Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers – 12th Special issue on Grapevine Trunk Diseases

Prevalence and pathogenicity of fungi associated with grapevine trunk diseases in Jordan

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Summary. Grapevines (*Vitis vinifera*) are important fruit producers in Jordan, and grapevine trunk diseases (GTDs) are suspected to cause problems in many Jordanian vineyards. This study aimed to estimate GTDs incidence and severity in selected vineyards, and to isolate and identify the causal agents associated with GTDs in this country. Field surveys were carried out and representative samples of diseased vines showing GTDs symptoms were collected to isolate and identify the causal organisms. Molecular analyses of DNA sequences of the Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) were used to confirm the morphological identifications of the fungal isolates. GTDs were present in all the surveyed vineyards. Mean GTD incidence was 44% across all the fields evaluated, ranging from 9 to 69% in individual vineyards. Disease severity ranged from 52–74% (mean = 62%) across all vineyards and locations. A total of 325 fungal isolates were recovered from infected grapevines. The most prevalent pathogens identified were those associated with Botryosphaeria dieback, including *Diplodia seriata*, *Lasiodiplodia theobromae* and *Neofusicoccum parvum*, followed by the Esca disease pathogens *Phaeoacremonium parasiticum*, *P. aleophilum*, *P. rubrigenum*, and *Fomitiporia* spp. *Ilyonectria liriodendri* and *I.* spp., known to be associated with black foot of grapevines, were also isolated. Plant pathogens causing vascular wilts and root rots, including *Fusarium proliferatum*, *F. oxysporum*, *Verticillium* spp., and *Rhizoctonia solani*, were also identified from diseased plant samples, and were found in mixed infections with GTDs pathogens. Most of the identified pathogens, except those associated with vascular wilt and root rot, are reported for the first time in Jordan. Results of this study indicate that GTDs are widespread in Jordan, and that there is urgent need to adopt a "national strategy" for GTD management in this country.

Keywords. GTDs, Botryosphaeria dieback, Esca, black foot.

OPEN ACCESS

Citation: F.M. Abu El Samen, M. Nasrallah, M.A. Alfaqih, K.M. Alananbeh (2023) Prevalence and pathogenicity of fungi associated with grapevine trunk diseases in Jordan. *Phytopathologia Mediterranea* 62(2): 255-268. doi: 10.36253/phyto-13766

Accepted: July 24, 2023

Published: September 15, 2023

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: José R. Úrbez Torres, Agriculture and Agri-Food Canada, Summerland, British Columbia, Canada.

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INTRODUCTION

Grapevine (*Vitis vinifera*. L) is an important fruit crop in Jordan and grape production ranks third among all fruit trees planted. The total area of cultivated grapevines in Jordan is 3,057.4 ha with total annual grape production of

53,886 tons (Department of Statistics, 2019). The AL-Mafraq region, with approx. 40% of the Jordanian production, is the leading grape-producing region, so this region was selected to conduct the present study.

During recent years, symptoms of grapevine decline were frequently observed in most grapevine production areas in Jordan. Several samples from farmers' vines from the Al Mafraq region were also sent to the Phytopathology laboratory at the Jordan University of Science and Technology (JUST), for diagnoses of unknown grapevine diseases that were causing decline and death of young and mature grapevines. Initial diagnoses from these samples suggested that most were infected with fungi known to cause the grapevine trunk diseases (GTD) complex.

GTDs are the most common fungal diseases of grapevines, and cause several symptoms in foliage and vascular tissue of these plants. Fungi causing GTDs primarily infect grapevines through pruning wounds, subsequently colonizing the vascular tissues (Bertsch *et al*., 2013; Mondello *et al*., 2018). GTDs are limiting factors that adversely affect production and longevity of vineyards, causing important economic losses (Agusti-Brisach *et al*., 2013; Gramaje and Dimarco, 2015). Major symptoms of GTDs include dieback, death of cordons or trunks, canker formation in vascular tissues, and necrotic wood (Mugnai *et al*., 1999; Úrbez-Torres *et al*., 2006). Over time, these infections gradually progress and spread within affected vines. When infection occurs, overall vine health begins to slowly deteriorate, making them susceptible to a wide range of other pathogens. Poor early growth and reduced vigour are prominent symptoms associated with these diseases (Trouillas *et al*., 2010; Van Niekerk *et al*., 2011; Fontaine *et al*., 2016). There are six major GTDs, each caused by different fungi, and include the Esca complex, Eutypa dieback, Botryosphaeria dieback, and *Phomopsis* dieback. The black-foot and Petri diseases affect primarily young grapevine (<5 years old) (Glawe and Rogers, 1982; Mostert *et al*., 2005; Van Niekerk *et al*., 2005; Bruez *et al*., 2013; Gramaje *et al*., 2018).

