesis, College of Science, University of Al-Qadisiyah, Iraq.

Burchett, S. and Burchett, S. (2017). *Plant Pathology*. Taylor and Francis, UK.

Da Cruz Cabral, L., Rodriguero, M., Stenglein, S., Nielsen, K. F. and Patriarca, A. (2017). Characterization of small-spored Alternaria from Argentinean crops through a polyphasic approach. *Int. J. Food Microbiol*. **257**: 206-215.

Dinodia, N., Sharma, R. K. and Gond, V. K. (2022). Endophytic fungi isolation and identification from stem of *Tinospora cordifolia*. *Res. Square* **2022**. *https://doi.org/10.21203/rs.3.rs-1483060/v1*.

Filiz, Ü. and Çakir, E. (2017). Molecular identification of sooty molds on wheat fields in central Anatolia region and effect of seed germination. *Eur. J. Agric. Res*. **1**: 73-81.

Grum-Grzhimaylo, A. A., Georgieva, M. L., Bondarenko, S. A., Debets, A. J. and Bilanenko, E. N. (2016). On the diversity of fungi from soda soils. *Fungal Diver*. **76**: 27-74.

Hoffmann, A., Lischeid, G., Koch, M., Lentzsch, P., Sommerfeld, T. and Müller, M. E. (2021). Co-cultivation of Fusarium, Alternaria and Pseudomonas on wheat-ears affects microbial growth and mycotoxin production. *Microorganism* **9**: 443.

Jaata, Ahmed Turki Nayef (2021) Evaluation of some fertilization treatments and the fungus *Trichoderma hazarium* in the control of Alternaria spot disease on wheat. Master's thesis, College of Agriculture, Tikrit University, Iraq.

Jun-Fu Li, Hong-Bo Jiang, Rajesh Jeewon, Sinang Hongsanan, Darbhe Jarayama Bhat, Song-Ming Tang, Saisamorn Lumyong, Peter Edward Mortimer, Jian-Chu Xu, Erio Camporesi,Timur S. Bulgakov, Gao-Juan Zhao, Nakarin Suwannarac and Rungtiwa Phookamsak (2023). Alternaria: Update on species limits, evolution, multi-locus phylogeny and classification. *Studies Fungi* **8**. *https://doi.org/10.48130/SIF-2023-0001*.

Lawrence, D. P., Rotondo, F. and Gannibal, P. B. (2016). Biodiversity and taxonomy of the pleomorphic genus Alternaria. *Mycological Prog*. **15**: 3. *https://link.springer.com/article/10.1007/s11557-015-1144-x*.

Ogada, A. R., Ezekiel, M. N., Jonah, K. B. and Amuka, O. (2021). Characterization of Alternaria species causing dark leaf spot disease on cabbages grown in Limuru and Nyeri, Kenya. *Plant Path. Quar*. **11**: 23-33.

Poursafar, A., Ghosta, Y. and Javan-Nikkhah, M. (2019). *Alternaria ershadii* sp. nov.–A new species isolated from wheat black head mold in Iran. *Phytotaxa* **422**: 175-185.

Tariq, M., Khan, A., Asif, M., Khan, F., Ansari, T., Shariq, M. and Siddiqui, M. A. (2020). Biological control: A sustainable and practical approach for plant disease management. *Acta Agric. Scand. Sect. B Soil Plant Sci*. **70**: 507-524. *https://doi.org/10.1080/09064710.2020.1784262*.

Turzhanova, A., Khapilina, O. N., Tumenbayeva, A., Shevtsov, V., Raiser, O. and Kalendar, R. (2020). Genetic diversity of Alternaria species associated with black point in wheat grains. *Peer J*. **8**, e9097.

Woudenberg, J. H. C., Seidl, M. F., Groenewald, J. Z, de Vries, M., Stielow, J. B., Thomm, B. P. H. and Crous, P.W. (2015). Alternaria section Alternaria: Species, formae speciales or pathotypes? *Stud. Mycol.* **82**: 01-21.