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ORIGINAL PAPER

Detection and Molecular Diagnosis of *Stemphylium vesicarium* Isolated from Wheat Spikes

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ABSTRACT

During the annual assessment of the National Wheat Program, Gemmeiza Agricultural Research Station, Gharbia governorate, March 2021, brown spots were observed on the spikes of A-ESWST and SAWYT experiments. These experiments were originated from International Maize and Wheat Improvement Center (CIMMYT) to assess the yield and wheat diseases under Egyptian environmental conditions. Disease severity on the infected spikes was up to 30%. At the end of the season, all the infected spikelets turned to dark brown color. The pathogen was identified morphologically as *Stemphylium vesicarium* in the Mycology Dept., Plant Pathology Institute, ARC, Giza and in the Regional Center of Mycology and Biotechnology Al-Azhar University. The culture includes conidiophores, 3.0 µm and conidia in short chains and conidia are ovoid and obclavate 32.6×9.6 µm, with 3-8 transverse and several longitudinal septa. For molecular identification of Stemphylium isolate, total genomic DNA was extracted and amplified using the primers Internal Transcribed spacer (ITS1 and ITS4) then sequenced. The sequenced DNA was aligned by BLAST and searched for homology in the GenBank nucleotide database. Molecular identification confirmed that the isolate is *Stemphylium vesicarium* with 100 % homology with other *Stemphylium* stains and various accession numbers in NCBI data Bank. The sequence was submitted to NCBI and has accession number OM722056. To fulfill Koch's postulates, wheat plants in 2022 growing season at flowering stage were sprayed with *Stemphylium vesicarium* suspension with concentration 2.5×10^4 conidia ml^{-1}. The same symptoms appeared on wheat spikes with 100% disease severity and *S. vesicarium* was reisolated and have the same microscopic characters as initial culture.

Keywords: Wheat, *Triticum aestivum*, *Stemphylium vesicarium*, Spikes, Morphology, ITS.

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INTRODUCTION

Wheat (*Triticum aestivum*) production is affected by diseases and pests, which cause 10 to 28% of yield losses (Figueroa *et al*., 2018 and Savary *et al*., 2019). *Stemphylium vesicarium* is considered entophytic, epiphytic, or saprophytic in nature (Puig *et al*., 2015 and Das *et al*., 2019). *Stemphylium* sp. survives in soil, crop residues, on many alternative hosts and plant debris (Hudson 1971; Woudenberg *et al*., 2017 and Wahdan *et al*., 2020). But some species are the main pathogens that cause leaf blight on various crops, resulting in economic and yield losses (Hanse *et al*., 2015 and Brahmanage *et al*., 2018). *Stemphylium vesicarium* is able to infect commercially significant plants including herbaceous crops, fruit trees, and vegetables (Lamprecht *et al*., 1984; Falloon *et al*., 1987; Aveling and Snyman 1993; Rossi *et al*. 2005; Suheri and Price 2000 and Sharma *et al*., 2020). Moreover, *Stemphylium* spp are among the fungi that associated with black (sooty) mold of wheat (Poursafar *et al*., 2016). Stemphylium leaf blight (SLB) of onion can cause reduction in onion crops in quality and yield up to 90% (Lorbeer 1993 and FAO/IPGRI 1997). Köhl *et al*. (2009) reported that insufficient information is available on the relationship between pathogenic and saprophytic populations of *Stemphylium* spp. furthermore on the probable pathogenic isolates' host specificity. The aim of the present work is to isolate and identify the causal organism that causes the brownish color on the infected wheat spikes collected from A-ESWST and SAWYT experiments cultivated at Gemmeiza Agricultural Research Station, Gharbia governorate.

MATERIALS AND METHODS

Survey of wheat fields:

In March 2021, during the seasonal assessment of the National Wheat Program at Gemmeiza Station, Gharbia governorate wheat spikelet's of A-ESWST and SAWYT experiments showed unusual brown spots. The Elite Spring Wheat Yield Trial (ESWYT) and Semi-Arid Wheat Yield Trial (SAWYT), wheat advanced lines were introduced from CIMMYT for evaluation in all agricultural stations including Gemmeiza Station. Each of the two experiments consisted of 50 lines with 4

replicates. Initial infection symptoms appeared as brown spots on wheat spikelets, then enlarged and finally the whole spike turned into brown color at the end of the season (Fig. 1 A and B). Fifty infected spikes from each experiment were collected and maintained in Glycine bags to isolate the causal organism.