GTDs cause economic losses to grape growers in most regions where grapes are produced. In Jordan, GTDs are becoming serious diseases affecting most vineyards, but etiology of these diseases has not been studied or identified. The identification of the fungi causing GTDs is important for implementing appropriate disease management strategies. The aims of the present study were to examine the extent of GTDs in grapevine growing areas of Jordan, and to identify the associated fungi and test their pathogenicity to grapevine.

MATERIALS AND METHODS

Field surveys, sample collection, and estimation of GTD incidence and severity

Field surveys were conducted in the spring of 2019, 4 to 6 weeks after grapevine bud break. Eleven vineyards were included in this surveys. Farms were selected to include the main grapevine cultivars grown in Jordan, including Black magic, Zeiny, Halawani, and Superior. Other cultivars, including Cabernet Sauvignon, Grenache, Gewurztraminer, Syrah, and Chardonnay used for processing purposes, were also included. At each farm, one or more fields were selected to estimate disease incidence. The number of vines showing typical symptoms of GTDs was counted in different vine rows to estimate the disease incidence, and these assessments covered 15 to 20% the total number of vines in each field. Disease incidence generally indicates prevalence in each area or host plant population. Disease severity for each infected vine was estimated based on the appearance of different known GTD symptoms, and each symptom was given a relative weighting in the overall severity score, as shown in Table 1. Representative samples (approx. 5%) from diseased vines showing GTDs symptoms in each field were collected, to isolate and identify the causal organisms. These samples were labeled, kept in paper bags, placed in ice boxes, and transferred to the plant pathology laboratory at Jordan University of Science and Technology (JUST) for analyses.

Fungal isolations

Tissue pieces (wood tissue, about 5.0 mm^2 diam.) from collected diseased samples were cut from the margins of necrotic wood tissues. Each wood tissue chip was disinfected in 70% ethanol solution for 2 min, soaked in sterile distilled water, then transferred to 1% sodium hypochlorite solution for 2–5 min, rinsed twice in sterile distilled water, and allowed to dry on Whitman no. 2 filter paper for 10 min inside a laminar flow cabinet (Cortesi *et al*., 2000; Larignon and Dubos, 1997). The tissue pieces were then placed into Petri plates containing either potato dextrose agar (PDA) or malt extract agar (MEA). The media in these plates had been amended with ampicillin and streptomycin (each at 50 ppm) after autoclaving to inhibit the growth of saprophytic bacteria (Abreo *et al*., 2013). The plates were then incubated at 25°C in the dark until the fungal mycelium growth was observed from wood sections. Pure cultures were obtained by transferring single fungal hyphal tips from

Table 1. Descriptions of different grapevine trunk disease symptoms, and scores and sub-scores used to calculate disease severity values for each grapevine examined in the present study.

Categories of symptoms	Weight (%) for total severity score, and symptom description	Sub-score (%) for each symptom in the total severity score
Foliar symptoms (20%)		
Slight yellowing	All or part of foliage showing slight yellowing.	7
Moderate yellowing	All or part of foliage showing moderate yellowing and stunting.	10
Severe yellowing	All or part of foliage showing severe yellowing and stunting.	15
Reddening	Foliage showing reddening symptoms	5
Tiger stripes	All or some leaves showing tiger stripes	5
Total defoliation	Most leaves on vines are absent (defoliated), or no leaves emerged after bud break.	20
Cordon symptoms (50%) (in trellis training method)		
One cordon with dieback symptoms or death	25% of the spurs on one cordon are affected	12.5
Two cordons with dieback symptoms or death	26 to 50% of the spurs on two cordons affected by the disease	25
Three cordons with dieback symptoms or death	>50% of the spurs on three cordons were affected. All primary cordons affected by the disease	37.5
Four cordons with dieback symptoms or death	All cordons dead, and buds did not break	50
Cordons symptoms (50%) (for two-cordon training systems)		
A few buds did not break in part of the cordon	25% of the spurs on one cordon are affected.	12.5
One main cordon with dieback symptoms or death	More than 25% but less than 50% of the spurs on the two cordons of the vine are significantly impacted by the disease,	25
Two main cordons with dieback symptoms or death	More than 50% of the spurs on two cordons affected. All primary cordons affected by the disease.	37.5
All main cordons with dieback symptoms or death	All cordons dead, and buds did not break	50
Crack Symptoms in the Main trunk (15%)		
No trunk cracks	No cracks present in the main vine trunk	0
Trunk with slight to moderate cracks	Some wood cracking extends downward and upward along the trunk	10
Trunk with severe cracks	Severe wood cracking extends downward and upward along the trunk	15
Cross-section discolouration inside trunk (15%)		
No discolouration	No vascular tissue showing dark discolouration	0
Partial discolouration	Some vascular tissues dark brown, black vascular streaking when the trunk is cut	10
Total discolouration	Vascular tissues with dark discolouration and dead woody tissue	15

each colony to individual plates of MEA.

