Morphological, molecular biology and pathological characterization of fungi causes root rot on grapevine

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Background
Grapevine is subjected to attack by several of soilborne fungi causing root-rot diseases that lead to limit the production of grapevine.
Objective
This study aimed to investigate the morphological, molecular identification, and pathogenic potential of fungi that causes root-rot disease of grapevine in Egypt.
Materials and methods
Isolation of fungi causing root rot of grapevine plants from the samples of diseased roots of different cultivars, i.e., crimson, superior, and flame seedless. Identification according to characterizations of morphological, cultural, and molecular biology based on internal-transcribed spacer 1 (ITS1). Pathogenicity tests of fungal isolates on grapevine plants under greenhouse conditions. Root-rot incidence, disease severity, and plant growth characteristics were determined.
Results and conclusion
Isolation traits from affected grapevine trees with different grape varieties in Egypt's El-Nobaria Province, El-Behira Governorate, yielded eighteen isolates of four fungal genera. *Fusarium* spp. was the most fungal genus highly frequent on all cultivars tested and colonization (100%) on root-rot tissue of superior and flame-seedless cultivars, then (75%) on Crimson cultivar. While *Lasiodiplodia* spp. was recorded with moderate frequency and high colonization (100%) on rotten tissue of Crimson cultivar followed by (75%) in both flame-seedless and superior cultivars. As opposed to that, fungi of *Macrophomina phaseolina* followed by *Rhizoctonia solani* had the least frequency and colonization percentage. Isolates of fungi were identified according to morphological characteristics, cultural, and molecular biology based on internal-transcribed spacer-1 (ITS1) sequencing and conserved in GenBank with accession numbers from (ON037457.1 to ON037474.1). Fungal isolates were varied for causes of root rot on grapevine plants and their reduction effect on the characteristics of growth of flame-seedless cultivar. *Fusarium solani* isolate (ON037462.1) was the most pathogenic isolate that caused (100%) of root rot, high disease severity, and highly significant reduced grapevine plant growth characteristics followed by isolates of *Lasiodiplodia theobromae* (ON037474.1). In this respect, a new isolate of fungal species was hosting grapevine plants, i.e., two isolates each of *F. chlamydosporum, F. brachygibbosum,* one isolate of *F. ipomoeae*, and one isolate of *L. exigua* are the new causal pathogens of root-rot disease on grapevine as the first report in Egypt.

Keywords:
Fusarium spp, grapevine, internal-transcribed spacer primer, molecular biology, root rot

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Introduction

One of the most widely cultivated fruit crops in the world, grape (*Vitis vinifera* L.), is Egypt's second-largest fruit producer behind citrus. Through the year 2020, Egypt will have planted 192934 feddan worth of grapevines, producing 1596169 tonnes of grape fruit. El-Behira has the largest agricultural areas, followed by the governorates of El-Minia and El-Gharbeia, in that order, while the area planted with grapes in El-Nobaria Province accounts for around 47% of all the land planted with grapes in Egypt [1].

Around the world, there is a disease called grapevine root rot that causes the plants to deteriorate and die. The symptoms of the disease root rot on the shoot of grape plants began on the bottom to above leaves as chlorosis, yellowing, stunting, and wilting, in addition, discoloration was observed on root system [2–6]. The most prevalent soilborne fungi that cause grapevine

root rot are *Fusarium oxysporum, F. solani, F. brachygibbosum, F. avenacum, F. moniliforme, Rhizoctonia solani*, *Lasiodiplodia theobromae, Phytophthora* spp., *Pythium* spp., and *Macrophomina phaseolina* in Brazil [7], in the United States, [8], in South Africa [9], in Poland [10], in Australia [11], in Japan [12], in Turkey [13], and in Egypt [2–6]. In addition, *Fusarium commune* was reported as the first time a causal of root-rot disease on grapevine in China [14].

There is currently no knowledge of the synergistic pathogen–pathogen interactions in plant diseases, although the fact that van Leeuwenhoek had recognized the significance of pathogenic microorganisms as a component of complex multispecies ecosystems during the 1600s [15]. In this manner, the synergistic result observed on fruits of bananas affected by crown rot was highly incident in case of the combination between pathogenic fungi of *Colletotrichum muse, F. proliferatum,* and *Lasiodiplodia theobromae* than each of the fungus [16]. Moreover, advanced studies were reported of different fungal infection types of grapevine by *Fusarium* spp., a single and in different combination with fungi of *Lasiodiplodia theobromae, R. solani,* and *M. phaseolina* [2,4]. Furthermore, two types of infection were reported on banana fruits after harvesting by fungi of *Fusarium* spp., *Thialoviopsis paradoxa,* and *Colletotrichum musae* [17].

The identification of fungi pathogens, and also insight about studying unknown species' DNA sequences, have all benefited from the insights generated by molecular biology. rRNA genes are appealing, and a quick test and precise fungal pathogen identification for initiating treatment in the first stages of infection [18]. Based on the variation of the ribosomal genes 5.8S, 18S, and 26S rRNA, the majority of molecular protocols is used to identify fungi [19,20]. The internal-transcribed spacer (ITS), which is used in a variety of systematic studies of plant taxa at the genus and species levels, has been widely used [21].

This study aimed to investigate morphological, molecular identification, and pathological potential of fungi that causes root-rot disease on grapevine in Egypt.

Materials and methods

Isolation and morphological identification of the causal organisms

The samples of diseased grape roots of different cultivars, i.e., Crimson, superior, and flame seedless, were collected from El Nobaria region, El-Behira Governorate, Egypt. The samples of diseased roots were cleaned with running tap water and cut into tiny pieces (0.5 cm2). The pieces were submerged in 1% sodium hypochlorite solution for 2 min to sterilize the surface before being repeatedly washed with sterile distilled water. The pieces were transferred individually to Petri dishes after being dried between layers of sterilized Watman (No. 1) filter paper. For the purpose of reducing bacterial contamination, 50 ug/L of streptomycin sulfate was added to each plate in the medium and incubated at 27±2°C. Utilizing single-spore or hyphal tip techniques, fungus isolates were purified. According to cultural, morphological, and microscopic characteristics described by [22–26] in Central Laboratories Complex at the Faculty of Agriculture, Cairo University, purified cultures of fungi were identified.

Molecular identification

DNA extraction

From pure cultures of 18 fungi strains, genomic DNA was extracted and isolated from diseased grapevine roots of different cultivars, which were collected from El-Nobaria, El-Behira Governorate, Egypt, following the manufacturer's instructions, after being grown on PD broth medium, using the i-genomic BYF DNA extraction Mini Kit [27].

Internal-transcribed spacer (ITS) sequencing and partial PCR amplification

Utilizing the initial ITS, molecular genetic analysis was used to identify the fungal isolates. A method based on [28] was used to obtain the isolate 18S rDNA partial sequences. The gene's divergent domain was amplified using two different primers: the ITS1 primer and the ITS4 primer. Operon Technologies Company, based in the Netherlands, provided two primers. There were 40 ng of pure DNA and 12 ng of the primer used in each polymerase chain reaction (PCR) bead. Sterile distilled water was used to bring the amplification reaction's total volume to 25 µl. The amplification procedure was carried out as follows. The following segments made up each of the 35 cycles. The PCR was kept at 4°C until analysis. The amplified DNA products were electrophoresed on a 1.0% agarose gel. The various band sizes were determined. Using the Gel Documentation System and UV Tran's illuminator, the separated bands were then stained with 0.5 g/ml ethidium bromide and photographed.

