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Response of various cultivars of Egyptian wheat (*Triticum aestivum* L.) to infection by *Stemphylium vesicarium*

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Abstract

A new fungal disease affecting wheat in Egypt, known as Stemphylium leaf spot, caused by *Stemphylium vesicarium* Wallr (Simmons) is reported. From all symptomatic wheat leaves, *S. vesicarium* was the most frequently isolated fungus (71.9%). The isolated pathogen was identified based on morphological characteristics together with molecular diagnosis. The *Stemphylium* isolate AUMC 15115 in this study was clustered at the same branch as *Stemphylium mali* CBS 122640, ex-type material (Synonym = *Stemphylium vesicarium*). At the Smart Agriculture Clinic Project, Sids Agricultural Research Station, Agricultural Research Centre, Beni Suef governorate, 12 cultivars of wheat were assessed in pot experiments for their resistance to Stemphylium leaf spot during the 2020 and 2021 growing seasons. Overall, distinct variations were observed in all examined cultivars in response to *S. vesicarium* infection. The Sakha 95 cultivar exhibited a minimal infection rate (disease incidence was 6.7, 3.3% and disease severity being, 0.7, 0.5%, respectively in the two growing seasons) and was classified as resistant. In contrast, Beni Suef 5 had the highest percent of infection (63.3, 66.7%), disease severity (38.5, 40.3%) and was classified as susceptible. In resistant cultivars, there was an increase in total phenol content, polyphenol oxidase (PPO), peroxidase (POD), and superoxide dismutase (SOD) activities. Conversely, there was a reduction in electrolyte leakage percentage and hydrogen peroxide (H_2O_2) accumulation. However, the number of protein bands in resistant wheat cultivars exhibited a more significant increase than susceptible ones, particularly in the Sakha 95, which displayed the highest number of proteins.

Keywords Fungal diseases, Molecular diagnosis, Stemphylium leaf spot, *Triticum aestivum*, Egypt

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops in the world and the first strategic crop in Egypt. It is consumed by about 40% of the world's population as human and animal food, which supplies a significant number of calories (19%) and protein (12.5–13.5%) (Giraldo et al. 2019). It is anticipated that by 2050 demand of wheat will go up by more than 60% from developing countries (FAO 2021). Nevertheless, the yield of wheat will be decreased by 29% because of climate change-related temperature fluctuations, floods, drought, insect infestations, and disease outbreaks (Omar et al. 2021). During the 2020 growing season, Stemphylium

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leaf spot, a new disease in Egypt caused by *Stemphylium vesicarium* Waller (Simmons), was more frequently noticed in wheat fields (cv. Beni Suef 5) in Beni Suef governorate, Egypt (Farag et al. 2022). The disease appears as brown-colored lesions, encircled by a yellow halo with a white spot in the center. The size of the circular to oval-shaped lesions on the leaves ranged between 5–14 mm (Farag et al. 2022).

Stemphylium vesicarium is known as a plant pathogenic fungus that causes leaf spot on a wide range of plant species (over 20 plant species globally), including tomato (Razak and Abass 2021), onion (Roylawar et al. 2021; Feitosa et al. 2023; Vally et al. 2024), chilli pepper (Vitale et al. 2017), parsley (Koike et al. 2013), garlic (Suheri and Price 2000), asparagus (Foster 2018), sugar beet (Khan et al. 2023), mango (Ahmed et al. 2019) and pear (Köhl et al. 2009). Four *Stemphylium* species viz., *S. alfalfae*, *S. eturminum*, *S. lycii* and *S. vesicarium* were first reported in Iran in 2016 as a causal agent of black (sooty) head mold of wheat and barley (Poursafar et al. 2016).

Classification and taxonomic identification of *Stemphylium* species mainly was based on morphological characteristics such as conidium shape, size, length/width ratio, septation, and ornamentation. Due to the significant variation in culture conditions, environmental factors, and host selection flexibility (Chowdhury et al. 2015; Subash and Sarawati 2016), it was a challenging task to identify the fungus up to the species level. For precise separation of *Stemphylium* spp., molecular methods and sequence analysis of multiple DNA regions for species identification were applied (Vaghefi et al. 2020). The nuclear ribosomal internal transcribed spacer (ITS) region is a well-conserved and repeated gene that is commonly used as a universal DNA barcode marker for different fungal taxa (Schoch et al. 2012).

The use of traditional fungicides to control plant diseases poses food safety issues, environmental pollution, and pesticide resistance issues, which necessitated the use of alternate pathogen control strategies. Resistant varieties that affect a plant's ability to tolerate disease or have the ability to resist pathogens may be considered as economically and environmentally safe biological control agents (Chen et al. 2009; Shreen et al. 2019; Mohdly et al. 2024). Since it is a new disease, no significant information is available on its germplasm for resistance against Stemphylium leaf spot pathogen in Egypt. Reactive oxygen species (ROS), causing oxidative stress on plants are frequently considered as an initial response to microorganism infection. This may have several effects on defense mechanisms, such as phytoalexins creation, lignin formation, induction of systemic acquired resistance, and direct antibacterial action (Al-Maaroof et al. 2014). However, excessive ROS initiates an oxidative process that damages macromolecules like proteins, pigments, lipids, and nucleic acids and causes lipid peroxidation (El-Komy 2014; Madkour 2020). As a result, plants have an antioxidant defense mechanism that restricts oxidative damage and controls the cytotoxic effects of these free radicals by increasing the activity of antioxidant enzymes including catalase (CAT), polyphenol oxidase (PPO), peroxidase (POD), and superoxide dismutase (SOD), which immunizes plants against disease infection (El-Komy 2014; Thabet et al. 2023). It was shown that the ability of a pathogen to prevent oxidative compound accumulation in host cells either directly or indirectly has been linked to the absence of a large oxidative burst (Unger et al. 2005; Macarisin et al. 2007).

A crucial method for analyzing genetic variability among cultivars is the electrophoretic profiles of soluble proteins (Barta et al. 2003). Plant species and organs have an impact on the protein variation (Kong-Ngern et al. 2005). Ghasempour and Kianian (2002), Ghasempour and Maleki (2003) stated that polyacrylamide gel electrophoresis (SDS-PAGE) is a biological analysis method that involves shifting detection of protein bands and signifying hormone changes, enzyme alterations, or any other stresses.

A visual representation of the relationship between genotypes, environments, and interactions is made possible by the genotype and genotype by environment (GGE) biplot, which makes use of the principal component analysis (PCA) technique to analyze the multi environment data (Kendal et al. 2019).

Therefore, the current study aimed to isolate and identify wheat leaf spot pathogens in Beni Suef governorate, Egypt, using morphological traits and molecular phylogenetic data; test wheat cultivars for pathogen resistance in greenhouse conditions; perform histologically and biochemical analysis to study wheat cultivar susceptibility and resistance mechanisms as well as evaluating the evolutionary relationship of the tested wheat varieties using SDS-PAGE.

Materials and methods

Isolation and purification of the causal pathogen

Leaves showing disease symptoms that appear as brown-colored lesions, encircled by a yellow halo with a white spot in the center were collected randomly from different wheat fields in Beni Suef governorate, Egypt during January, 2020 growing season. The leaves were chopped and the pieces (0.5 cm) were disinfected with 2% sodium hypochlorite solution for 2 min. The chopped leaves were rinsed with sterilized distilled water three times and left on sterilized filter paper to dry. Once the leaves were dried, they were then placed on Potato Dextrose Agar (PDA) plates (9 cm diam.).

The plates were incubated for seven days at 25 °C in dark and growth was monitored daily. The resultant fungal colonies were then purified using the single spore method and kept at 5 °C on PDA slants (Zhang et al. 2013).

Morphological assessment

Using the standardized criteria of Simmons (2001), morphological traits were assessed at Assiut University's Mycological Centre (AUMC), Faculty of Science, Assiut, Egypt. Purified culture was grown for seven days at 25 °C in the dark on PDA plates. Slide preparation was done using the Sellotape technique with a 25% lactic acid solution as mounting fluid (Schubert et al. 2007). Morphological characteristics of the causal pathogen structures such as conidiophores, conidiogenous, and conidia form, color, size, and septation were noted and compared to the available literature.