Morphological identification of fungi

Isolates having colony morphology representative of pathogenic species were examined microscopically. Different techniques were employed to identify the isolated microorganisms including direct examination of Petri plates with a stereo microscope and a compound microscope (Olympus Corp.). Initial identification of isolates to the genus level was determined using appropriate mycological identification keys. Culture features, including colony texture, colony colour, conidium morphology (shape, colour, presence or absence of septa), conidiophore morphology, and conidiation characteristics were recorded. General and specific mycological identification keys were used to determine the identity of each fungal culture. For *Botryosphaeriaceae* spp. the identification keys of Crous *et al*. (2006) and Phillips *et al*. (2006) were used. For *Phaeoacremonium* spp., keys of Mostert *et al*. (2006) and Essakhi *et al*. (2008) were used. Isolated and identified fungi were recorded, and each isolate was stored at 4°C in long-term storage for further identification using molecular techniques and for testing of pathogenicity on grapevine seedlings.

Molecular identification of fungal isolates

DNA extraction, PCR amplification and sequencing of amplification products

Fungal isolates were cultured on MEA for 2 weeks for DNA extraction. The DNeasy Plant Mini Kit (CAS NO. 69104; Qiagen group) was used to extract DNA from mycelium of different fungal isolates, following the manufacturer's instructions. The DNA concentration in each sample was estimated using NanoDrop 2000 (Thermo Scientific) from a 2 µL DNA sample. DNA from fungal isolates were used to amplify the Internal Transcribed Spacers (ITS region) by the primers ITS1 and ITS4 (White *et al*., 1990). PCR reactions were each carried out in a total final volume of 25 µL, using FIREPOL® Master mix ready to load 5x (Solis BioDyne), containing 3 ng of DNA template and 1µM of each of forward and reverse primers. The PCR was conducted using T100™ Thermal cycler (Biorad) at the following parameters: 95°C for 3 min, then 34 cycles each of 95°C for 30 sec, 60°C for 1 min, and 72°C for 1 minute, followed by 72°C for 5 min and 4°C. PCR reaction products were stored at -20°C before being electrophoresed on a 2% agarose gel with a Tris-Borate-EDTA (TBE) buffer. Amplification products were visualized using an ultraviolet light box.

The identification of each fungal isolate was confirmed by sequencing of the PCR products of approx. 300-750 bp (depending on the genus) by Genetics Company. Sequences were uploaded to SnapGene v5.0 software and to the GenBank database (http://www.ncbi.nlm.nih.gov). The BLAST tool was used to compare these sequences with published sequences of previously identified fungi in the database (Kaliterna *et al*., 2012).

Pathogenicity tests of isolated fungi

The pathogenicity of 88 fungal isolates was confirmed by inoculating each isolate onto 1-year-old self-rooted grapevine canes (two canes per isolate), and recoding the observed disease symptoms. Self-rooted canes from different grapevine cultivars (Zeiny, Halawani, Baladi, or Cabernet Sauvignon) were inoculated with the fungi identified in this study. Each isolate was grown on PDA or MEA plates for 2 weeks at 25C in the dark. Each cane was wounded using a 5 mm cork borer, and the wound was then inoculated with a 5 mm diam. isolate colonized PDA plug from a 2-week-old culture. Control treatments were inoculated with non-colonized PDA plugs. Each inoculation point was covered with Parafilm to prevent inoculum desiccation. The grapevine canes were then covered with plastic bags (to maintain humidity) and were placed in a growth chamber at 25°C, 90% relative humidity and a 12 h light 12 h dark cycle for 3 weeks. The plants were then moved to a greenhouse bench and were monitored for disease development.

A final pathogenicity assessment was made 1 to 3 months' post-inoculation. Development of GTD symptoms on plant foliage and stems were monitored and recorded twice each week, and the final assessment was carried out by removing the bark of each cane and observing wood tissue discolouration and necrosis. All the inoculated canes were subjected to re-isolation and identification of the inoculated pathogens, to determine fulfilment of Koch's postulates.

Assessments of disease development in inoculated self-rooted grapevine canes

To assess the extent of disease development within woody tissues of grapevine self-rooted canes, 200 inoculated canes from previous pathogenicity tests were evaluated. The aim of this assessment was to determine the development of necrotic wood tissue and discolouration during 3 months period after inoculation. Inoculated canes showing typical GTD symptoms (yellowing, stunting, leaf tiger stripes, stem cracking) were selected and cut above the soil surface line. Three sections, each approx. 10 mm thick, were taken from each inoculated self-rooted cane, at 1 cm intervals above and below the inoculation point (i.e., a total of six sections). The sections were then photographed using a dissecting microscope equipped with a digital camera.