Purification and sequencing of fungus DNA

PCR purification kit Gene JETTM (Thermo K0701) was used to clean up the PCR products. ABI 3730xl

DNA sequencer (GATC Company, Germany) was used to sequence the purified PCR products' DNA using ITS primers.

Taxa evolution of relationships

The Neighbor-Joining method was used to infer the evolutionary history [29]. The bootstrap consensus tree produced from 1000 replications is taken to represent the evolutionary history of the taxa under investigation [30]. Branch collapse occurs for partitions that are repeated of bootstrap replicates. A duplicate tree with connected taxa clustered together is shown next to the branches in the bootstrap test (1000 repetitions). Using the Jukes–Cantor method, the evolutionary distances, which are expressed as base substitutions per site, were calculated [31]. A gamma distribution with a shape parameter of 1 was used to simulate the rate variance between sites. There were 22 nucleotide sequences in this investigation. For each set of sequences, all ambiguous locations were eliminated. The final dataset contained 848 locations altogether. Evolutionary analyses were conducted in MEGA11 [32].

Pathogenicity test

The National Research Center's Plant Pathology Department in Egypt tested the pathogenic potential of isolated fungi under greenhouse conditions. Pots (25 cm in diameter) were sterilized in a 5% formalin solution for 20 min. Formaldehyde was removed from sandy loam soil by sterilizing it with 5% formalin solution, covering it for seven days to trap the gas, and letting it air-dry for two weeks. Sterilized soil was used to fill the pots. Each isolate of the tested fungi was grown in glass bottles using a sterilized medium of corn meal and sand. One disk (1 cm in diameter) from a mycelial culture that was 7 days old and from each of the tested fungal isolates was used to inoculate a bottle. Each of the tested fungi was inoculated into pots at a rate of 5% of the soil weight (w/w) and were regularly watered three times per week before planting. Each pot had one grapevine transplant that was a one-year-old flame-seedless cultivar. As duplicates, five pots were used. A control was grown in five pots without any fungi infestation. The incidence and severity of the root-rot disease were both recorded at 3 months following grape plant cultivation. Root-rot disease severity was determined according to [2], based on a linear scale from 0 to 4 on the shoot system of the grape plant as follows: 0 indicates a healthy plant, 1 a yellowish +1/3 wilted plant, 2 a 2/3 wilted plant, 3 a whole plant, and 4 plants dead showed severe wilt. Additionally, the linear scale from 0 to 3 was used to rate the severity of the root-rot disease that was determined according to [2].

$$\textbf{Disease incidence} = \frac{\textbf{No.of infected plants}}{\textbf{total of plants}} \times 100$$

Estimation of morphological growth characteristics of grapevine plants

At the end of the experiment of (3 months after cultivation), the morphological properties of the grapevine plants in pots were measured in accordance with [6].

Statistical analysis

All previously planned experiments underwent statistical analysis in accordance with the (ANOVA) methods described by [33].

Results

Frequency and colonization of fungi associated with diseased grapevine plants

Isolation trials during 2019 fungal genera of roots diseased grapevine of root-rot-diseased grapevine plants of various cultivars, i.e., flame seedless, Crimson, and superior of El-Nobaria, El-Behira Governorate, Egypt. Data in (Table 1) proposed that several fungal genera that included *Fusarium* spp., *Lasiodiplodia* spp., *Rhizoctonia solani,* and *M. phaseolina* were the common fungi associated with grapevine cultivars, flame seedless, Crimson, and superior cultivars. *Fusarium* spp. became the most fungi highly colonized (100%) on root-rot tissue of superior and flame-seedless cultivars followed by (75%) on Crimson cultivar with a high frequency on all cultivars tested, while *Lasiodiplodia* spp., recorded high colonization (100%) of rotten tissue on Crimson followed by (75%) in both cultivars of flame seedless and superior. As opposed to that, the fungus of *Rhizoctonia solani* was the least in frequency.

Infection types of fungi associated with grapevine cultivars

Flame seedless, crimson, and superior grapevine cultivars were used in isolation tests of diseased root-rotten tissues in El-Nobaria Province, El-Behira Governorate, Egypt. Four soilborne fungal genera were associated with diseased roots, i.e., as *Lasiodiplodia* spp., *Fusarium* spp., *M. phaseolina,* and *Rhizoctonia solani*. Table 2 shows that *Fusarium* spp. recorded a high percentage of single infections in all grapevine cultivars followed by *Lasiodiplodia* spp. on flame-seedless and crimson cultivars. However, neither *M. phaseolina* nor *R. solani* caused a single infection. Meanwhile, the double infection by the combination by fungi of *Fusarium* spp.+ *Lasiodiplodia* spp. and

Table 1 Frequency and colonization of fungi on root-rot tissues of grapevine plants under natural infestation

Cultivar	Fungal name	Total isolates	Fungal frequency %	Fungal colonization%
Flame seedless	*Fusarium spp.*	18	15.4	100
	Macrophomina phaseolina	9	7.7	75
	Lasiodiplodia spp.	14	12.0	75
	Rhizoctonia solani	2	1.7	25
Crimson	*Fusarium spp.*	14	12.0	75
	Macrophomina phaseolina	10	8.5	75
	Lasiodiplodia spp.	13	11.1	100
	Rhizoctonia solani	5	4.3	25
Superior	*Fusarium* spp.	12	10.2	100
	Macrophomina phaseolina	7	6.0	75
	Lasiodiplodia spp.	10	8.5	75
	Rhizoctonia solani	3	2.6	25
Total		117	100%	

Table 2 Percentage of combination of four fungi causing root-rot disease for different grapevine cultivars under natural field infestation

		Grapevine cultivars		
Infection types	Fungi	Crimson	Flame seedless	Superior
Single	*Fusarium* spp. (F)	40.0	40.0	60.0
	Lasiodiplodia spp. (L)	20.0	10.0	0.0
	Rhizoctonia solani (R)	0.0	0.0	0.0
	Macrophomina phaseolina (M)	0.0	0.0	0.0
Double	F+L	10.0	10.0	20.0
	F+R	0.0	0.0	0.0
	F+M	10.0	15.0	10.0
	L+M	10.0	0.0	0.0
Third	F+L +M	10.0	15.0	10.0
	F+L+ R	0.0	0.0	0.0
Fourth	F+L+M+R	0.0	10.0	0.0

F, Fusarium; L, Lasiodiplodia; M, Macrophomina; R, Rhizoctonia.