Molecular identification of *Stemphylium* isolate AUMC 15115 DNA extraction, PCR and sequencing of ITS

The DNA extraction was performed following the method outlined by Moubasher et al. (2019). In which, a small portion from fungal growth of 7-day-old culture of *Stemphylium* isolate AUMC 15115 grown on potato dextrose agar (PDA) at 25 °C were collected and transferred to 2 ml-Eppendorf tube. PCR was conducted according to CTAB method described by Al-Bedak and Moubasher (2020). The universal primers ITS1 and ITS4 (White et al. 1990) were used for amplification of the internal transcribed spacer (ITS) region.

Alignments and phylogenetic analyses

Contiguous sequence of *Stemphylium* isolate AUMC 15115 was produced using DNASTAR (version 5.05). Sequence of *Stemphylium* isolate AUMC 15115 along with the closest similar sequences in GenBank, which included type and ex-type specimens, were aligned by MAFFT (version 6.861b) with the default parameters (Katoh and Standley 2013). Alignment gaps and parsimony uninformative characters were treated by BMGE (Criscuolo and Gribaldo 2010). Maximum-likelihood (ML) and Maximum parsimony (MP) phylogenetic analyses were performed using MEGA X (version 10.2.6) (Kumar et al. 2018). The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Felsenstein 1985). Utilizing Model test 3.7's Akaike Information Criterion (AIC), the optimum nucleotide substitution model for ML analysis was selected (Posada and Crandall 1998). The obtained phylogenetic tree was edited and saved as TIF format.

Screening of some wheat genotypes

Twelve Egyptian wheat cultivars, i.e., Beni Suef 1, Beni Suef 5, Beni Suef 6, Beni Suef 7, Sakha 95, Sids 14, Sohag 4, Gemmeiza 12, Giza 171, Misr1, Misr 2, and Morocco (Table 1), were examined for Stemphylium leaf spot resistance in pot experiments. At the Smart Agriculture Clinic Project, Sids Agricultural Research Station, Agricultural Research Centre, Beni Suef governorate, where the geographical location is 30° 94445′ N latitude, 28° 89901′ E longitude and 25 m above sea level, in North Upper Egypt. Experiments were conducted under greenhouse conditions during the 2020–2021 growing seasons. Twenty healthy-looking wheat grains from each cultivar

Table 1 Names, pedigree and the year of release of some tested Egyptian wheat cultivars in the current study

Cultivars	Pedigree	Release year
Beni Suef 1	JO"S"/AA"S"//FG"S"	1987
Beni Suef 5	DIPPERZ/BUSHEN3.CDSS92B128-1M-0Y-3B-0Y-0SD	2007
Beni Suef 6	BOOMER-21/BUSCA-3. CDSS95Y01185-8Y-OM-0Y-0B-1Y-0B0SD	2010
Beni Suef 7	CBC509CHILE//SOOTY_9/RASCON-37/9/USDA595/3/D67.3/RABI//CRA/4/ALO/5/HUI/YAV_1/6/ARDENTE/7/HUI/YAV79/8/POD/9	2017
Sids 14	Bow"s"/Vee"s"/TSI/3/Bani Sewef1 and SD293-1SD293-ISD-2SD-4SD-OSD	2017
Sohag 4	AJAIA-16//HORA/JRO/3/GAN/4/ZAR/5/SUOK-7/6/STOT//ALTAR84/ALDCDSS99B00778B-0SHS-0T0PY-0M-0Y-129Y-0M-0Y-1	2016
Sakha 95	PASTOR//SITE/MO/3/CHEN/AEGILOPS SQUARROSA (TAUS)//BCN/4/WELL1CMA0/Y00158S-040POY-040M-030ZIM-040SY26M-0Y-0B-0ET	2019
Giza 171	SAKHA93/GEMMEIZA 9S.6-1GZ-4GZ-1GZ-2GZ-0S	2013
Gemmeiza 12	OTUS/3/SARA/THB//VEECMSS97Y00227S-5Y-010M-010Y-010M-2Y-1M-0Y-0GM	2011
Misr 1	OASIS/SKAUZ//4*BCN/3/2*PASTORCMSS00Y01881T-050M-030Y-030M-030WGY-33M-0Y-0S	2011
Misr 2	SKAUZ/BAV92CMSS96M03611S-1M-010SY-010M-010SY-8M-0Y-0S	2011
Morocco	–	–

were grown in pots with a 25 cm diameter. After germination, the plant density was reduced to 10 plants/pot, and replications were made with three pots with each cultivar. The pots were arranged in a randomized complete block design (RCBD). The planting date for the wheat genotypes, which is the first of November (close to the normal planting date), and the harvesting date as the first of May were applied during the two growing seasons.

Stemphylium vesicarium was cultured in a conical flask, containing PD broth medium, and incubated for 15 days at 25 °C. A hemocytometer was used to adjust the conidial suspension concentration to 3×10^4 conidia/ml (Köhl et al. 2009). Artificial inoculation was carried out by spraying wheat seedling (5-true-leaf stage) with spore suspension. Periodic observations were made for developing symptoms. Re-isolation was done from infected plants in order to follow Koch's postulates for the processes of etiology research. To establish the pathogen's identification, the resulting cultures were compared to the original culture.

Disease assessment

Disease incidence

The percentage of disease incidence was calculated by dividing the number of infected plants by the total number of growing plants for each cultivar. The average of disease incidence was calculated.

Disease severity

According to Sharma (1986), a 0 to 5 visual scale was used to assess the severity of the disease, where 0: indicates no disease; 1: minute pinhead size spots, 1–10% diseased leaf area; 2: 11–20% diseased leaf area; 3: 21–40% diseased leaf area; 4: breaking of leaves from center, 41–75% diseased leaf area; 5: coalescing lesions with >75% diseased area. The percentage of disease severity was determined using the formula shown below:

$$\text{Disease severity\%} = \left[\sum (\text{n} \times \text{c})\right] / (\text{N} \times \text{C}) \times 100$$

whereas n is the number of infected leaves; c is the category number; N is the total number of examined leaves and C is the the highest category number of infections.

Biochemical studies

Levels of hydrogen peroxide (H2O2) and Electrolyte leakage

Levels of hydrogen peroxide (H_2O_2) and electrolyte leakage percent were measured in the Food Safety & Quality Control Department, Faculty of Agriculture, Cairo University, Egypt, as described by Brudzynski et al. (2011); Szalai et al. (1996), respectively.

Antioxidant enzymes and phenol content

The Worthington enzyme manual's approach was used to measure peroxidase activity (Worthington 1971). Polyphenol oxidase and superoxide dismutase activities were determined using the techniques described by Esterbaner et al. (1977); Nishikimi et al. (1972), respectively. Total phenol was assessed using the Folin ciocalteau reagent procedure, according to Lafka et al. (2007). Activities of peroxidase, polyphenol oxidase enzymes as well as total phenol were estimated on Soil, Water & Environment Research Institute, ARC, Egypt while superoxide dismutase activity was analyzed in the Food Safety & Quality Control Department, Faculty of Agriculture, Cairo University, Egypt.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The total soluble proteins for each wheat cultivar artificially inoculated with *S. vesicarium* as well as control plants were analyzed by SDS-PAGE at the Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Centre, Egypt. To separate proteins according to their molecular weight, SDS-PAGE was used as described by Laemmli (1970).

Genetic similarity or difference between twelve wheat cultivars was determined by dendrogram constructed by un-weighted pair group of arithmetic means (Sneath and Sokal 1973).

Histological examination

Three different wheat cultivars (Beni Suef 5, Sids 14, and Sakha 95) which represent a susceptible, moderately resistant, and resistant cultivars, respectively were selected to examine the anatomical structure of diseased leaves using a transmission electron microscope (TEM). Microtechnique procedures were carried out at Cairo University's Agric. Botany Dep. Fac. Agric. Wheat cultivars leaves (4 mm^2) were taken, killed immediately, and fixed in formalin aceto alcohol solution (F.A.A.) for at least 48 h. Leaf samples were cleaned in 50% ethanol and then dried in a succession of butyl alcohols before being combined into paraffin wax (melting point 56 °C). Sections were cut using a rotary microtome at a thickness of 15–20 microns. Stained using crystal violet-erythrosine mixture, cleared in xylene and mounted in Canada balsam (Nassar and El-Sahhar 1998). Using a microscope, the slides were examined and captured on camera.