Digital images of the wood sections were analyzed using the Image J program, provided by the National Institutes of Health (NIH) (Schneider *et al*., 2012). The image analysis software measured different levels of section darkening, which were converted to disease severity values on a scale from 0 to 100% relative to the total area of each section (Table 4). Colour of the wood tissue was assessed by measuring the reflective light intensity of each section. The change in light intensity was then used to calculate the disease severity relative to the light intensity of healthy sections, as reported by Niemira *et al*. (1999). The resulting values were reported in pixels, and included the total area of each section, total darkened area, and mean darkened area. These values were used to calculate disease severity in the grapevine sections using the following formula:

Disease severity relative to area of healthy section = (Total area of section darkened tissue / Total area of section) × 100.

RESULTS

Field surveys, sample collection, and GTD incidence and severity

GTD symptoms were observed and recorded in all the surveyed vineyards. Symptoms included leaf chlorosis, stunting, wedge-shaped cankers in trunks and cordons, discolouration and browning of the vascular tissues in the main trunks and cordons, and trunk cracks (Figures 1 and 2). The main Esca-complex symptoms were observed in most fields and included: general decline, reduced foliage and leaf size, tiger stripe symptoms on leaves (chlorosis and necrosis), dark brown to black streaking in trunk cross-sections, pathogen signs including black pycnidia in vascular tissues.

Wedge-shaped cankers and discolouration of wood tissues were the most common vascular symptoms observed, and these were associated with foliar symptoms, including lack of spring growth in the spur positions. Browning and black streaking of wood tissues were the second most common vascular wood discolouration symptoms (Figure 2), followed by light brown discolouration of wood and central stem necrosis. Black streaking of wood tissues was often found in vines showing characteristic Esca symptoms and in samples collected from vines showing general dieback with no characteristic foliar symptoms.

A total of 38,600 vines in the 11 fields were surveyed in this study. Among these, 7558 vines (approx. 20%) were evaluated for the presence of GTDs symptoms. The mean overall GTD disease incidence (11 fields), based on visual observation of symptoms, was 43.6%. Mean GTD incidence ranged from 9.5% to 68.8% in the 11 fields (Table 2).

The greatest mean GTD incidence (68.8%) recorded at Sama Al-Sarhan, in the grapevine cultivar Grenache, followed by Zamlat Al-Amir Ghazi, North Badia location (64.9%) in Chardonnay, and at Thagrat Al-Gubb (62.5%) in Zeiny. Incidence at the other locations and fields was generally less than 60% (Table 2). GTD severity ranged from 52 to 74% (overall mean = 62.3%) across all fields and locations (Table 2). The greatest mean GTD severity was 74% for Chardonnay at Zamlat Al-Amir Ghazi, North Badia, and least severity (52.5%) was in the cultivar Zeiny at Thagrat Al- Gubb (Table 2).

Morphological identification of fungal isolates

Morphological identification of fungal isolates was carried out using several steps. Fungal isolates were divided into groups. The first group had dark green fast-growing mycelium on PDA and MEA. The cultures developed single or clustered black, globose pycnidia from which two types of conidia (pigmented or hyaline) were identified. These characteristics were consistent with descriptions of species of *Botryosphaeriaceae* (Phillips, 2002; Úrbez-Torres *et al*., 2006). Fungal cultures with pigmented conidia were preliminarily divided into two categories, *Lasiodiplodia* spp., and *Diplodia* spp. Cultures with hyaline conidia were similarly identified as *Neofusicoccum* spp., (Table 3) (Crous *et al*., 2006; Phillips *et al*., 2006; Alves *et al*., 2008).

The second group of isolates developed mycelial bundles, simple or branched conidiophores, slender phialides in three size types and bearing narrow funnel-shaped collarettes, and conidia aggregated in slimy heads and that were oblong-ellipsoidal to allantoid in shape on PDA and MEA. These isolates were typical of the *Phaeoacremonium* spp. Some cultures had white colour and cottony texture, producing dense aerial hyphae after 2 to 3 d incubation and developing white to yellowish mycelia after 10 d, and later becoming yellow or brown. These isolates were typical of the *Fomitiporia* spp. (Table 3) (Mostert *et al*., 2006; Essakhi *et al*., 2008).

Identification of fungal isolates from samples collected from different grapevine cultivars and details of isolates associated with different GTD symptoms are presented in Table 3.