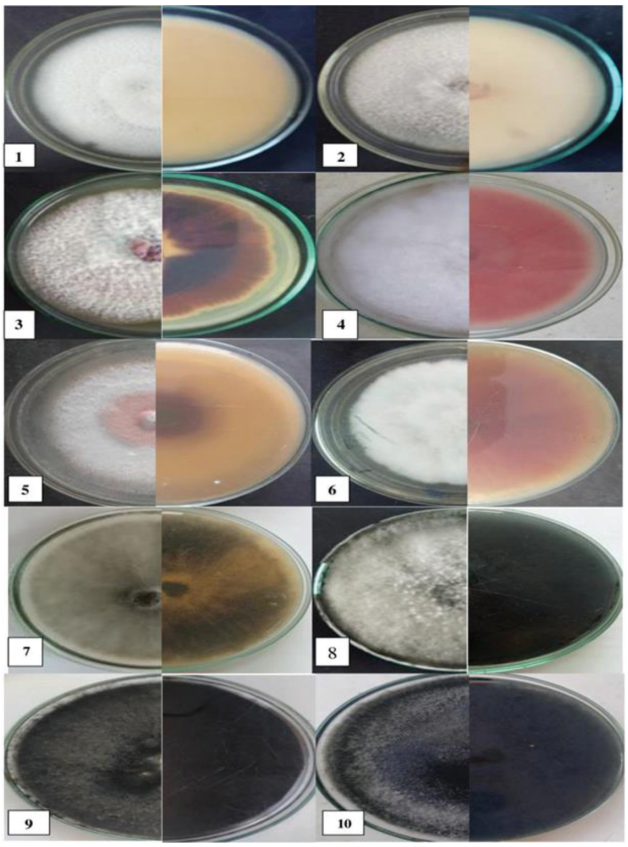
Fusarium spp.+*M. phaseolina* on all grapevine cultivars, followed by *Lasiodiplodia* spp.+ *M. phaseolina,* was only observed on Crimson cultivar. Only one-third of infection combinations were noted between *Fusarium* spp.+*Lasiodiplodia* spp.+ *M. phaseolina* on all grapevine cultivars. Meanwhile, the fourth infection type was recorded between four fungi *Fusarium* spp.+ *Lasiodiplodia* spp.+ *M. phaseolina* + *R. solani,* which only recorded on flame-seedless cultivar in this study.

Morphological identification of fungal isolates

Isolates of fungi were identified morphologically according to the basis of cultural traits of mycelial growth and pigmentation, as well as microscopic observations of macroconidia, microconidia, sclerotia, and their morphological characteristics. Five isolates were found of *F. solani*, one isolate of *F. oxysporum*, two isolates each of *F. chlamydosporum, F. brachygibbosum*, and one isolate each of *F. ipomoeae* and *Gibberella moniliformis*. In addition, three species of *Lasiodiplodia,* i.e., one isolate each of *Lasiodiplodia crassispora*, *L. theobromae,* and *L. exigua*, in addition to one isolate of *M. phaseolina* .

Data in Figs. 1 and 2 and Table 3 indicated that based on morphological characteristics, such as colonies, macroconidia, microconidia, and chlamydospores, different species of *Fusarium* were identified. These isolates were identified as *F. solani, F. oxysporum, F. ipomoeae, F. chlamydosporum, F. brachygibbosum,* and *Gibberella moniliformis*. The colony of *F. solani* formed on PDA white to cream, the microconidia range in shape from fusiform to oval, septated into 0–2 septa, and size of microconidia measures (7–15×2–4 μm), macroconidia is straight to almost cylindrical-shaped, septated into 3–6 septa with dimension (33–72×4–8 μm), as well as chlamydospores formed in globose and subglobose. The colony of *F. oxysporum* formed white and a deep purple underside on the PDA, the microconidia range in form from ellipsoid to oval, septated into 0–2 septa, macroconidia

Figure 1



Mycelial growth of fungal isolates causing root rot of grapevine plants grown on Potato Dextrose Agar (PDA) i. e. *Fusarium solani* (1), *F. oxysporum* (2), *Gibberella moniliformis* (3), *F. brachygibbosum* (4), *F. ipomoeae* (5), *F. chlamydosporum* (6), *M. phaseolina* (7), *Lasiodiplodia theobromae* (8), *L. crassipora* (9) and *L. exigua* (10).

is fusiform, pointed at both ends shaped, and chlamydospores were formed in chains. The colony of *F. brachygibbosum* formed pink with yellow-orange and red pigment on PDA, the oval-shaped microconidia, macroconidia is tapered and pointed-shaped, septated into 3–5 septa, and the formation of chains of chlamydospores. *F. chlamydosporum* white mycelium usually with grayish rose to burgundy pigment on PDA, the microconidia are spindle-shaped, and the macroconidia is sickle-shaped. The colony of *F. ipomoeae* was pinkish-white and grayish-orange at the center on PDA, the macroconidia smooth, and hyaline septated into 3–5 septa and there were no chlamydospores. The colony of *Gibberella moniliformis* was pale-pinkish color on PDA, the microconidia are oval-shaped, and the macroconidia gradually pointed or sickle-shaped, septated into 3–5 septa.

Isolates of the fungus genera of *Lasiodiplodia* spp. were done, identification based on the structural properties of the fungus and its cultural traits, initially produced white colonies, which later turned gray to black. The mycelium was quickly branching, submerged, spreading, and septets. Conidia were initially unicellular, ellipsoidal, hyaline, and granular in composition; a thick-walled, mature conidia had one septum, dark brown with longitudinal striations, and size of the conidia measuring from (20 to 25 ×10–12.8 μm). Identification of *Macrophomina phaseolina* was based on the cultural traits and morphological characteristics of sclerotia. The fungus's young hyphae were found to be hyaline, color is light brown to dark, and more septa. The sclerotia's color initially started out as light brown but eventually darkened to brown to black. The sclerotia's shapes ranged from irregular to spherical to oval to oblong, measuring from (90 to 110 ×60–90 m).

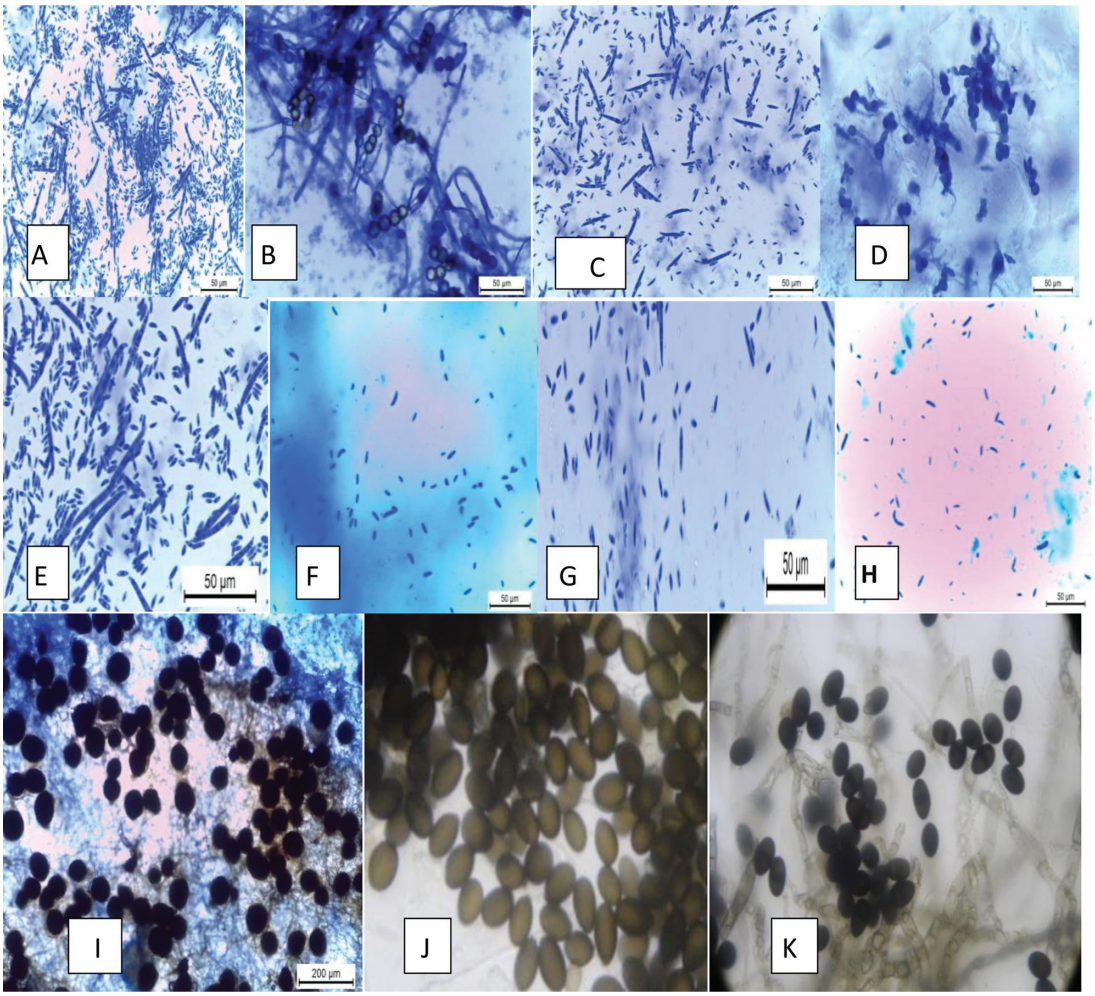
Molecular identification of fungal isolates

