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First record of *Stemphylium vesicarium* associated with oat grains in Iraq: Phenotypic and molecular characterization

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ABSTRACT

This study was conducted on three oat seed cultivars (Shifa, Kenzania, and Carlop) obtained from to detect the most significant fungi associated with the seeds. T from Ministry of Agriculture, Department of Agricultural Research, Cereals and Legumes Department - Abu Ghraib) he study revealed that *Stemphylium* sp. was the most frequently observed fungus, with a 100% occurrence in the Kenzania cultivar. This fungus was identified morphologically based on the shape and color of the fungal colony and the appearance of the conidial spores, as well as molecularly at the species level using nucleotide sequences of the ITS1 and ITS4 regions within the 5.8S rRNA gene. The species was identified as *Stemphylium vesicarium* and was recorded for the first time in Iraq. And registered in NCBI (National Center for Biotechnology Information) with accession No. PP907787.1. The results also showed that the viability test of oat seeds from the cultivars used in the study indicated that the Shifa cultivar significantly outperformed the others, with a germination rate of 86.7% compared to the other to the other cultivars Carlop at 83.33% and Kenzania 80%.

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Introduction

Oat (*Avena sativa*) is a winter cereal crop belonging to the Poaceae family. Its significance arises from its diverse uses, as oat grains are commonly included in the human diet due to their high content of vitamins and unsaturated fatty acids (Leszczyńska *et al*., 2023) Additionally, oats are used in animal feed, especially for horses, cattle, and poultry, while oat straw is utilized as bedding for livestock (Dvořáček et al., 2021). Globally, oats rank seventh in importance among cereal crops, following wheat, rice, and maize (Acarlson & Kaeppler 2007).

This crop is susceptible to various seed-borne fungal diseases, including *Alternaria* spp., *Aspergillus*, and *Fusarium* spp. (Wang *et al* ,2021). These fungi negatively impact seed viability, nutritional content, and quality, while also reducing storage life and protein content in the grains (Isaura Martín *et al*. 2022). This leads to significant direct and indirect economic losses for oat crops. The fungi cause seed rot and decay, impair germination capacity, reduce storability, and result in damage or death of seedlings (Wang *et al*. 2022).

It was reported that *Stemphylium vesicarium* is a highly pathogenic fungus that causes considerable yield losses in another range of crops, including wheat, across the world (Ameer & Mohammed, 2023). It attacks vegetables, fruits, grains, and other crops including cotton, lentils, pears, and onions, among others (Hanna & Grażyna 2024). This fungus prefers soil and other hosts apart from crops, crop stalks, and plant debris (Mohamed, 2022). Being a seed-borne fungus, it becomes destructive when seeds are in storage since it affects germination

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capability as well as virtues associated with seed qualities like germination, nutritive value, and moisture content. Furthermore, infected seeds remain a substrate for plant pathogens and are more easily reinfected by other fungi, so they cannot be used for human food or animal feed purposes (Kochiieru *et al*, 2021). In general, other seed-borne fungi can produce secondary metabolites known as mycotoxins which are dangerous fungal toxins such as *Stemphylium* that are likely to affect the consumer diet (Kochiieru *et al.* 2021.; Stricker *et al*.2021)

Some taxa of *Stemphylium* are important plant pathogens, showing diseases of different crops. These fungi also can produce secondary metabolites including ferric chelates, aromatic polyketones, and glucosides, which in their toxic forms help promote crop diseases (Stricker *et al*.2021; Abd Oun &Abass 2023).

This study aimed to identify the main fungi associated with oat seeds in Iraq, with a focus on their potential impact on seed viability.

Materials and methods

Isolation medium

Oat seed viability test

For the laboratory experiment, three oat seed cultivars Shifa, Kenzania, and Carlop, were obtained from the Agricultural Research Directorate in Abu Ghraib. The seeds were surface sterilized by washing in 1% Sodium hypochlorite solution for 2 minutes then rinsed twice in sterile distilled water for 2 minutes each. The seeds were then dried using sterilized filter paper (Roberts & Punja 2021)