Fig. (1): Symptoms of an infected wheat spikes with *Stemphylium vesicarium*.

Isolation and identification of the fungus:

For isolation, the infected spikelets were surface disinfected for 2 minutes in a 1% sodium hypochlorite solution, rinsed three times with sterile distilled water, dried, and plated on PDA (Potato Dextrose Agar) supplemented with 0.01% streptomycin. For 7 days, plates were kept in the dark at 23°C. Microscopic examination and identification of the fungal isolate was carried out in the Mycology Dept., Plant Pathology Institute, Agricultural Research Center, Giza. The fungus was identified morphologically according to Ellis (1976) and Barnett and Hunter (1987).

Pathogenicity test:

The wheat cultivar Morocco was chosen to carry out the pathogenicity test at the field in Gemmeiza Research Station. Seeds of Morocco were sown in rows, 30 cm apart and 1.2 m long, in three replicates. Each row was sown by twenty seeds for each line. To attain *S. vesicarium* conidial suspensions for inoculation, cultures were stored at -80°C then re-cultured in PDA petri dishes with 12h black light (350 nm) at 18-22°C per day. Cultures were flooded with sterile water and, after gently rubbing with a rubber spatula, the resulting suspensions were filtered through sterile nylon gauze with a mesh of 200 µm. A haemocytometer was used to determine the conidial suspensions concentration and adjusted to 2.5×10^4 conidia ml^{-1} with sterile tap water. The conidial suspensions were maintained in ice-water until used on the same day. In 2022 season, wheat spikes were sprayed with *Stemphylium* suspension at flowering stage, and the control plants were sprayed with sterile distilled water only until runoff.

Disease severity assessment:

Disease was recorded after 15 days of inoculation and at the end of the season using James scales for foliar diseases (James, 1971).

Re-isolation of the fungus:

To fulfill the Koch postulates, the fungus was reisolated onto MEA (Malt Extract Agar). Identification of the fungal isolate was carried out in the Regional Center of Mycology and Biotechnology Al-Azhar University. The fungus was identified morphologically in the second time according to Ellis 1976; Ellis and Ellis, 1986.

Molecular Characterisation:

Genomic DNA from a purified isolate of the fungus was extracted according to Quick-DNA™ Fungal/Bacterial Miniprep Kit (SIGMA Company). DNA was measured and diluted with nuclease-free water to 50 nanograms/microliter to perform PCR (polymerase chain reaction) in a thermocycler. According to White *et al*. (1990), specific primers were used to amplify regions of ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'TCCTCCGCTTA TTGATATGC-3'). PCR reaction set-up contains 25 µL, 8 µL of MyTaq Red Mix, DNA template, 1 µl of each primer (20 pmol) and 15 µL nuclease-free water. The following were the PCR amplification conditions, initial denaturation was for 6 min at 94°C, denaturation for 45s at 94°C, annealing for 45s with 35 cycles at 56°C, Extension for 1 min at 72°C, and a final extension was for 5 min at 72°C. The sequenced DNA was aligned using the BLAST to identify the species and searched for homology with other species of fungi deposited in the GenBank nucleotide database. Molecular phylogenetic and evolutionary analysis was conducted using the Neighbor-Joining method by BLAST Tree viewer.

RESULTS

As a results of the detection and molecular diagnosis of *S. vesicarium* isolated from wheat spikes, the obtained results revealed that:

1- The isolated fungus was identified as *Stemphylium vesicarium* fungus.
2- The colonies grown on MEA at 25°C were effuse, dark brown with black reverse attaining a diameter of 5.0 cm in 7 days.

3- Microscopic examination showed conidiophores, 3.0 µm, and conidia in short chains and conidia are ovoid and obclavate 32.6×9.6 µm, with 3-8 transverse and several longitudinal septa (Fig. 2 A, B, C and D).

4- Disease severity on brownish spikes reached to 30% as natural infection.

5- Using ITS region, the isolated fungus identified as *Stemphylium vesicarium*. The sequence was submitted to NCBI and has accession number OM722056. The amplified PCR products were sequenced, aligned, and the BLAST analysis showed 100% homology based on ITS1 and ITS4 region from the GenBank nucleotide database with various accession numbers (Fig. 3). The clusters were carried out by maximum likelihood and showed maximum similarity with other *Stemphylium* spp., *Botrytis* spp. and *Pleospora allii* (100%).