After the pure fungus strains' DNA was isolated and determination of the concentration by a spectrophotometer, the rDNA repeat unit's ITS-containing region from using the ITS1 and ITS4 primers, the genomic DNA of the fungal strain was amplified. Approximately 500–600 bp was obtained shown in Figs. 3 and 4 after amplification. After using forward and reverse primers to sequence PCR products using a German company called GATC's ABI 3730xl DNA sequences, the obtained DNA sequences with the identified fungal strains from Kh1 to Kh18 were obtained in Table 4 and conserved in the Gen Bank under the accession numbers from ON037457.1 to ON037474.1, respectively.

Evolutionary relationships of the identified strains

The results of the molecular identification revealed four groups of isolates. The first group consists of eleven isolates, all of which belong to the *Fusarium* genus. The *Fusarium solani* was also discovered to be the biggest, with five species. While *Fusarium chlamydosporum* and *F. brachygibbosum* represent the second place with two numbers each. There are *Fusarium oxysporum* and *F. ipomoeae*, with one type of each of them, as in Fig. 5. In addition, it was discovered that the fungus *Lasiodiplodia* has three species: three isolates of them belong to *L. theobromae*, one isolate to *L. crassispora*, and one isolate to *L. exigua*, as demonstrated in Fig. 6. Finally, it was discovered that the two genera, *Gibberella moniliformis* and *Macrophomina phaseolina*, each have their own taxonomic position, as demonstrated in Figs. 7 and 8. Phylogenetic dendrogram illustrating the taxonomic position of the identified fungal isolates (Kh9) isolated from grapevine roots.

Figure 2

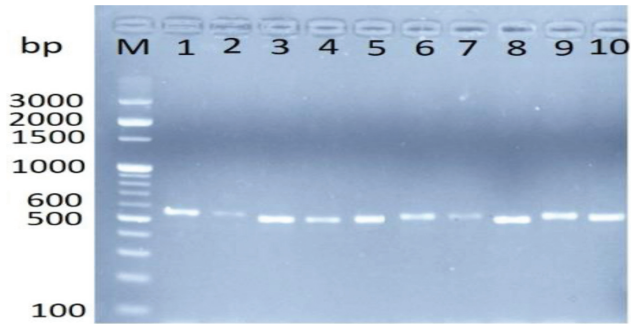


Macroconidia, microconidia, sclerotia and chlamydospores of fungal isolates causing root rot of grapevine plants grown on Potato Dextrose Agar (PDA) i. e. *Fusarium solani* (A-B), *F. oxysporum* (C-D), *Gibberella moniliformis* (E), *F. brachygibbosum* (F), *F. chlamydosporum* (G), *F. ipomoeae* (H), *M. phaseolina* (I) and *Lasiodiplodia theobromae* (J-K). (Scale bar 50 µm).

Table 3 Morphological characters of *Fusarium* spp., the causal of root rot on grapevine plants grown on PDA medium

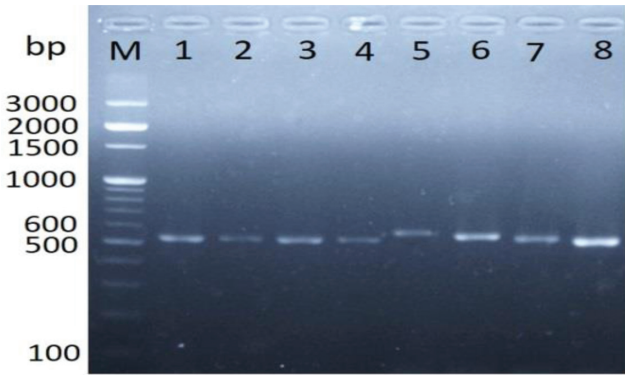
		Microconidia			Macroconidia			
Character isolates	Colony on PDA	Shape	Septa	Size (µm) length×width	Shape	Septa	Size (µm) length×width	Chlamydospores
Fusarium solani	White to cream	Oval – fusiform	0–2	7–15×2–4	Straight to almost cylindrical	3–6	33–72×4–8	Present
Fusarium oxysporum	White and a dark purple	Oval – ellipsoid‘	0–2	5–12×2.2–3	Fusiform, pointed at both ends	3–5	28–45× 3.5–4.5	Present
Fusarium chlamydosporum	White mycelium with grayish rose to burgundy pigment	Spindle	0–2	6–22× 2–4	Sickle-shaped	3–5	30–38×3–4.5	Present
Fusarium brachygibbosum	Pink with yellow-orange and red pigment	Oval	0–2	5–11×2–4	Tapered and pointed	3–4	26–68×3–5	Present
Fusarium ipomoeae	Pinkish white and grayish orange at the center	Oval	0	4–10×2–3	Smooth and hyaline	3–5	36–57× 2–4.5	Absent
Gibberella moniliformis	Pale pinkish	Oval – kidney	0	5–12× 2–3.5	Gradually pointed or sickle	3–5	15–60×2–5	Present

Figure 3



Photograph of internal-transcribed spacer-DNA amplified bands by polymerase chain reaction for fungal isolates (Kh1 to Kh10) isolated from diseased grapevine roots (lanes 1 to 10) using internal-transcribed spacer1 and internal-transcribed spacer4 primers against 100 bp ladder DNA marker (lane M).

Figure 4



Photograph of internal-transcribed spacer-DNA amplified bands by polymerase chain reaction for fungal isolates (Kh11 to Kh18) isolated from diseased grapevine roots (lanes 1 to 8) using internal-transcribed spacer1 and internal-transcribed spacer4 primers against 100 bp ladder DNA marker (lane M).