Statistical analysis

Statistical analysis of the data was done according to Snedecor and Cochran (1989) by using the suggested methodology to compute the L.S.D. test with a 5% probability.

According to Gabriel (1971), Yan et al. (2000), the formula of GGE model that used to assess the resistance of wheat cultivars across environment as follows:

$$Y_{ij} - \acute{Y} = \lambda_1 \xi i_1 \eta j_1 + \lambda_2 \xi i_2 \eta j_2 + \varepsilon ij$$

where Y_{ij} is the expected value for genotype (i) in environment (j); $\acute{Y}_j$ is the average data over all genotypes in environment (j); $\lambda_1 \xi i_1\, \eta\, j_{1+}\, \lambda_2 \xi i_2\, \eta\, j_2$ are collectively called the first principal component (PC1) and the second principal component (PC2); $\lambda_1 + \lambda_2$ are the singular values for PC1 and PC2, respectively; $\xi i_{1+}\ \xi i_2$ are the PC1and PC2 scores respectively, for genotype (i); $\eta\ j_1 + \eta\ j_2$ are the PC1and PC2 eigenvectors, respectively and εij is the residual of the model associated with the genotype (i) in environment (j).

Results

Isolation and purification of the causal pathogen

From naturally infected wheat leaves that were randomly taken from several wheat fields in the Beni Suef governorate, many fungal species were isolated and identified as *Stemphylium vesicarium* Wallr (Simmons), *Cladosporium* sp., *Alternaria* sp., *Plectosphaerella cucumerina* (Lindfors) W. Gams, *Curvularia lunata* (Wakker) Boedijn, *Acremonium* sp., *Nigrospora sphaerica* (Sacc.) E. Mason, *Aspergillus niger* Tieghem and *Penicillium* sp. (Table 2). A high number of colonies (95 colonies) with a high frequency of 71.9%, *S. vesicarium* was isolated from all symptomatic wheat leaves followed by *Cladosporium* sp. (12.1%) and *Plectosphaerella cucumerina* (5.3%).

Morphological assessment

Microscopic examination showed that the hyphae of *S. vesicarium* were light brown, 4–6 µm in width, branched and septate. Conidiophores ranged in size from 18–71×4–7 µm, pale brown, septate straight or curved. Conidiogenous cells were dark brown in color, 6 to 8 µm broad and swollen at the apex bearing single conidia which had the shapes of ovoid, oblong and ovoid to oblong, rarely globose and their color were dark brown at maturity, mostly constricted at the median septum, their length varied from 12.0 µm to 14.8 µm×1.74 µm to 2.86 µm in width (average 2.3×13.4 µm). However, *S. vesicarium* isolates displayed noticeable variations in their horizontal and longitudinal conidial septation as illustrated in (Fig. 1).

Stemphylium vesicarium AUMC15115 GenBank: MZ944879 (576 letters) from wheat

Phylogenetic analysis of ITS dataset was employed to determine the taxonomic status of *Stemphylium* isolate AUMC 15115 relative to other members of *Stemphylium*. The entire ITS dataset comprised 36 sequences. The maximum parsimony dataset consisted of 523 characters with 445 could aligned correctly (no gaps, no N), 68 characters were counted as variable characters, and 21 characters as parsimony informative. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter using a discrete Gamma distribution (K2 + G) as the perfect model for nucleotide substitution. The dataset for maximum parsimony yielded 7 trees, with the most parsimonious one with a tree length of 122 steps, the highest log likelihood (− 1405.10), a consistency index (CI) of 0.666667, a retention index (RI) of 0.803571, a rescaled consistency index (RCI) of 0.658665, is shown in Fig. 2. In the phylogenetic tree, the *Stemphylium* isolate AUMC 15115 in this study was clustered at the same branch as *Stemphylium mali* CBS 122640, ex-type material (Synonym = *Stemphylium vesicarium*). Consequently, the *Stemphylium* isolate AUMC 15115 in this study is identified here as *Stemphylium vesicarium*. ITS sequence of the *Stemphylium* isolate AUMC 15115 was deposited in GenBank database as MZ944879.

Table 2 Frequency % of isolated fungi from wheat infected leaves obtained from Beni Suef governorate

Fungi	No. of colonies	Frequency (%)
Stemphylium vesicarium Wallr (Simmons)	95	71.9
Cladosporium sp.	16	12.1
Alternaria sp.	5	3.8
Plectosphaerella cucumerina (Lindfors) W. Gams	7	5.3
Curvularia lunata (Wakker) Boedijn	3	2.3
Acremonium sp.	2	1.5
Nigrospora sphaerica (Sacc.) E. Mason	1	0.8
Aspergillus niger Tieghem	2	1.5
Penicillium sp.	1	0.8
Total	132	–

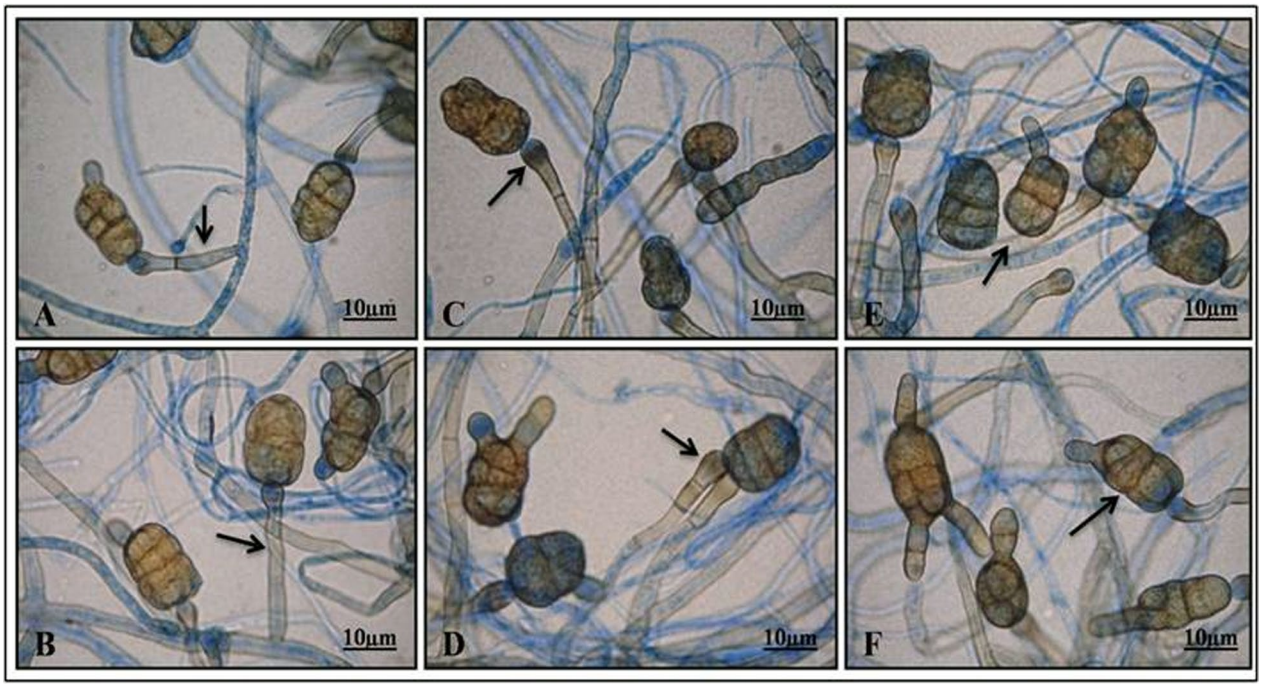

Fig. 1 Morphology of *S. vesicarioum* (AUMC15115, accession numbers MZ944879). **A**, **B** Conidiphores; **C**, **D** Conidiogenous cells and **E**, **F** conidia

Response of various Egyptian wheat cultivars to S. vesicarium infection

Table 3 demonstrates significant variations in responses of all wheat cultivars tested to *S. vesicarium* under artificial inoculation**.** Sakha 95 significantly showed a low percent of infection (6.7, 3.3%) and disease severity (0.7, 0.5%), respectively in 2020 and 2021 growing seasons, followed by Giza 171, Gemmeiza 12, Misr 1, Misr 2 and Sohag 4. Sids 14 and Morocco both gave a moderate level of infection with notable variances between them. The average of disease incidence was 20.0 and 26.7% and disease severity was 10.5 and 13.3%, respectively in the 2020 growing season and 16.7, 23.3% and 8.7, 11.6%, respectively in the 2021 growing season. Meanwhile the highest percent of infection (63.3, 66.7%) and disease severity (38.5, 40.3%) were noticed with Beni Suef 5 followed by Beni Suef 6 (53.3, 63.3%) & (31.2, 36.5%), Beni Suef 1 (43.3, 46.7%) & (23.2, 27.5%) and Beni Suef 7 (36.7, 40.0%) & (17.6, 20.3%), respectively in the two growing seasons with significant differences among them.