Vascular wilt pathogens, including *Verticillium* spp., and *Fusarium* spp. (Table 3), known to cause wilt and decline of fruit tree crops, were also isolated. Other fungi unrelated to GTDs were also identified, including *Alternaria* spp., *Rhizoctonia* spp., *Penicillium* spp., and *Aspergillus* spp. Many other cultures were difficult to identify based on morphological characteristics, and were kept for further identification using molecular methods.

Molecular identification of fungal isolates

Among the 325 isolates obtained from all the sampled plants, 216 were classified as fungi associated with GTDs, 139 (42.8%) were associated with Botryosphaeria dieback [*Diplodia seriata* (51 isolates); *Lasiodiplodia theobromae* (42 isolates); and *Neofusicoccum* spp. (46 isolates)], 47 isolates (14.5%) were associated with the Esca disease complex [*Phaeoacremonium* spp. (41 isolates); *Ilyonectria liriodendri* (four isolates); *Fomitiporia* spp. (two isolates). Seventy-nine isolates associated with vascular wilt and root rot pathogens were obtained, including *Fusarium* spp. (19.7%), *Verticillium* spp. (1.5%) and the root rot pathogen *Rhizoctonia* sp. (1.2%). The other

Figure 1. Foliar Symptoms of grapevine trunk diseases observed in mature vines in Jordan including, stunted shoots with chlorotic leaves and necrotic margins, stunted shoots, tiger-stripe symptoms on leaves of a red cultivar and apoplectic symptom which is a sudden wilting of the plant or one arm or several shoots.

Figure 2. Vascular symptoms of grapevine trunk diseases in cross sections cut from symptomatic vines. The different pattern of dark brown to black discoloration.

49 fungal isolates (15.1%) included many saprophytic fungi (e.g. *Aspergillus niger*, *Penicillium* sp.), endophytes, or potential biological control agents.

Pathogenicity tests of isolated fungi

Results of pathogenicity tests for selected isolates (14 isolates of *Phaeoacremonium* spp., eight of *Diplodia seriata*, 58 of *Fusarium* spp; six of *Lasiodiplodia theobromae*, nine of *Neofusicoccum parvum*, and five isolates of *Rhizoctonia solani*) (Figure 3) showed that all the tested isolates were pathogenic on self-rooted grapevine canes. These inoculations produced symptoms typical of GTDs. These symptoms varied depending on isolate and grapevine cultivar source, and include, chloroses, leaf yellowing, tiger stripes, and discolouration (purple, light tan, dark tan, brown), stunting, stem cracking, and different patterns of vascular tissue discolouration in stem cross sections (Figure 3). None of the control plants inoculated with plugs of non-colonized PDA showed any symptoms, and these plants continued to grow normally until the end of the experiment (Figure 3).

Disease development in inoculated grapevine canes

Analyses of cross sections cut from each inoculated cane showed that thickness of the cross sections did not affect light intensity measurements using image analysis software (Figure 3). Therefore, a section thickness of 10 mm was used for the analysis of discolouration caused by the different fungal isolates (Table 4). The mean disease severity values of stem cross sections varied among the different fungal isolates used in this experiment, ranging from 27.9 to 100% (Table 4).

The greatest mean disease severities occurred in canes inoculated with *L. theobromae*, *D. seriata*, *P. parasiticum*, *P. rubrigenum*, *I. liriodendra*, *N. parvum*, or two isolates of *F. oxysporum*. Disease severity values for these fungi were either 100% or close to 100%, indicating complete discolouration of stem sections above and

Table 2. Disease incidence (DI) and mean disease severity of grapevine trunk diseases (GTDs) in different locations and vineyards surveyed in Al Mafraq, Jordan.

Field number	Vineyard location	Cultivar	Total No. of vines in the vineyard	No. of observed vines	No. of symptomatic vines	Mean incidence (DI %)	Mean severity (%)
1.	Sama Sirhan	Gewurztraminer	10,000	3000	284	9.5	66.0
2.	Sama Sirhan	Grenache	5000	800	550	68.8	57.0
3.	Ad-Dafyanah	Superior seedless	2450	700	224	32.0	67.0
4.	Sabha	black magic	800	250	94	37.6	60.0
5.	Sabha	Zeiny	4300	572	165	28.9	59.2
6.	Zamlat Al-Amir Ghazi	Cabernet Sauvignon	4600	550	138	25.1	70.0
7.	Zamlat Al-Amir Ghazi	Syrah	2500	490	238	48.6	63.0
8.	Zamlat Al-Amir Ghazi	Chardonnay	3000	330	214	64.9	74.0
9.	Thagrat Al- Gubb	Zeiny	2000	160	100	62.5	52.0
10.	Umm Al-Jimal	Zeiny	1450	216	114	52.8	62.0
11.	Umm Al-Jimal	Zeiny	2500	490	243	49.6	59.0
Total			38,600	7558	2364		
Overall mean						43.6	62.3

Table 3. Fungi associated with different grapevine trunk diseases symptoms, and numbers of plant samples from which each fungus was isolated.