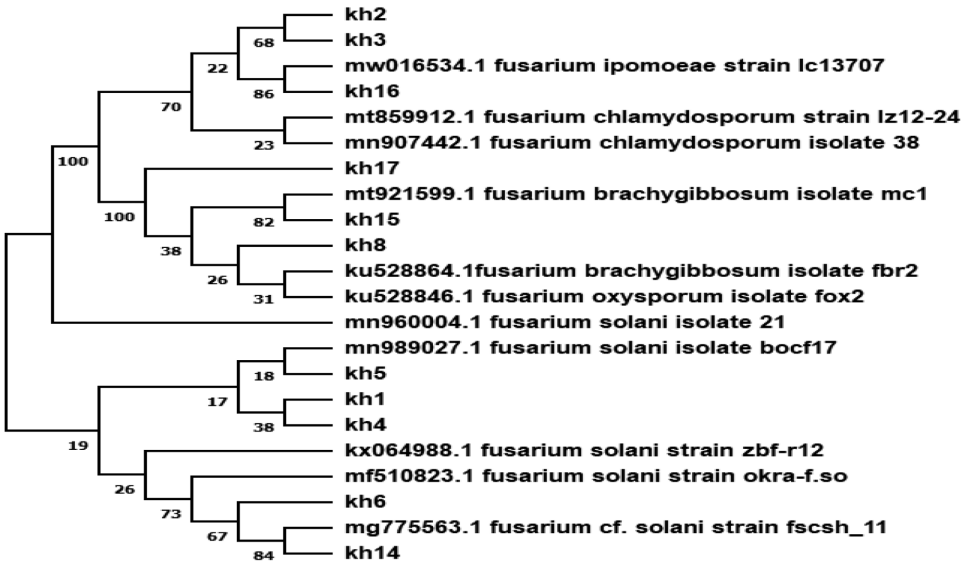
Pathogenicity test of fungal isolates on grapevine

As shown in Table 5, the El Nobaria–El Behira Governorate in Egypt produced 18 different fungi that were isolated from different grapevine cultivars. All isolated fungi, including *Fusarium* spp., *Macrophomina phaseolina,* and *Lasiodiplodia* spp., were examined for their pathogenic potential on grapevine flame-seedless cultivars in soil that was infested with (5%) each isolate's weight in soil (w/w). According to the information in Table 5, root rot was a problem for grapevine plants. On the shoot system of grapevine plants, symptoms of root-rot syndrome were found as illustrated in Fig. 9. While *F. oxysporum* and *F. solani* were mostly induced to cause leaf necrosis for a length of shoot, isolates of *L. theobromae, M. phaseolina,* and *F. solani* were induced to cause the bottom-to-top leaves of grapevine plants to become yellowish from chlorosis, which causes the plants to wilt and die. The secondary and feeder roots of all fungal isolates rotted, and brown discoloration was seen in Fig. 10. In addition, all fungal isolates had decreased root system growth. The percentage of root rot and the severity of the disease were noted 60 and 90 days after grapevine plants were cultivated. Isolated fungi, i.e., *Fusarium solani* (kh6), *M. phaseolina* (kh13), *F. oxysporum* (kh17), and *Lasiodiplodia theobromae* (kh18), were recorded for the percentage of grapevine plants with severe root rot and the severity of the disease. The majority of fungal isolates, *Lasiodiplodia theobromae* (kh18), recorded high percentages of root rot (100%) and

Table 4 Accession number, closest phylogenetic relative, and identity presents eighteen fungal isolates (Kh1– Kh18) obtained from diseased grapevine roots

Isolate	Name of fungal isolates	Accession number	Closest phylogenetic relative and accession number	Identity %
Kh1	*Fusarium solani*	ON037457.1	*Fusarium solani* MN989027.1	99.81
Kh2	*Fusarium chlamydosporum*	ON037458.1	*Fusarium chlamydosporum* MT859912.1	*99.23*
Kh3	*Fusarium chlamydosporum*	ON037459.1	*Fusarium chlamydosporum* MN907442.1	99.03
Kh4	*Fusarium solani*	ON037460.1	*Fusarium solani* KX064988.1	95.81
Kh5	*Fusarium solani*	ON037461.1	*Fusarium solani* MN960004.1	99.07
Kh6	*Fusarium solani*	ON037462.1	*Fusarium solani* MF510823.1	99.62
Kh7	*Lasiodiplodia crassispora*	ON037463.1	*Lasiodiplodia crassispora* AB873032.1	99.61
Kh8	*Fusarium brachygibbosum*	ON037464.1	*Fusarium brachygibbosum* KU528864.1	100.0
Kh9	*Gibberella moniliformis*	ON037465.1	*Gibberella moniliformis* EU314975.1	99.80
Kh10	*Lasiodiplodia theobromae*	ON037466.1	*Lasiodiplodia theobromae* MK696048.1	89.19
Kh11	*Lasiodiplodia theobromae*	ON037467.1	*Lasiodiplodia theobromae* MK860754.1	99.80
Kh12	*Lasiodiplodia exigua*	ON037468.1	*Lasiodiplodia exigua* MT663295.1	99.60
Kh13	*Macrophomina phaseolina*	ON037469.1	*Macrophomina phaseolina* MG772648.1	99.09
Kh14	*Fusarium cf. solani*	ON037470.1	*Fusarium cf. solani* MG775563.1	98.72
Kh15	*Fusarium brachygibbosum*	ON037471.1	*Fusarium brachygibbosum* MT921599.1	99.61
Kh16	*Fusarium ipomoeae*	ON037472.1	*Fusarium ipomoeae* MW016534.1	99.41
Kh17	*Fusarium oxysporum*	ON037473.1	*Fusarium oxysporum* KU528846.1	99.61
Kh18	*Lasiodiplodia theobromae*	ON037474.1	*Lasiodiplodia theobromae* MT199153.1	99.80

Figure 5

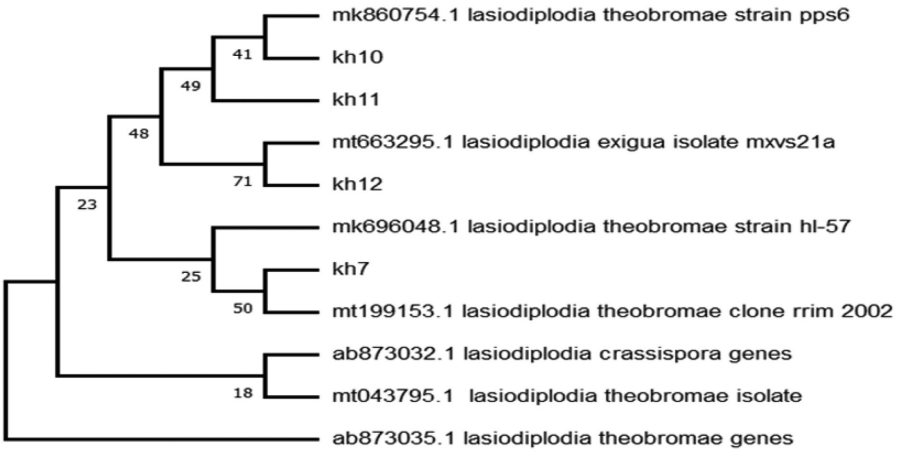


A phylogenetic tree of the taxonomic position of fungal isolates (Kh1, Kh2, Kh3, Kh4, Kh5, Kh6, Kh8, Kh14, Kh15, Kh16, Kh17) isolated from diseased grapevine roots was created using the Neighbor-Joining method with 1000 bootstrap replicates, based on the internal-transcribed spacer sequences and other closely related species in database.

disease severity (3.8), (2.8), respectively, on the shoot and root. Next was a *Fusarium solani* isolate (kh6), then *F. oxysporum* (kh17) proceeded by *M. phaseolina* (kh13) recorded root-rot percentage (100%) and disease severity (2.6), (3.2) on root and shoot, respectively. The most prevalent *Fusarium* fungal isolate, *F. solani* (kh6), caused a high percentage of root rot (100%) and high disease severity in the shoot and root (3.8) and (2.8), respectively.