Viability test of the sterilized oat seeds from the three cultivars used in this study was done using the blotter paper method. Only sterilized seeds were sown, in a manner that ten seeds were placed in one plate, and three such plates were used for each treatment. The seeds were sown on 9 cm Petri dishes with a layer of sterile filter paper with moisture content that was incubated at 25°C for five days. This test was performed to determine the viability of the seeds used in this study, and the germination percentage was done by the following formula (ISTA, 1999):

$$\text{Percentage of seed germination} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \text{x} 100$$

Investigation seeds borne fungi and estimate occurrence percentage

A standard method for fungal isolation was used to detect the fungi associated with oat seeds (from the three cultivars). Five seeds from each cultivar (in three replicates) were implanted in sterilized nine-centimeter Petri dishes comprising sterilized PDA medium supplemented with chloramphenicol (50 mg/L) according to Abdel-Azeem et al. (2024). These plates then were incubated at 25 ± 2°C for 5 days. The associated fungi were isolated and purified individually after their growth and were identified phenotypically according to Domsch *et al*. (2007). The percentage of fungal colony occurrence in the plates was calculated for each fungus using the following formula:

$$Occurrence\ of\ the\ fungus\ (\%) = \frac{\text{No. of samples in which the fungus appeared}}{Total\ no.\ of samples} x100$$

Fungal colonies were purified individually on a sterilized PDA medium and incubated for five days at a temperature of 25 ± 2°C.

Molecular identification

The most frequent taxon was identified at the species level using molecular methods, specifically through nucleotide sequence analysis of the *5.8S rRNA* gene. A small amount (100 mg) from a freshly grown 5-day-old pure fungal colony was used to extract the genomic DNA using the ZR Fungal, Bacterial, Yeast DNA Mini Prep™ kit, provided by Zymo company, USA (Cat. No, D6005, USA, following the steps described by the manufacturer. Purity of DNA=The average Absorbance at 260 nm / The average Absorbance at 280 nm (Sambrook *et a*l,.1989).

PCR mixture and DNA amplification

DNA extraction was performed using the Maxime PCR PreMix kit (i-Taq) 20µl rxn (Cat No. 25025 LiStarfish, Company. English). The ITS genomic region was PCR-amplified using the primer pair (ITS1: 5′-TCCGTAGGTGAACCTGC GG; ITS4: 5′ TCCTCCGCTTATTGATATGC) (White *et al*,1990). PCR mixture was prepared in a 25µL volume containing Taq PCR PreMix (5 µl), one µL of each primer, DNA (1.5µl), nuclease-free water (16.5 µl). The PCR amplification was carried out using the following parameters: 94°C for one min, followed by 35 cycles, each consisting of denaturation for 94°C for 45 sec, primers annealing at 58°C for 45 sec, then initial extension for 1 min, and final extension at 72°C for 5 min that was common in all PCR cycling programs. DNA products amplified by PCR were then separated by agarose gel electrophoresis and then checked under UV illumination.

Analysis of nucleotide sequence

A BLAST search was conducted to find linked nucleotide sequences on the GenBank database. Alignment of the ITS sequence generated from the fungal isolate and comparison with other isolates previously

deposited at NCBI using the BLAST program were carried out.

Statistical analysis

Therefore, the complete randomized design (CRD) was used in statistical analysis in order to carry out experiments. These data means were equated using the Least Significant Difference at a probability level of 0.05, as determined by the GenStat statistical software (Al-Rawi ,1980).

Results and Discussion

The germination capacity of the studied seed of the three oat cultivars used in the study was determined using the Blotter Paper (BP) technique. The findings proved that seed-borne fungi affected viability of seeds in the following ways. From the figure (1) it could be seen that germination rates of the three oat cultivars were significantly affected by the seed-borne fungi. The highest germination percentage was recorded in Shifa cultivar with 86.7 % while the second was Carlop cultivar with 83.33%. However, the Kenzania cultivar yielded the lowest germination rate at 80 percent in the experiment.

In different crops, the population density of saprophytic and pathogenic fungi associated with seeds has been found to reduce germination percentage (Nagaraja, & Krishnappa, 2009). Furthermore, pathogenic fungi are recognized with their capacity to secrete enzymes and toxins, and with their potential of degrading pectin and cellulose that may impair or arrest germination (Hatim *et al* 2022).