Fungus	Grapevine vascular symptoms [a]							
	Wedge-shape canker [b]	Black wood streaking	Yellowish soft wood	Brown discolouration	Central necrosis	Black spots	Total	Proportion (%) of total isolates
Diplodia seriata	23[b]	17			11		51	15.7
Lasiodiplodia theobromae	14	11		10	7		42	12.9
Neofusicoccum parvum	16	8		13	9		46	14.2
Fusarium oxysporum	18		9		20		47	14.5
Fusarium proliferatum					15		15	4.6
Fusarium perseae				2			2	0.6
Phaeoacremonium spp.		19	11			10	41	12.6
Ilyonectria liriodendra						4	4	1.2
Fomitiporia spp.			2				2	0.6
Verticillium spp.				5			5	1.5
Rhizoctonia spp.				10			10	3.07
Simplicillium obclavatum			1				1	0.31
Nectria spp.		7				3	10	3.1
Others							49	15.1
Total							325	100

[a] Description of the typical GTD symptom observed in vineyards.
[b] Number of samples that had typical GTD symptoms.

below the inoculation points (Figure 3). The least stem cross section disease severity values were in plants inoculated with *F. oxysporum*, *F. perseae*, *F. proliferatum*, or *R. solani*, with disease severity values less than 50% (Table 4).

DISCUSSION

This study was the first survey of grapevines affected by GTDs in Jordan, carried out to determine the prevalence of these diseases in vineyards, and to iden-