6- In 2022 season, the wheat spikes artificially inoculated with *S. vesicarium* showed brown spots after 2 weeks of inoculation. At the end of the season wheat spikes showed 100% disease severity (Fig. 4. A, B and C).

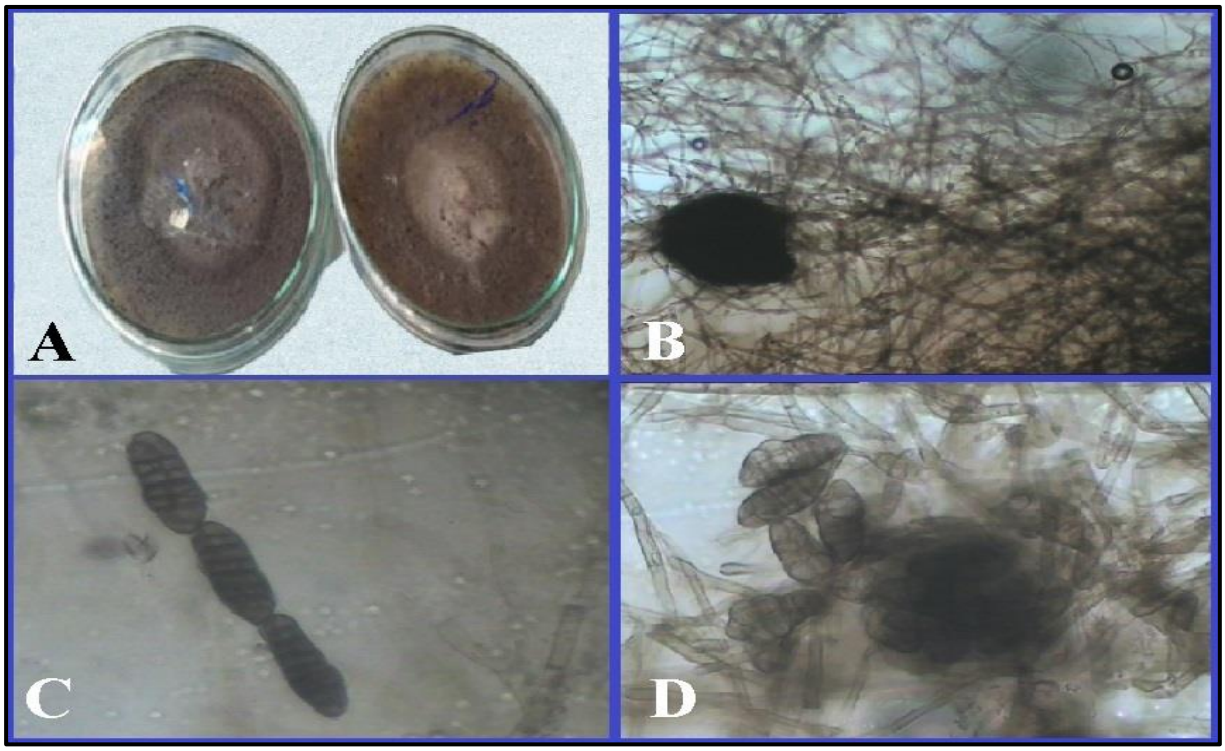

Fig. (2): Colonies, mycelium and spores of *Stemphylium vesicarium* (A, B, C and D).