Biochemical studies

Levels of hydrogen peroxide (H2O2)

Levels of hydrogen peroxide (H_2O_2) varied in all tested wheat cultivars due to *S. vesicarium* inoculation, which recorded the highest values in Beni Suef 1 (0.07 mM/g), Beni Suef 5 (0.07 mM/g) and Beni Suef 6 (0.07 mM/g) followed by Beni Suef 7 (0.06 mM/g) as illustrated in (Table 4). Moderate levels were recorded in Morocco (0.05 mM/g), Sohag 4 (0.05 mM/g) and Sids 14 (0.04 mM/g). Cultivar of Sakha 95 showed the lowest value of H_2O_2 (0.01 mM/g) followed by Giza 171 (0.02 mM/g), Gemmeiza 12 (0.02 mM/g), Misr 1 (0.02 mM/g) and Misr 2 (0.02 mM/g).

Electrolyte leakage

When compared to resistant cultivars of wheat, susceptible cultivars had a much higher percentage of electrolyte leakage (Table 4). Maximum percent was noticed in Beni Suef 5 cultivar (98.10%) followed by Beni Suef 1 (97.80%), Beni Suef 6 (97.65%), and Beni Suef 7 (97.30%). Regarding other cultivars, it was observed that there was a descending order in the percentage of electrolyte leakage which reaching the lowest percentage in Sakha 95 (76.44%).

The GGE biplot analysis

Relationship between different cultivars at different biochemical parameters and disease response is shown using GGE biplot analysis (Fig. 3). The first two principal components (PCs) together explained 99.94% of the total variance, with PC1 accounted for 61.0% and PC2 for 38.93% of the variance. The vertex cultivars Beni Suef 1 (BS1), Beni Suef 5 (BS5) and Sakha 95 (Sa 95) were the most interaction ones to the disease either positively or negatively. Whereas the cultivars in the right side were the highest positively response and the cultivars in the

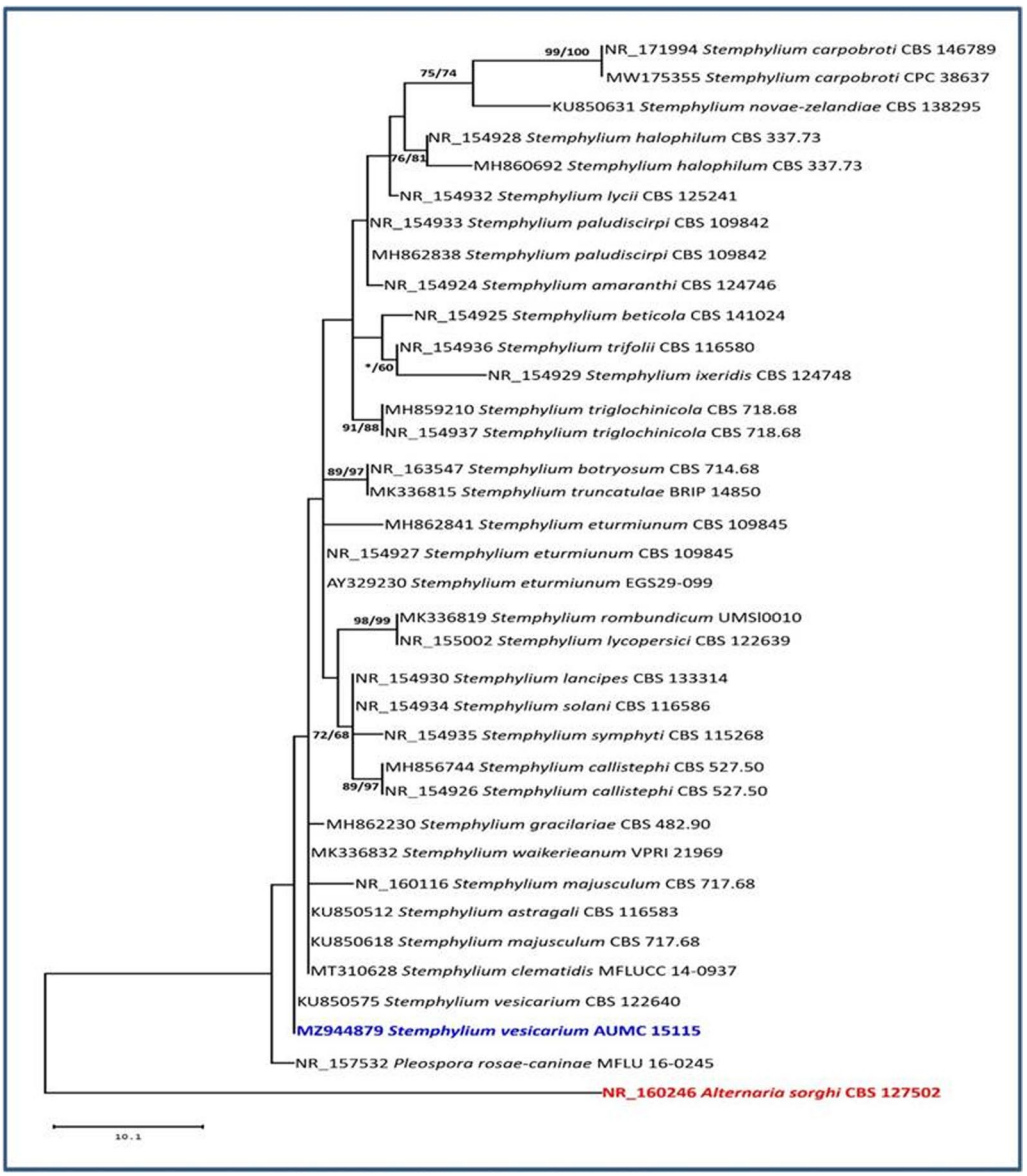

Fig. 2 Maximum likelihood phylogenetic tree generated from ML/MP analysis based on ITS sequence data of *Stemphylium* isolate AUMC 15115 (in bold blue color) compared to the most similar ITS sequences belonging to *Stemphylium* in GenBank. Bootstrap support values (*1000 replications*) for ML/MP combination equal to or greater than 50% are indicated above/below the respective nodes. The tree is rooted to *Alternaria sorghi* CBS 127502 as outgroup (in bold red color)

left side with a negative response. The vertex cultivar Sakha 95 (Sa 95) was placed at the opposite side and did not exhibit any disease characteristics, which indicates that it was the most tolerant cultivar. Contrarily, Beni Suef 5 (BS5) was the vertex cultivar for disease incidence and severity, which was the most susceptible cultivar to Stemphylium leaf spot. Beni Suef 6 (BS6) show the same finding. The vertex cultivar Beni Suef 1 (BS1) was considered susceptible to Stemphylium leaf spot since it was best performing for electrolyte leakage (EL) and hydrogen peroxide (H_2O_2), which indicate the highly oxidative stress due to *S. vesicarium* infection.