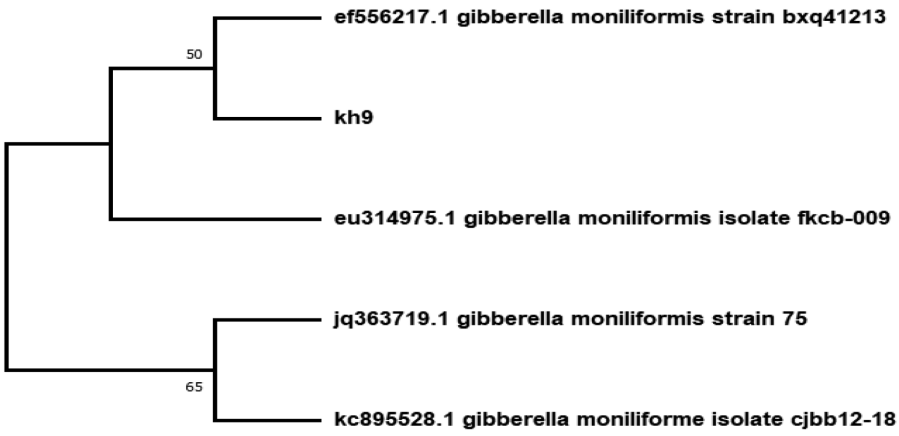
As opposed to that, isolates, i.e., *F. chlamydosporum* (kh2), *F. brachygibbosum* (kh8), and *Lasiodiplodia crassispora* (kh7), the severity of the disease and the percentage of root rot in grapevine plants that were recorded were the lowest. In this respect, several new fungal species induce root-rot syndromes of grapevine plants such as chlorosis, yellowish, and wilt on the shoot system of grapevine plants as shown in Fig. 9, these isolates significantly caused 60% root rot at 90 days after cultivation of grapevine plants as shown in Table 5, these isolates were two isolates of *F. chlamydosporum*, two isolates of *F. brachygibbosum,* one isolate of *F. ipomoeae,* and one isolate of *Lasiodiplodia exigua* that are the new causal pathogens of root rot on grapevine as the first report in Egypt.

Figure 6



A phylogenetic tree of the taxonomic position of fungal isolates (Kh7, Kh10, kh11, Kh12) isolated from diseased grapevine roots was created using the Neighbor-Joining method with 1000 bootstrap replicates, based on the internal-transcribed spacer sequences and other closely related species in database.

Figure 7



A phylogenetic tree of the taxonomic position of fungal isolates (Kh9) isolated from diseased grapevine roots was created using the Neighbor-Joining method with 1000 bootstrap replicates, based on the internal-transcribed spacer sequences and other closely related species in database.

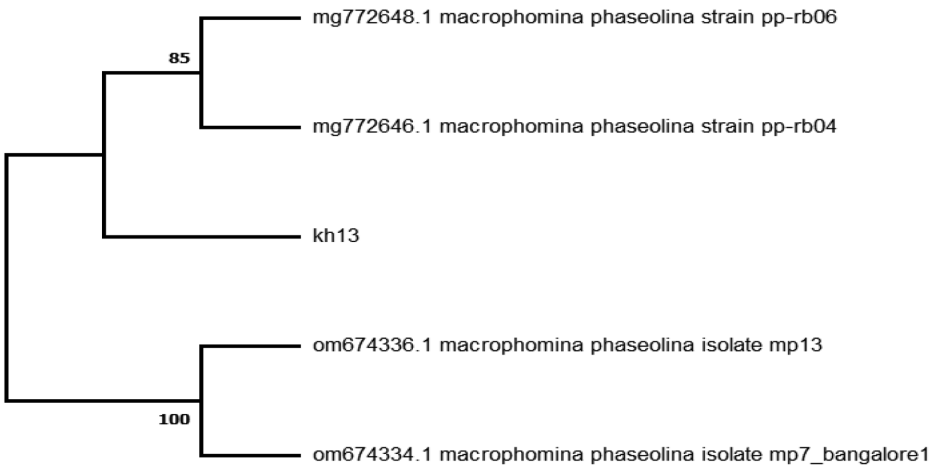
Growth parameters of grapevine plants are affected by fungal isolates in greenhouse conditions

According to data in Table 6, *Fusarium* spp., *Lasiodiplodia theobromae,* and *Macrophomina phaseolina* isolates all had a significant impact on grapevine plants 90 days after each fungus cultivation in this study. All fungi isolates showed decreased growth characteristics, such as shoot and root length, fresh and dry root weight, and root size of each treatment compared with control. *F. solani* (kh6), *Lasiodiplodia theobromae* (kh18), *F. oxysporum* (kh17), and *Macrophomina phaseolina* (kh13) were the majority of fungus isolates shortening the shoot and root length of grapevine plants, meanwhile, two isolates, i.e., *Lasiodiplodia theobromae* (kh18) and *F. oxysporum* isolate (kh17) significantly reduced fresh and dry weight of shoot and root than other isolates. In general, *Fusarium solani* (kh6) was the most significant and fungal isolate that significantly decreased the majority of growth parameters, including shoot length, dry and fresh root weight, shoot weight, and root system size.

Discussion

Soilborne pathogenic fungi communities of *Fusarium* spp., *Lasiodiplodia theobromae, M. phaseolina,* and *R. solani*, associated with roots of grapevine orchard, were increased with increasing age development of grapevine growth that caused root-rot syndromes during several years, which developed as epidemic disease causing, i.e., *Fusarium* spp., *L. theobromae,* and *M. phaseolina*, which significantly loses quality and quantity of fruit yield [2–6,11,13,14,34].