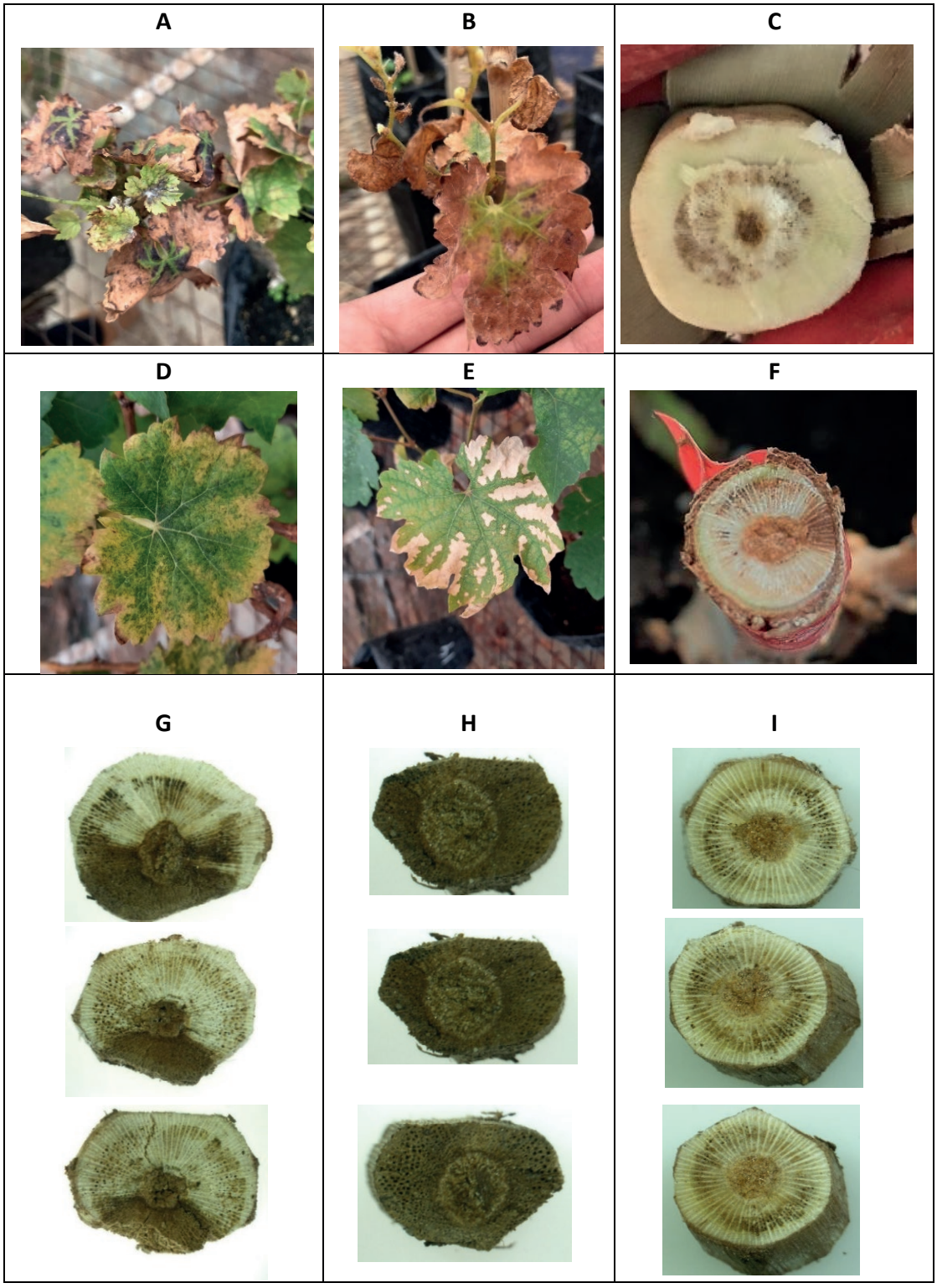

Figure 3. Grapevine self-rooted canes inoculated with GTDs pathogens. Panels **A**, **B**, and **C** display foliar and wood symptoms of grapevine canes after inoculation with *Phaeoacremonium parasiticum* isolate. Panels **D**, **E**, and **F** show foliar and wood symptoms of grapevine canes after inoculation with *Diplodia seriata* isolate. Panels **G**, **H**, and **I** present cross sections cut at 1.0 - 3.0 cm (top to bottom) below the inoculation point from grapevine canes inoculated with different fungal isolates. Panel **G** represents canes inoculated with *Fusarium oxysporum* isolate, panel **H** with *Lasiodiplodia theobromae* isolate, and panel **I** with *Fusarium proliferatum* isolate.

Table 4. Mean disease severities (MDS) of grapevine trunk diseases caused by isolates of different fungi, estimated using digital image analyses of cross sections from inoculated grapevine self-rooted canes. The sections were cut from locations, 1, 2 or 3 cm above or below each inoculation point.

Fungus isolate	MDS [b] (%) above inoculation point			MDS (%) below inoculation point			MDS % (all sections)
	3 cm	2 cm	1 cm	1 cm	2 cm	3 cm	
Lasiodiplodia theobromae (n = 4)[a]	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Diplodia seriata (n = 2)	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Phaeoacremonium parasiticum (n = 5)	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Phaeoacremonium rubrigenum (n = 1)	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Ilyonectria liriodendra (n = 1)	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Neofusicoccum parvum (n = 2)	97.6	98.4	100.0	97.5	96.6	96.6	97.8
Fomitopsis spp. (n = 1)	67.5	70.9	74.8	72.2	70.3	69.8	70.9
Fusarium oxysporum (n = 40)	40.3	42.7	45.0	40.3	37.1	35.6	40.2
Fusarium proliferatum (n = 15)	38.8	41.2	43.3	38.8	36.5	33.6	38.7
Rhizoctonia solani (n = 8)	35.4	37.3	40.1	34.9	32.0	30.3	35.0
Valsa sordida (n = 2)	29.8	31.9	34.0	29.9	28.7	27.5	30.3
Fusarium perseae (n = 2)	28. 0	30.3	32.8	27.0	25.3	23.8	27.9

[a] Number of isolates of each fungus included in the pathogenicity tests.
[b] Mean disease severities (DS) were calculated using the following formula: Disease severity relative to the healthy sections (%) = (total area of darkened tissue in a section / total area of the section) × 100.

tify the fungal pathogens present in affected plants. This one-year survey found that average GTD incidence was 44%, observed and recorded in eleven commercial vineyards in the Al Mafraq region, the main grape production area in Jordan. Plants were visually assessed, based on symptoms that are internationally associated with GTDs. Overall mean disease severity of 63% was estimated, based on visual observation of foliar symptoms and wood tissue discolouration in the grapevine trunks and affected cordons. The disease severity scale used in this field survey was detailed, and considered all previously described symptoms of all GTDs. Symptoms were divided into major and minor categories (including foliar, cordon, and main trunk symptoms, and vascular tissue discolourations). Each category was given a specific weight from the total severity value, which reflected progression of GTDs in affected vines. The purpose of this detailed scale was to give accurate estimations of overall disease severity in each assessed vine.

Results of the field survey demonstrated that GTD incidence and severity varied among different cultivars in the different vineyards. Average disease incidences were 74% for the cultivar 'Chardonnay' and 70% for 'Cabernet Sauvignon'. On these cultivars, A 2-year survey of vineyards of these cultivars in central Italy by Quaglia *et al*. (2009) showed that GTD incidence on 'Cabernet Sauvignon' was greater than 50%, and was 15% for 'Chardonnay'. Marchi *et al*. (2006) reported 10 to 25% incidence of GTDs in 'Cabernet Sauvignon' Italian vineyards, depending on the region and age of the vineyards. Lesser incidences than those recorded in the present study were reported by Andreini *et al*. (2014), where incidence of symptoms in vineyards in Tuscany (Italy) during 6 years averaged 45% for 'Cabernet Sauvignon' and 8% for 'Chardonnay'. These studies were made for emerging GTD situations in the surveyed regions, so the reported incidence levels probably did not reflect the situation throughout Italy.