Antioxidant enzymes and phenol content

Activities of peroxidase, polyphenol oxidase, and superoxide dismutase enzymes as well as phenols increased in Sakha 95 after *Stemphylium* inoculation, being 13.80, 12.00, 345.35, and 6.27, respectively (Table 5). In comparison to Sakha 95 and Sids 14 cultivars, Beni Suef 5 had the lowest levels of these enzymes and phenols,

Table 3 Host response of some Egyptian wheat cultivars to *S. vesicarioum* under artificial inoculation conditions in the green house during 2020/21 season

Cultivars	Season of 2020		Season of 2021	
	Disease incidence (%)	Disease severity (%)	Disease incidence (%)	Disease severity (%)
Beni Suef 1	43.3	23.2	46.7	27.5
Beni Suef 5	63.3	38.5	66.7	40.3
Beni Suef 6	53.3	31.2	63.3	36.5
Beni Suef 7	36.7	17.6	40.0	20.3
Morocco	26.7	13.3	23.3	11.6
Sids 14	20.0	10.5	16.7	8.7
Sohag 4	13.3	5.2	16.7	7.2
Sakha 95	6.7	0.7	3.3	0.5
Giza 171	10.0	2.0	10.0	2.3
Gemmeiza 12	10.0	2.8	13.3	3.1
Misr 1	13.3	5.2	13.3	4.3
Misr 2	13.3	4.1	10.0	3.1
L.S.D. at 0.05	1.7	1.2	2.1	1.1

Table 4 Levels of hydrogen peroxide and electrolyte leakage in different wheat cultivars artificially inoculated with *S. vesicarium* during 2021 growing season

Cultivars	Hydrogen peroxide (mM/g)	Electrolyte leakage (%)
Beni Suef 1	0.07	97.80
Beni Suef 5	0.07	98.10
Beni Suef 6	0.07	97.65
Beni Suef 7	0.06	97.30
Sids 14	0.04	96.73
Sohag 4	0.05	95.56
Sakha 95	0.01	76.44
Giza 171	0.02	82.54
Gemmeiza 12	0.02	82.63
Misr 1	0.02	83.60
Misr 2	0.02	84.98
Morocco	0.05	96.82

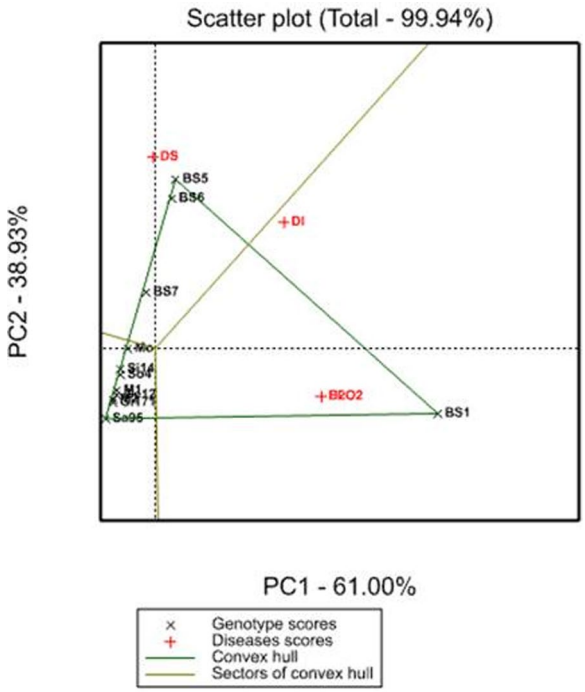

Fig. 3 GGE biplot based on relationship among tested wheat cultivars, biochemical parameters and Stemphylium leaf spot disease response. Beni Suef 1 (BS1); Beni Suef 5 (BS5); Beni Suef 6 (BS6); Beni Suef 7 (BS7); Morocco (Mo); Sids 14 (S14); Sohag 4 (So4); Sakha 95 (Sa 95); Giza 171 (Gi171); Gemmeiza 12 (Ge12); Misr 1 (M1); Misr 2 (M2); Disease incidence % (DI); Disease severity % (DS); hydrogen peroxide (H_2O_2); Electrolyte leakage (EL)

Table 5 Activities of the peroxidase, polyphenol oxidase, superoxide dismutase and total phenols in Beni Suef 5, Sids 14 and Sakha 95 cultivars artificially inoculated with *S. vesicarium* during 2021 growing season

Cultivars	Enzymatic activities			Total phenols (g)
	Peroxidase	Polyphenol oxidase	Superoxide dismutase (µg/g)	
Bani Suef 5	10.56	7.50	271.40	2.84
Sids 14	10.80	11.40	287.10	4.92
Sakha 95	13.80	12.00	345.35	6.27

with respective activities of 10.56, 7.50, 271.40, and 2.84, respectively.

Figure 4 show relationship between three wheat cultivars at different biochemical traits such as peroxidase (POD); polyphenol oxidase (PPO); superoxide dismutase (SOD); Total phenol (TP) as well as disease incidence and severity. Based on the GGE biplot technique, the main principal components (PCs) analysis revealed that the measured traits of wheat plants explained about 100.00% of the observed variance between the tested three wheat cultivars. PC1 accounted for 89.46% of the total, whereas PC2 contributed 10.54%. Sakha 95 (Sa 95) was the vertex cultivar for peroxidase (POD), superoxide dismutase (SOD) and total phenol (TP) indicating its resistance to *S. vesicarium* infection. Meanwhile, Sids 14 (Si14) exhibited favorable behavior for polyphenol oxidase (PPO) enzyme. Beni Suef 5 (BS5) was the vertex cultivar for disease incidence and severity, and did not exhibit any resistance characteristics.

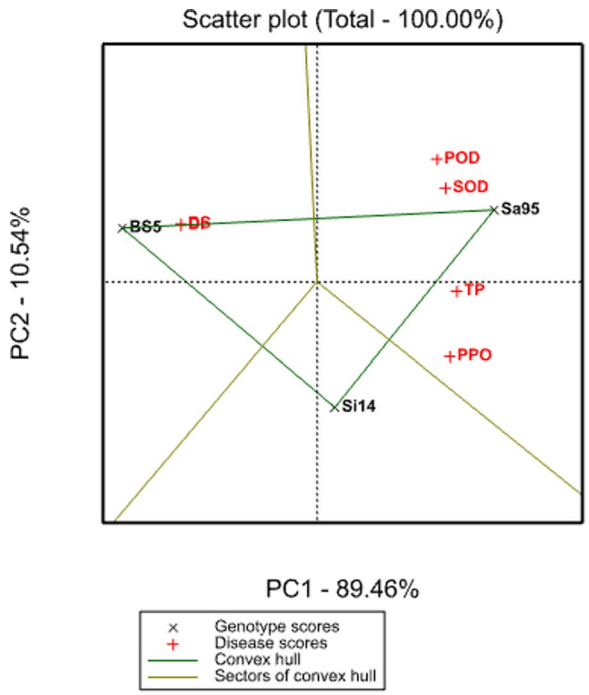

Fig. 4 GGE biplot based on relationship among tested wheat cultivars, biochemical parameters and Stemphylium leaf spot disease response. Beni Suef 5 (BS5); Sids 14 (S14) and Sakha 95 (Sa 95); Disease incidence % (DI); Disease severity % (DS); peroxidase (POD); polyphenol oxidase (PPO); superoxide dismutase (SOD); Total phenol (TP)

Soluble proteins in wheat leaves

Data in Table 6 and Fig. 5 illustrated that there were changes in different protein bands and the appearance new bands in infected wheat plants of different cultivars compared with control plants due to inoculation by *S. vesicarium*. It is obvious from the SDS-PAGE study of the total soluble leaf proteins that resistant wheat cultivars had more bands than susceptible ones. In Sakha 95, five novel proteins with molecular weights of 10, 20, 50, 100, and 130 kDa were induced. Giza 171 and Gemmeiza 12 both produced four novel bands of molecular weights 10, 20, 100, and 130 kDa. Whereas Misr 1 and Misr 2 participate in a new protein band with molecular weights of 10, 20, 50, and100 kDa. While susceptible wheat cultivars showed the lowest expressed bands compared to resistant cultivars. In this regard, Beni Suef 1 and Beni Suef 5 gave a new protein band with molecular weights of 20, 80 kDa and molecular weights of 20, 230 kDa, respectively. While Beni Suef 6 and Beni Suef 7 gave a new protein band with molecular weights of 20, 220 kDa and molecular weight of 10 kDa, respectively.