Figure 8



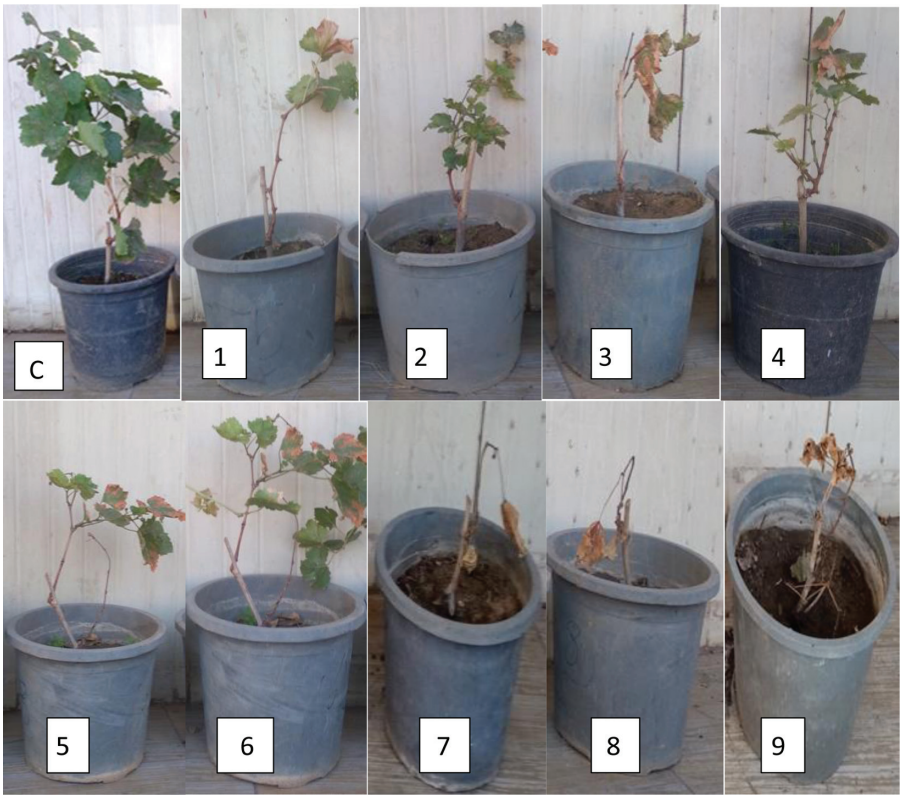
A phylogenetic tree of the taxonomic position of fungal isolates (Kh13) isolated from diseased grapevine roots was created using the Neighbor-Joining method with 1000 bootstrap replicates, based on the internal-transcribed spacer sequences and other closely related species in database.

Table 5 Pathogenicity test of fungal isolates on grapevine plants in pot experiment

Fungi		Root-rot incidence on grapevine plants (days)				
		60		90		
		Infection %	Disease severity	Infection %	Disease severity	
No	Isolate name				Shoot	Root
Kh1	*Fusarium solani*	40c	1.4de	60c	1.8fg	1.6ef
Kh2	*F. chlamydosporum*	40c	1.2ef	60c	1.6g	1.4f
Kh3	*F. chlamydosporum*	40c	1.2ef	60c	1.8fg	1.6ef
Kh4	*Fusarium solani*	40c	1.4de	60c	1.8fg	1.6ef
Kh5	*Fusarium solani*	60b	1.6cd	80b	2.2d	2.0d
Kh6	*Fusarium solani*	80a	2.4a	100a	3.8a	2.8a
Kh7	*Lasiodiplodia crassispora*	40c	1.4de	60c	1.6g	1.6ef
Kh8	*F. brachygibbosum*	40c	1.4de	60c	1.8fg	1.6ef
Kh9	*Gibberella moniliformis*	60b	1.8bc	80b	2.2d	1.8de
Kh10	*Lasiodiplodia theobromae*	40c	1.2ef	60c	2.0ef	1.8de
Kh11	*Lasiodiplodia theobromae*	60b	1.8bc	80b	2.4c	2.2c
Kh12	*Lasiodiplodia exigua*	40c	1.6cd	60c	1.8fg	1.8de
Kh13	*Macrophomina phaseolina*	60b	2.0b	100a	3.2b	2.6b
Kh14	*Fusarium cf. solani*	40c	1.2ef	60c	2.2d	1.8de
Kh15	*F. brachygibbosum*	40c	1.4de	60c	1.8fg	1.8de
Kh16	*Fusarium ipomoeae*	40c	1.4de	60c	2.2d	2.0d
Kh17	*Fusarium oxysporum*	60b	2.0b	100a	3.2b	2.6b
Kh18	*Lasiodiplodia theobromae*	80a	2.4a	100a	3.8a	2.8a
	Control	0.0d	0.0g	0.0d	0.0h	0.0g

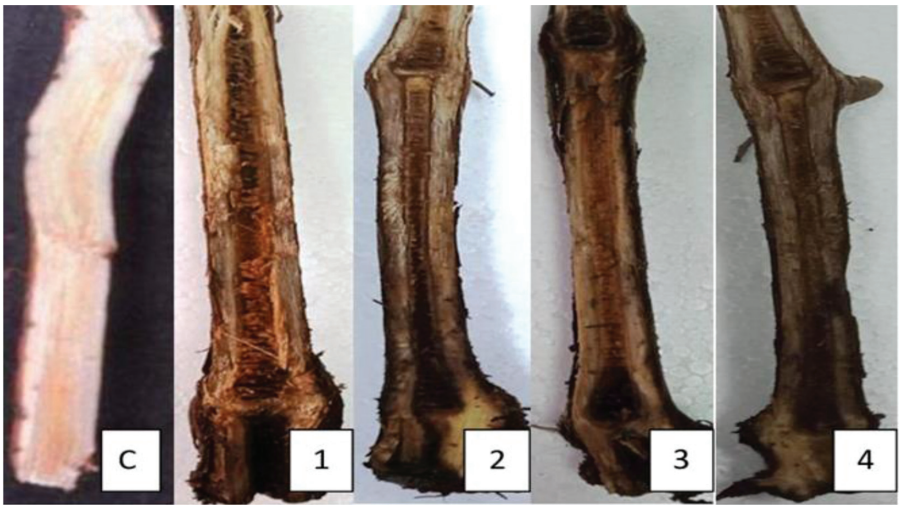
Means with the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple-range test.

Figure 9



Disease symptoms of grapevine on shoot system of Cv. flamseedless, under artificial infested soil by *Fusarium oxysporum* (1), *F. chlamydosporum* (2), *F. solani* (3), *F. brachygibbosum* (4), *F. ipomoeae* (5), *M. phaseolina* (6), *Lasiodiplodia exigua* (7), *L. crassispora* (8) and *L. theobromae* (9) compare ith control (c).

Figure 10



Root rot discoloration and maceration of grapevine root system by *Fusarium solani* (1), *Fusarium oxysporum* (2), *Macrophomina phaseolina* (3), *Lasiodiplodia theobromae* (4) and control (C).

Moreover, the new fungal species were first recorded in Egypt in this investigation, i.e., *Fusarium chlamydosporum*, *F. brachygibbosum*, *F. ipomoeae*, and *Lasiodiplodia exigua*, *L. crassispora* caused root rot of grapevine that induced root-rot symptoms similar to that produced by major root-rot fungal pathogens in economic plants, i.e., *F. brachygibbosum* was first identified as the cause of watermelon wilt disease in Mexico [35], soybean root-rot disease in China [36], and the demise of young grapevines brought on by *F. brachygibbosum* and *F. solani* in Turkish vineyards [13]. *F. chlamydosporum* causes guava wilt disease in India and damping of on Aleppo pine in Algeria [37,38], *Fusarium ipomoeae* was originally reported as the causal of Fusarium wilt on soybean in South Korea [39], *Lasiodiplodia exigua*, *L. crassispora*, and *L. theobromae* are linked to grapevine dieback in Italy, Algeria, and Tunisia [40], and mango stem-end rot in the Northeastern region of Brazil and Brazil's *Jatropha curcas* biofuel plant has collar and root rot [41,42].