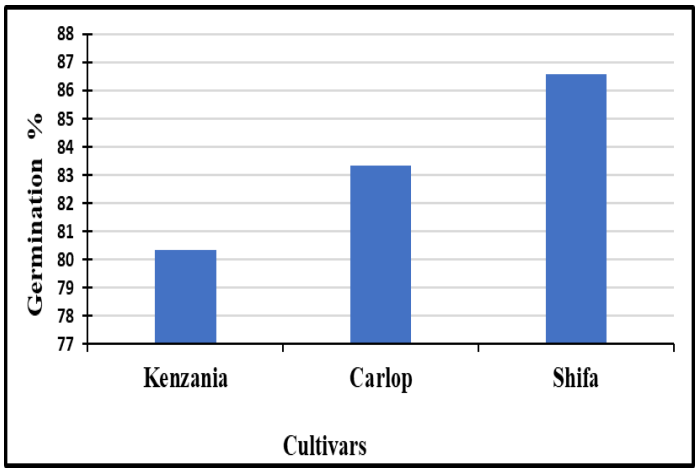

Fig 1. Test of the Seed viability for three studied oat cultivars (Shifa, Carlop, and Kenzania).

***Isolation and identification of fungi associated with oat* seeds**

The analyses carried out on the samples yielded six genera of fungi in connection with the sterilized oat seeds, as shown in Table (1). The fungi were identified and classified at the genus level depending on the morphological features of the fungal clusters on the culture medium, including colony color, mycelial growth, and spore morphology. Identification was conducted using a compound light microscope and with the aid of taxonomic keys (Ellis, 1971; Booth, 1971, Abdel-Azeem et al. 2020).

Table 1 Fungi associated with oat seeds

No.	Fungi	Kenzania	Carlop	Shifa
1	*Alternaria* sp. .	**	*	-
2	*Aspergillus* sp.	**	*	*
3	*Fusarium* sp..	*	*	-
4	*Penicillium* sp1.	**	**	*
5	*Penicillium* sp2.	**	*	*
6	*Stemphylium* sp	***	**	*

Where: - no appearance, *low appearance, ** moderate appearance, ***high appearance.

Our results align with those of (Limnord,1968) who indicated that seed sterilization with NaOCl reduced the activity of fungi associated with the seeds. Similar observations were made by (Bhutta *et al*,.1998; Sharfun-Nahar *et al*,.2005), where *Stemphylium* sp. was the most frequently observed fungus, appearing in all cultivars in the study.

Determination of the most prevalent fungus

The results in figure (2) show that *Stemphylium* sp. was the most frequently occurring fungus compared to other fungi. A significant increase in the occurrence rate of *Stemphylium* sp. was observed in the Kenzania cultivar, reaching 100%, followed by 96.7% in the Carlop cultivar, compared to 86.7% in the Shifa cultivar.

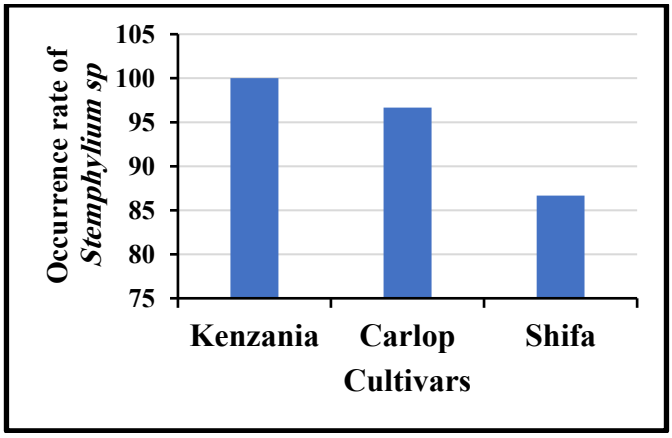

Fig 2. Occurrence rate of *Stemphylium* sp among the three varieties of oat.

Mycological identification of Stemphylium sp.

Figure (3) shows the colony of *Stemphylium* sp. grown on PDA medium after 5 days of incubation at 25°C. The colony is characterized by its dark color, ranging from light brown to green, with the conidia pigmented by melanin, giving them a dark brown color. The fungus produces conidial spores on conidiophores, which are usually unbranched. The conidia are oval to rectangular, often nearly spherical, and typically have transverse septa, with up to three main transverse septa. The base of the conidia is rounded this agree with (Koike *et al,* 2013) The current morphological identification results align with those of (Mohamed 2022; de Souza Feitosa 2023) who reported similar colony color, conidial shape, and size, commonly used for the morphological diagnosis of the pathogenic fungus.