Genetic similarity analysis based on SDS-PAGE

In Table 7, and dendrogram data analysis Fig. 6 based on similarity matrix showed two main clusters at 91.4 level of similarity. The first cluster exhibits the highest degree of similarity level between Sakha 95, Giza 171, Gemmeiza 12, Misr1, and Misr 2 that grouped at 95.6% level of similarity. The second cluster gave a high degree of similarity but lower than the first one, between Sids 14, Morocco, Beni suef 7, Beni suef 5, Beni suef 1, and Sohag 4 that grouped at 94.8% level of similarity.

Histological examination

Cross-sections and epidermal strips in the infected leaves indicated that the establishment of a compatible anatomical interaction showed in resistant cultivar (Sahka 95) as indicated by the dense of cuticle layers and increasing of epidermal layers that prevents the penetration of the *S. vesicarium* (Fig. 7A, B). On the contrary, the absence of the cuticle layer in the inoculated susceptible cultivar (Beni suef 5), along with the thinness of the epidermis layer, which facilitates the penetration process and thus destroys the leaf tissue and severely deformed (Fig. 7E, F). In case of moderately resistant cultivar (Sids 14), the tissue appears less affected due to the infection (Fig. 7C, D).

Discussion

A new wheat disease known as Stemphylium leaf spot, caused by *S. vesicarium*, has recently been identified in Egypt. *Stemphylium vesicarium* was isolated at high frequency (71.9%) from all symptomatic wheat leaves randomly collected from different wheat fields in Beni Suef governorate, Egypt. The identification of wheat isolate as *S. vesicarium*, which belonging to the *S. vesicarium* species group, was confirmed based on morphological characteristics such as hyphae, conidiophores, conidiogenous cells and conidia shape, color, diameters, and septation. The microscopic examination revealed the presence of light brown, branched, and septate hyphae. Pale brown, septate straight or curved conidiophores. Conidiogenous cells were dark brown, broad, and swollen at the apex, bearing single conidia, which were ovoid to oblong in shape, and brown in color. These morphological observations align with previous reports (Dangi et al. 2019; Gedefaw et al. 2019; Hassan et al. 2020; Chandel et al. 2022; Farag et al. 2022). The mega blast search using the ITS sequence of the *Stemphylium* isolate AUMC 15115 revealed that the closest species match was *Stemphylium botryosum* CBS 714.68 [(GenBank accession number NR_163547 (type species); identities = 570/576 (98.96%); gaps = 2/576 (0%)] and *Stemphylium gracilariae* CBS 482.90 [(GenBank accession number MH862230; identities = 564/568 (99.3%); gaps = 3/568 (0%)]. In the

Table 6 SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) protein patterns extracted from leaves of different wheat cultivars growing under artificial inoculation conditions

Molecular weight (KD)	Beni Suef 1	Beni Suef 5	Beni Suef 6	Beni Suef 7	Morocco	Sids 14	Sohag 4	Sakha 95	Giza 171	Gemmeiza 12	Misr 1	Misr 2
250												
240												
230		==										
220			==		==	==						
210												
200												
190												
180												
170												
160												
150												
140												
130								==	==	==		
120												
110												
100								==	==	==	==	==
90												
80	==						==					
70												
60												
50								==			==	==
40												
30												
20	==	==	==					==	==	==	==	==
10				==		==	==	==	==	==	==	==
0												

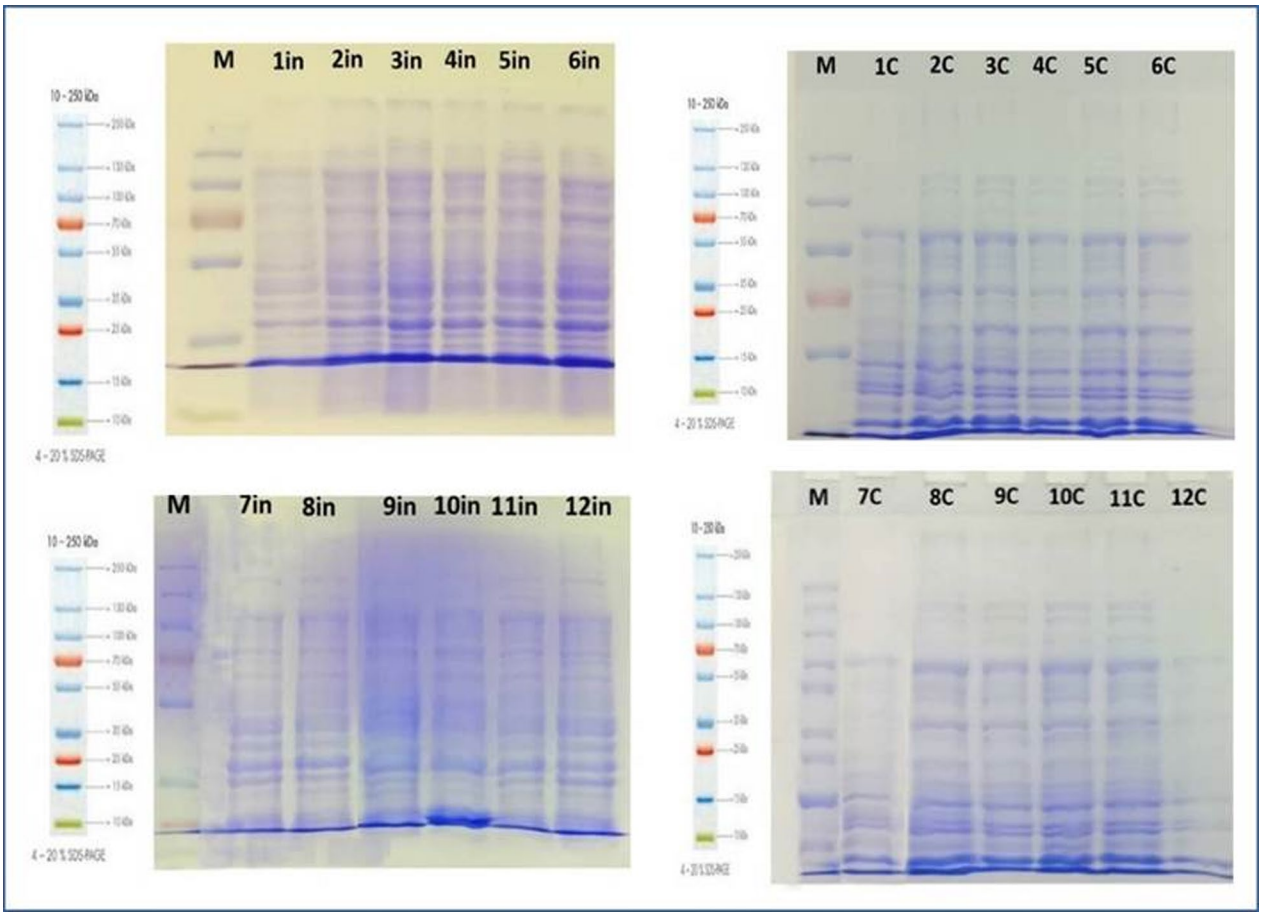

Fig. 5 SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) protein patterns extracted from leaves of different wheat cultivars growing under artificial inoculation conditions. 1. Beni Suef 1, 2. Beni Suef 5, 3. Beni Suef 6, 4. Beni Suef 7, 5. Morocco, 6. Sids 14, 7. Sohag 4, 8. Sakha 95, 9. Giza 171, 10. Gemmeiza 12, 11. Misr 1 and 12. Misr 2. In = Infected; C = Control

phylogenetic tree, the *Stemphylium* isolate AUMC 15115 in this study was clustered at the same branch as *Stemphylium mali* CBS 122640, which is ex-type material (Synonym = *Stemphylium vesicarium*). Consequently, the *Stemphylium* isolate AUMC 15115 in this study is identified as *Stemphylium vesicarium*. The Stemphylium isolate AUMC 15115 ITS sequence was deposited in the GenBank database as MZ944879. Our results align with the findings of Koike et al. (2013), Poursafar et al. (2016), Vitale et al. (2017), Gedefaw et al. (2019), Karbowy-Thongbai and Götz (2023).