Table 6 Effect of fungal isolates on grapevine plants' morphological characters in pot experiment

Fungi		Morphological characters of grapevine plant (90 days)						
		Length (cm)		Fresh weight (g)		Dry weight (g)		
Code	Fungal name	Shoot	Root	Shoot	Root	Shoot	Root	Root size (cm^3)
Kh1	*Fusarium solani*	28.4ghi	16.4fg	13.8h	16.0e	11.5ef	12.0c	7.9c
Kh2	*F. chlamydosporum*	33.6e	20.3c	23.8b	19.6b	10.5g	11.8c	9.3b
Kh3	*F. chlamydosporum*	36.0c	21.6b	21.3cd	18.6c	15.0b	11.5c	9.0b
Kh4	*Fusarium solani*	27.4i	20.6c	20.4d	20.0b	15.0b	15.1b	9.0b
Kh5	*Fusarium solani*	29.0g	20.0c	15.0g	15.3e	7.8i	8.6def	5.8e
Kh6	*Fusarium solani*	16.4l	11.8k	8.0k	10.2j	4.5k	6.0hi	2.2j
Kh7	*Lasiodiplodia crassispora*	34.4de	17.6de	18.4e	15.4e	10.8fg	8.2ef	4.3f
Kh8	*Fusarium brachygibbosum*	35.0d	18.4d	21.6c	17.6d	11.0fg	9.2d	4.3f
Kh9	*Gibberella moniliformis*	28.6gh	16.4fg	11.5i	11.8gh	6.0j	7.9fg	3.2gh
Kh10	*Lasiodiplodia theobromae*	27.8hi	15.8ghi	13.0h	12.0gh	8.1hi	7.2g	2.7hij
Kh11	*Lasiodiplodia theobromae*	28.6gh	16.8ef	13.5h	12.7g	8.9h	8.2ef	3.2gh
Kh12	*Lasiodiplodia exigua*	31.8f	16.0fgh	18.8e	18.5cd	13.2c	12.3c	3.2gh
Kh13	*Macrophomina phaseolina*	26.2j	15.2hi	10.0j	11.8gh	6.0j	6.4h	2.9hi
Kh14	*Fusarium cf. solani*	28.2ghi	16.6fg	10.8ij	10.6ij	7.8i	6.0hi	3.3gh
Kh15	*F. brachygibbosum*	39.2b	18.4d	16.4f	12.6g	12.0de	8.9de	3.7fg
Kh16	*Fusarium ipomoeae*	38.6b	19.8c	20.8cd	14.2f	12.6cd	8.5def	6.4d
Kh17	*Fusarium oxysporum*	21.6k	13.8j	7.6k	11.5hi	5.3jk	5.9hi	2.7hij
Kh18	*Lasiodiplodia theobromae*	25.8j	15.0i	7.2k	10.2j	5.9j	5.4i	2.4ij
	Control	55.2a	33.6a	39.4a	27.4a	27.9a	21.0a	10.8a

Means with the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple-range test.

In this respect, in China, *Fusarium commune,* a new species of *Fusarium* genera, was recently recorded as the causal pathogen of root rot on grapevine [14].

Little information about the etiology and epidemiology of causal pathogens needs a long period 3–7 years to developing on roots of grapevine from latent infection to visual syndrome observation [2,43]. In this respect, four infection types by causal pathogens causing root-rot disease were recorded on grapevine cultivars of superior, flame seedless, and Crimson. The first model of single-infection type by single of each fungal genera of *Lasiodiplodia theobromae* and *Fusarium* spp., was recorded on all grapevine cultivars, which were absent by either *Rhizoctonia solani* or *M. phaseolina*. Double-infection type was caused by *Lasiodiplodia theobromae* and *Fusarium* spp. in all grapevine cultivars, with a high percentage preceded by *Fusarium* spp.+*M. phaseolina* on flame seedless and superior. The third infection type was only caused by *Lasiodiplodia theobromae* + *Fusarium* spp.+*M. phaseolina* on all grapevine cultivars. Meanwhile, fourth infection types were recorded between four fungi *Fusarium* spp.+ *L. theobromae* + *M. phaseolina* + *R. solani* only on flame-seedless cultivar in this study. These results align with those mentioned by [2,4].

Fungal isolates obtained in this study were identified according to morphological, cultural, and molecular biology tools. Isolates were molecular identification with accession numbers ON037457.1, ON037458.1, ON037459.1, ON037460.1, ON037461.1, ON037462.1, ON037463.1, ON037464.1, ON037465.1, ON037466.1, ON037467.1, ON037468.1, ON037469.1, ON037470.1, ON037471.1, ON037472.1, ON037473.1, and ON037474.1. The results of the molecular identification revealed four groups of isolates. The first group consists of eleven strains, all of which belong to the *Fusarium* genus. The *Fusarium solani* was also discovered to be the biggest, with five species. While *F. chlamydosporum* and *F. brachygibbosum* represent the second place with two numbers each. There are *Fusarium oxysporum* and *F. ipomoeae*, with one type of each of them. In addition, it was discovered that the fungus *Lasiodiplodia* has four species: three of them belong to the *L. theobromae*, the third to *L. crassispora*, and the fourth to *L. exigua*. Finally, it was discovered that the two genera, *Gibberella moniliformis* and *Macrophomina phaseolina*, each have their own taxonomic position. The above results concur with those found by [20,32,44].

The majority of growth indices, such as plant height, root length, the weight of the shoot and root both fresh and dry, as well as the size of the root, were considerably reduced by all fungal isolates linked with symptoms of grapevine root rot in this study when compared with controls of grapevine plants, i.e., *Fusarium oxysporum*, *F. solani, Lasiodiplodia theobromae,* and *M. phaseolina*. The high incidence of root rot, the severity of the disease, and the diminished growth characteristics of grapevine plants were obtained with fungi of *Fusarium solani* (ON037462.1), *F. oxysporum* (ON037473.1), *Lasiodiplodia theobromae* (ON037474.1), and *M. phaseolina* (ON037469.1), which were isolated from El Nobaria province at El Behira Governorate, Egypt. These results are in agreement with [4–6,14]. Furthermore, this investigation first reported of new pathogens that were identified based on morphological and cultural characterizations confirmed by molecular tools of fungal isolates, i.e., *F. chlamydosporum* (ON037458.1 and ON037459.1), *F. brachygibbosum* (ON037464.1 and ON037471.1), and *Lasiodiplodia exigua* (ON037468.1) .

Because there is little knowledge of the ecological and pathological relationships between the causative organisms on grapevine and microbial communities in plant, rhizoplane, rhizosphere, and soil .This investigation has focused on plant pathogens' identification by different tools, study, their infection types by pathogens for understanding the mechanisms for implications, the plant diseases' epidemiology and their management [45,46], and therefore, developmental methodology for detection of microbial and their pathological activities for enhancing management on plant disease incidence for the health of grapevine orchids and good quality as well as high productivity.

Conclusions

In Egypt, root-rot disease on grapevine plants was caused by numerous common and newly first-recorded soilborne fungi. Four single and various synergistic combinations of infection types by fungi associated with grapevine plants were recorded. Fungal isolates were completely identified based on sequencing of the internal-transcribed spacer 1 (ITS1), cultural, morphological, and molecular biology characterizations, and conserved in International GenBank, these fungi are included in a new fungal species, i.e., *F. chlamydosporum*, *F. brachygibbosum, F. ipomoeae,* and *Lasiodiplodia exigua* first recorded in this

study as the causal pathogens of root-rot disease on grapevine in Egypt.

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Ethical statement: The paper has not been submitted to other journals and any part of data has not been published previously.

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