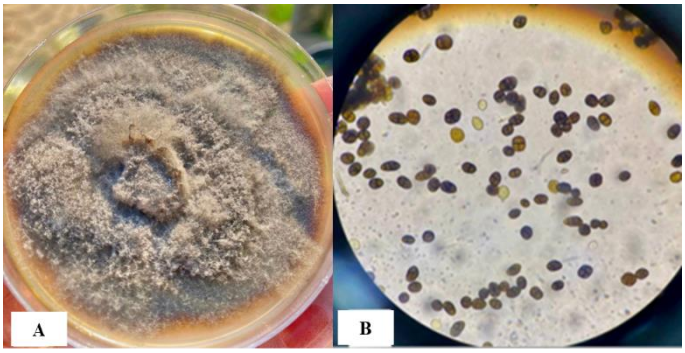

Fig 3. *Stemphylium* sp. A: Colony grown on PDA medium, B: Conidia under a compound microscope.

Molecular Identification

Nucleotide sequence analysis was used to characterize the most frequent isolated fungus at the species level. Electrophoresis of the PCR product revealed bands of 550 bp, confirming the accuracy of identification and classifying the identified fungus as a true fungus (*Eumycota*) (Figure 4).

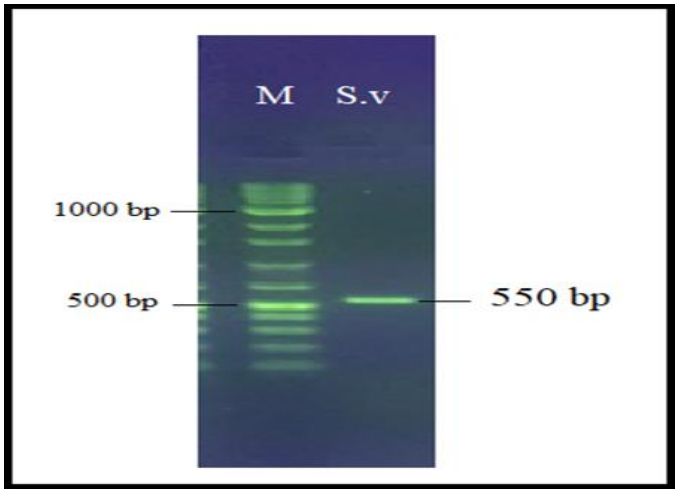

Fig 4. A 550 bp-PCR product electrophoresed on a 2% agarose gel) alongside a 100 bp DNA ladder marker (M). The sample, *Stemphylium vesicarium* (S. v), showed the expected band size.

Table (2) presents the molecular identification of these isolates at the species level, showing the similarity percentages between the pathogenic fungal isolates and their alignment with globally registered fungal species, along with their accession numbers and the countries from which they were isolated. The results show a 99.05% similarity with globally registered strains. The species *Stemphylium vesicarium* was recorded for the first time in Iraq and deposited in the Genbank under accession number PP907787.1.

Table 2. Molecular identification of the *Stemphylium vesicarium* isolate based on the percentage similarity of the 5.8S rRNA gene sequences with fungal strains in the global genetic bank at the NCBI database.

Fungal species with highest similarity	Global Accession Number	Country	Similarity Percentage %	Fungal species and isolate registered in the global genetic bank	Accession Number of fungi identified in this study
S. vesicarium 18-028	LC512756.1	South Korea	99.05	*S. vesicarium* isolate M-1	PP907787.1

A living culture was deposited at the Fungarium of the Suez Canal University (https://ccinfo.wdcm.org/details?regnum=1180) under accession number SCUF000000912

Conclusion

In our current study, the fungus *Stemphylium vesicarium* was isolated from oat seeds and identified based on morphological and microscopic characteristics, in addition to nucleotide sequencing using the ITS1 and ITS4 primers. The sequences were analysed through the NCBI database, confirming that the fungus belongs to *S. vesicarium*. This is the first study in Iraq to isolate and identify this fungus from oat seeds**.**

Conflicts of interest

The authors declare that there are no conflicts of interest

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