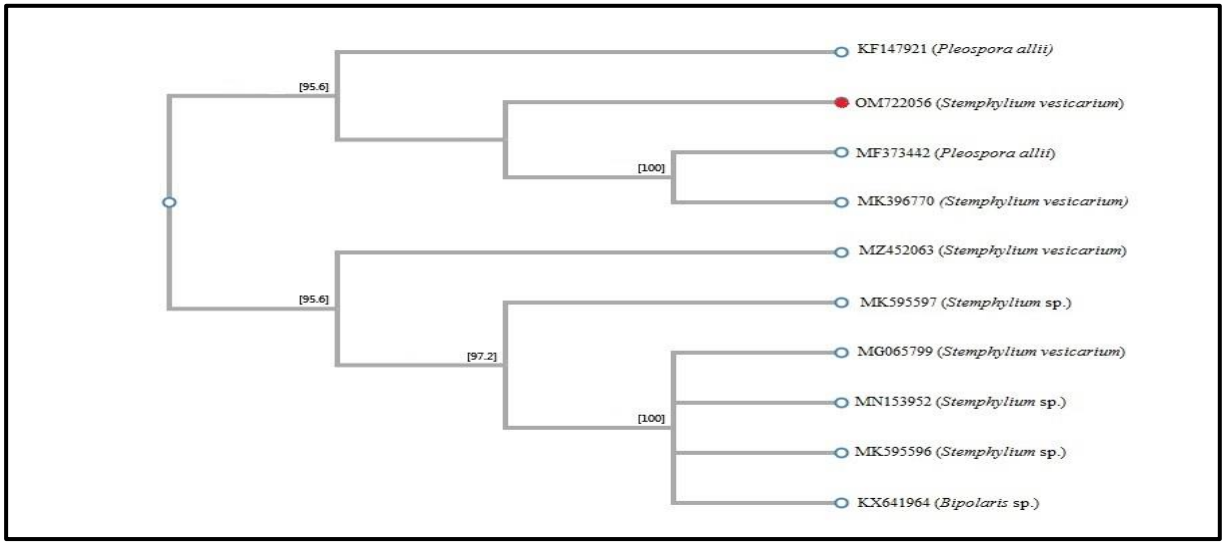

Fig. (3): Multiple Sequence Alignment and phylogenetic tree of *Stemphylium vesicarium* GenBank (GenBank Accession: OM722056), showing its identity with the most similar *Stemphylium vesicarium*.

Fig. (4): Symptoms of an infected wheat spikes with *Stemphylium vesicarium* (A, B and C).

DISCUSSION

Wheat (*Triticum aestivum* L.) is vulnerable to infection by many diseases like rusts, bunt, smuts, tan spot, foot rot, fusarium head blight, and false eyespot which threat its production around the world (Kayim *et al*., 2022 and Singroha *et al*., 2017). However, recently some diseases with minor economic importance infect wheat and may cause economic losses (Kayim *et al*., 2022). In 2021 during the annual assessment of the National Wheat Program at Gemmeiza Research Station to wheat diseases, wheat spikes of the A-ESWST and SAWYT experiments showed brown spots. These unusual brown spots turned to be enlarged, and finally become brownish blight. The causal fungus was isolated and identified morphologically as *Stemphylium vesicarium*. *Stemphylium vesicarium* causes onion blight and reduces onion production (Hay *et al*., 2021). The fungus was stored in slants for further using in the next season. In 2022 season, wheat spikes were inoculated artificially with *S. vesicarium*, and disease severity was recorded. The disease severity reached 30% as natural infection after 2 weeks of inoculation and at the end of the season the disease severity became 100%. The fungus was re -isolated and identified by morphology and molecular techniques as *Stemphylium vesicarium*. The results of the present study include isolation and identification of *Stemphylium vesicarium* from wheat spikes only in both initial infection and second time of artificial inoculation to fulfill Koch postulates. The sequence was submitted to NCBI and has accession number OM722056. The amplified PCR products were sequenced then aligned, and the BLAST analysis revealed 100% homology based on ITS1 and ITS4 region from the Gene Bank nucleotide database with various accession numbers. The clusters were carried out by maximum likelihood and showed maximum similarity with other *Stemphylium*, *Botrytis* sp. and *Pleospora allii* (100%). Generally, there are increasing in number of pathogens that infect non host and causes diseases around the world (Das *et al*., 2019). In Egypt, Farag *et al*. (2022) detected *S. vesicarium* on leaves and petioles of wheat plants during 2020 growing season at Beni Suef governorate, Egypt. Also, for *Stemphylium* spp., Das *et al*. (2019) reported that there are many reports about pathogenic *Stemphylium* spp. in different countries on current and new hosts from 1988 to 2019. Köhl *et al.* (2009) reported that various necrotrophic pathogens have a life-cycle that alternates between a pathogenic stage and a saprophytic stage like Stemphylium. Host tissues are colonized after necrosis induction with pathogens, as well as during senescence when pathogens spread subsequently within the plant. Crop residues are frequently colonized by necrotrophic pathogens of the relevant crop and are regarded as inoculum sources for future epidemics. In competition with other naturally occurring saprophytic microbes, necrotrophic pathogens can colonize non-host tissue. The development on non-host residues may play an underrated role in the diseases epidemiology.

The ability of Stemphylium to cause brown spots or blight on wheat may be related to cultivate the susceptible wheat varieties many times in the same area, changing environmental conditions and or emergence of new isolates which can infect wheat spikes instead of being colonize plant debris.

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