Utilizing resistant cultivars is an effective method for controlling plant diseases. However, due to the novelty of this particular disease, it was crucial to determine the resistance levels of different wheat cultivars grown under Egyptian conditions. The current study observed notable differences between resistant and susceptible wheat cultivars in terms of disease incidence and severity percentages. These variations were found to be significant among the wheat cultivars, which were artificially inoculated with *S. vesicarium*. Out of the 12 tested wheat cultivars, the Sakha 95 exhibited a low percentage of infection and was classified as resistant. In contrast, Beni Suef 5 had the highest infection rate and was classified as susceptible. These outcomes are in agreement with Koike et al. (2013), Mishra et al. (2013), Mishra and Singh (2019). Dangi et al. (2019) screened 59 onion accessions for resistance to Stemphylium blight and discovered that Red Creole 1 is susceptible to *S. vesicarium* infection, whereas Pusa Soumya (*Allium fistulosum* L.) and Red Creole 2 (*A. cepa* L.) are moderately resistant.

Understanding the biochemical mechanisms can provide valuable information regarding resistance against *S. vesicarium*. The rapid production of hydrogen peroxide (H_2O_2), a type of reactive oxygen species (ROS), is an early cellular response during plant-pathogen interactions. This process plays a crucial role in strengthening the cell wall through the formation of papillae, lignin formation, and the crosslinking of proteins high in hydroxyproline. Callose, proteins, and phenolic chemicals

Table 7 Similarity matrix among twelve studied wheat cultivars as computed according to Jaccard' Coefficient as revealed by protein marker

	Beni Suef 1	**Beni Suef 5**	**Beni Suef 6**	**Beni Suef 7**	**Morocco**	**Sids 14**	**Sohag 4**	**Sakha 95**	**Giza 171**	**Gemmeiza 12**	**Misr 1**	**Misr 2**
Beni Suef 1	1.00											
Beni Suef 5	0.94	1.00										
Beni Suef 6	0.94	0.94	1.00									
Beni Suef 7	0.97	0.97	0.97	1.00								
Morocco	0.94	0.94	*1.00*	0.97	1.00							
Sids 14	0.94	0.94	*1.00*	0.97	1.00	1.00						
Sohag 4	*1.00*	0.94	0.94	0.97	0.94	0.94	1.00					
Sakha 95	0.89	0.89	0.89	0.91	0.89	0.89	0.89	1.00				
Giza 171	0.91	0.91	0.91	0.94	0.91	0.91	0.91	0.97	1.00			
Gemmeiza 12	0.91	0.91	0.91	0.94	0.91	0.91	0.91	0.97	*1.00*	1.00		
Misr 1	0.91	0.91	0.91	0.94	0.91	0.91	0.91	0.97	0.94	0.94	1.00	
Misr 2	0.91	0.91	0.91	0.94	0.91	0.91	0.91	0.97	0.94	0.94	*1.00*	1.00

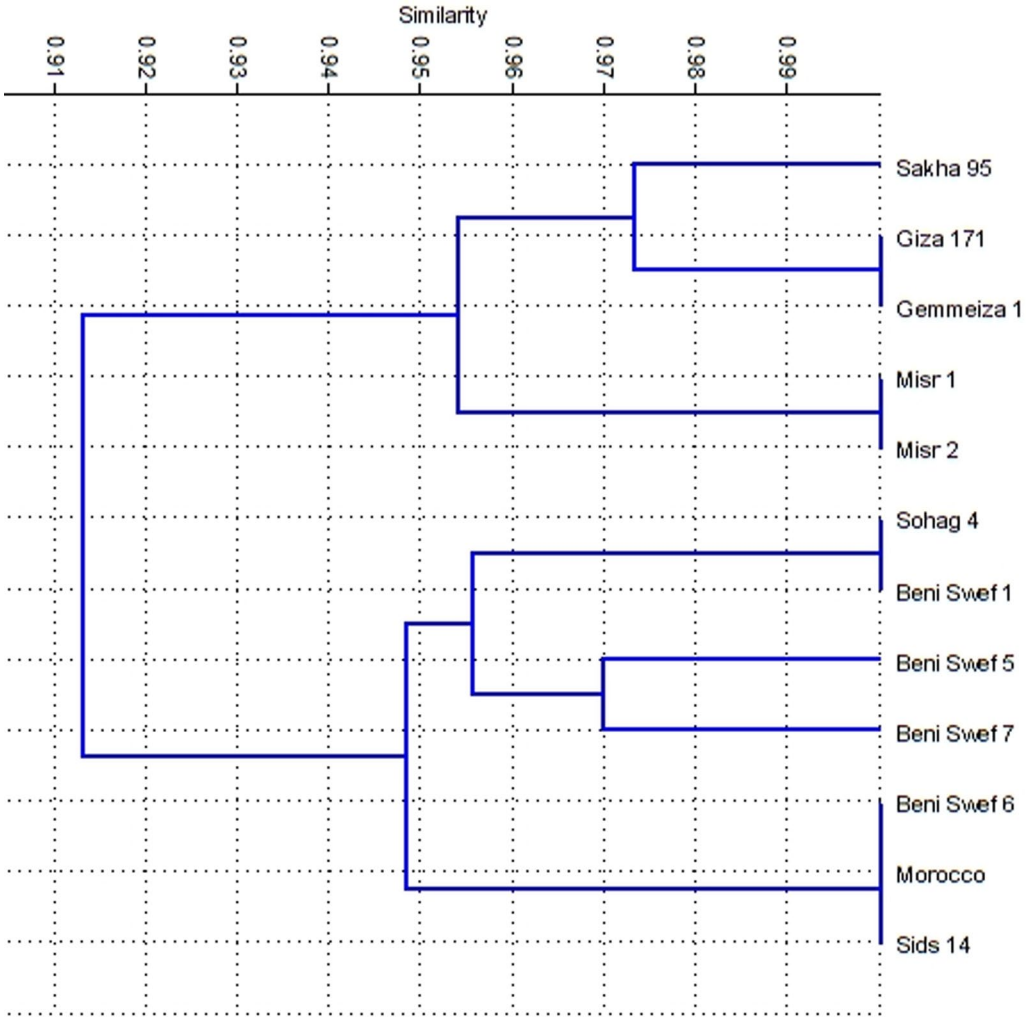

Fig. 6 The dendrogram of the twelve studied wheat cultivars as constructed using protein patterns and similarity matrices computed according to Jaccard' coefficient

accumulate in papillae-cell wall appositions (Hückelhoven et al. 1999; Garcia-Limones et al. 2002; Mellersh et al. 2002). All the wheat cultivars tested in the study exhibited significant production of H_2O_2 in response to *S. vesicarium* inoculation, albeit to varying levels. Our data suggests that the induction of H_2O_2 into wheat leaves during inoculation could potentially serve as a defense mechanism against the invasive pathogen. On the other hand, the cultivar Beni Suef 5, which is highly susceptible to infection, exhibited a high accumulation of H_2O_2. This suggests that there were elevated levels of ROS, which are associated with a leaf tissue's susceptibility to *S. vesicarium* infection. Hydrogen peroxide is a potent oxidizer produced in plants under oxidative stress and a hazardous substance. von Tiedemann (1997) found that increased levels of H_2O_2 later in the disease progression caused harmful changes in cells and facilitated the invasion and dissemination of necrotrophs via dead leaf tissues.

The current investigation revealed a linear relationship between peroxidase, polyphenol oxidase, superoxide dismutase activities, total phenol content, and resistance level. In contrast to the resistant cultivar, susceptible ones could not respond quickly to the pathogen onslaught. This may explain why the infection established in the susceptible cultivar and the disease developed. This outcome is consistent with Mydlarz and Harvell (2007), Raimbault et al. (2011), Kunos et al. (2022).

Increased lignin formation may be associated with higher peroxidase activity in resistant cultivars. According to Almagro et al. (2009), peroxidase is involved in physiological processes like lignin production and is essential for scavenging ROS. Polyphenol oxidase exhibits various mechanisms that contribute to its

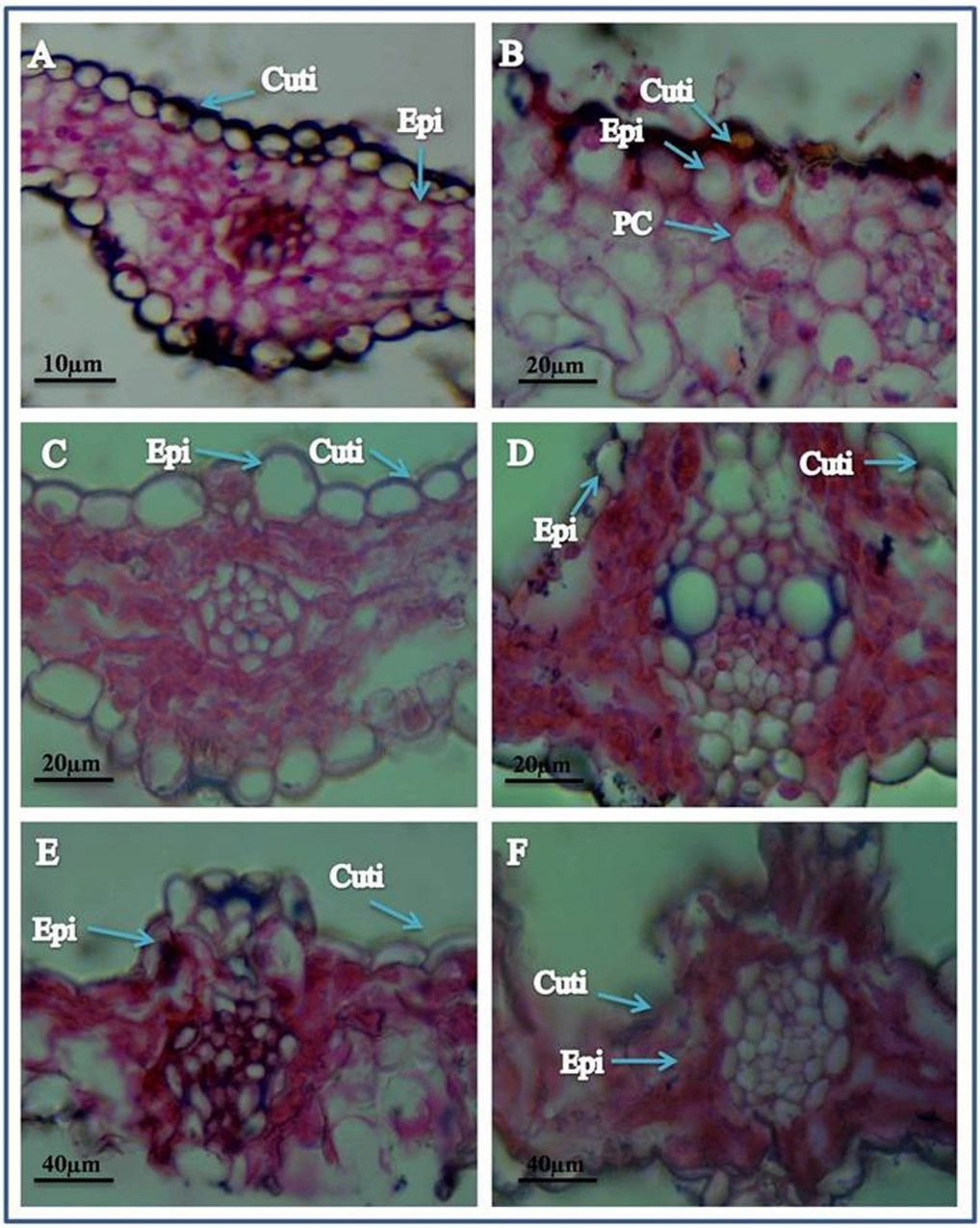

Fig. 7 Host cell responses as illustrated by TEM in resistant, (**A**, **B**) moderately resistant (**C**, **D**) and susceptible (**E**, **F**), wheat cultivars (Sakha 95, Sids 14 and Beni Suef 5, respectively) due to inoculated with *S. vesicarium*. Cuticle (Cuti); Epidermis (Epi) and Parenchyma cells (PC)

anti-pathogen effects. One hypothesis suggests that it converts phenols into quinones, which are highly reactive and possess more potent antibacterial properties compared to phenols. Additionally, it is believed to create a physical barrier within the cell wall against pathogens. Consequently, these enzymes can actively contribute to the prevention of pathogen development by increasing the rate of cell death close to the infection site, inhibiting the spread of infection, or creating a toxic environment within the cells that hinders pathogen propagation (Bi and Felton 1995; Melo et al. 2006; Shimzu et al. 2006). Superoxide dismutase converts the extremely hazardous oxygen radicals into oxygen and significantly less harmful hydrogen peroxide. Superoxide dismutase activity is associated with higher resistance to oxidative stress caused by infections and different abiotic stress conditions (Ehsani-Moghaddam et al. 2006; Gill and Tuteja 2010; Youssef et al. 2020).

The severity of cell membrane damage is indicated by the electrolyte leakage percentage. The percentage of electrolyte leakage in susceptible wheat cultivars (Beni Suef 5) significantly increased when compared to resistant cultivars. This demonstrates the extent to which a fungal infection causes significant damage to the membrane and reduces its integrity. The phenomenon could be attributed to the correlation between cellular components and the outflowing quantity, which invasive pathogens may exploit as a nutritional source. These findings are supported by Houimli et al. (2010), Hafez et al. (2014), Aslam et al. (2019). Abdelaal et al. (2014) found that when susceptible wheat cultivars were exposed to *Puccinia striiformis* f. sp. *tritici*, their cell membranes were damaged, and their components were lost. In contrast, resistant cultivars could resist the pathogen's attack and maintain their cell membranes.

Also, the multiple environments studies aim to determine which genotypes are better under different conditions. In term of resistance to Stemphylium leaf spot, eight of the twelve wheat cultivars examined in this study by the GGE biplot analysis fell to the left of the biplot origin, indicating that they are either resistant or moderately resistant. Sakha 95 was the vertex cultivar for peroxidase and superoxide dismutase enzymes as well as total phenols, placed at the opposite side and did not exhibit any disease characteristics, indicating its resistance to *S. vesicarium* infection. While Beni Suef 5 was the vertex cultivar for disease incidence and severity and did not exhibit any disease defense characteristics, indicating its susceptibility to *S. vesicarium* infection. Similar evaluations were conducted using this method for various diseases (Lillemo et al. 2010; Rubiales et al. 2012; Pande et al. 2013; Sharma et al. 2016; Akcura et al. 2017; El-Taweel et al. 2017).

Our results indicated that the protein bands of infected wheat plants from different cultivars exhibited variations in comparison to the control plants. This finding could be attributed to the interplay between diseases and plants. There has been an increase in the number of bands in resistant wheat cultivars compared to susceptible ones. This increase may be linked to resistance, particularly in Sakha 95, a resistant wheat cultivar, which exhibited a high number of new bands with molecular weights of 10, 20, 50, 100, and 130 kDa. In contrast, susceptible wheat cultivars showed the lowest expressed bands. These outcomes are consistent with Taheri and Tarighi (2012), Khairy et al. (2021), Elkobrosy et al. (2022). The dendrogram of the SDS-PAGE maker revealed two main clusters. The first cluster exhibited the highest degree of similarity level between Sakha 95, Giza 171, Gemmeiza 12, Misr1, and Misr 2, grouped at a 95.6% level of similarity. The second cluster exhibited a significant level of similarity among Sids 14, Morocco, Beni suef 7, Beni suef 5, Beni suef 1, and Sohag 4, which were grouped together with a similarity level of 94.8%. The findings of our study align with the findings of Hussein et al. (2019), Rayan et al. (2019), Atia et al. (2021), Omar et al. (2021), Khairy et al. (2021) Declarations: Ethics approval and consent to participate: Not applicable. Consent for publication: Not applicable. Competing interests The authors declare that they have no competing interests.

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Author's Contributions
The authors confirm their contribution to the paper as follows: study conception and design: Eman W. Ghebrial, Farag M. Farag, Mohamed A. Abou Zeid, and Atwa A. Atwa; analysis and interpretation of results: Eman W. Ghebrial, Farag M. Farag, Mohamed A. Abou Zeid, Nourhan A. Atwa, Shreef T. Eissa and Atwa A. Atwa; draft manuscript preparation: Eman W. Ghebrial, Farag M. Farag, Mohamed A. Abou Zeid, Nourhan A. Atwa, Shreef T. Eissa and Atwa A. Atwa; All authors reviewed the results and approved the final version of the manuscript.

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Declarations

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