In the present study, mean GTD incidences of 63% in 'Syrah' grapevines and 66% in 'Gewurztraminer' were recorded. These values are similar to those recorded by Chacón-Vozmediano *et al*., (2021), who reported these two grape cultivars among those with the most severe GTD symptom and with the greatest numbers of infected plants. The local grapevine cultivar "Zeiny" had an incidence of 58%, which was less than most of the imported grapevine cultivars surveyed in the present study. In this survey, most 'Baladi' grapevine fields were recently established in Mafraq (less than 5 years old), and disease symptoms were not common in most fields visited. Therefore, and although only a few samples from this cultivar indicated presence of GTD pathogens, further surveys are required to assess these diseases in this important cultivar. Furthermore, Pathogenicity tests conducted with this cultivar using fungal isolates identified in the present study as GTD causal agents demonstrated high susceptibility to most isolates tested. Cultivar susceptibility based on visual assessments of external symp-

toms, mainly foliar symptoms associated with GTDs, has the limitation that the causative pathogens often occur in mixed infections with those causing other diseases such as vascular wilts and root rots. Fungi causing vascular wilts and root rots, including *F. proliferatum*, *F. oxysporum, F. perseae, Verticillium* spp.*,* and *R. solani*, were frequently isolated and identified in diseased plant samples showing GTD symptoms. These pathogens were found in mixed infections with other GTD pathogens, and they made up approx. 25% of the identified fungal pathogens.

Among different fungal pathogens identified in this study, several genera are known to cause GTDs, including *Diplodia* spp., *Lasiodiplodia* spp., *Neofusicoccum* spp., *Phaeoacremonium* spp., *Ilyonectria* spp., and *Fomitiporia* spp. (Table 3).

The most prevalent fungal pathogens identified in the present study are those associated with *Botryosphaeriaceae* dieback, including *D. seriata*, *L. theobromae*, and *N. parvum*. These pathogens made up 43% of the total isolates identified in this study. *Diplodia seriata*, *L. theobromae,* and *N. parvum* have been reported as main causal agents of *Botryosphaeriaceae* dieback of grapevines in many countries, including Tunisia (Chebil *et al*., 2014), Iran (Mohammadi *et al*., 2013a), Turkey (Akgul *et al*., 2014), Spain (Elena, and Luque, 2016), China (Yan *et al*., 2013), France, Italy, Portugal, Egypt, India, Mexico, Chile, and Brazil (Úrbez-Torres, 2011, Alves *et al*., 2008).

In the present study, different *Botryosphaeriaceae* species were isolated from grapevines showing similar vascular symptoms but diverse foliar symptoms. Additionally, these fungi were isolated along with other pathogens from the same or different symptoms in individual vines. This observation has been documented by other researchers working with GTDs (Úrbez-Torres *et al*., 2006; Luque *et al*., 2009; Úrbez- Torres, 2011). Consequently, diagnoses of grapevine diseases caused by *Botryosphaeriaceae* can be difficult based only on observations of vascular and external symptoms, so accurate diagnosis utilizing isolations and/or molecular techniques are required to confirm the presence of these species.

The second most prevalent fungal pathogens isolated from grapevines in this study were fungi known to cause the Esca disease complex, including *Phaeoacremonium* spp., which made up approx. 13% of all isolates identified. Three species of *Phaeoacremonium* were identified, morphologically and using molecular methods, and these were *P. parasiticum, P. aleophilum, and P. rubrigenum*. Both *P. parasiticum* and *P. aleophilum* have been frequently reported as causal agents of Esca in mature grapevines (Bertelli *et al*., 1998; Scheck *et al*., 1998; Mugnai *et al*., 1999; Aroca and Raposo, 2009; Halleen *et al*., 2007), while *P. rubrigenum* has been recently reported from Croatia as a cause of Esca (Essakhi *et al*., 2008). In the present study, *P. parasiticum, P. rubrigenum, and P. aleophilum* were almost exclusively isolated from dark streaked or soft-yellowish wood. This observation is consistent with previous reports of association of these pathogens with discoloured softwood vascular tissue (Mugnai *et al.,* 1999; Úrbez-Torres *et al.,* 2006; Luque *et al.,* 2009; Úrbez-Torres *et al.,* 2009; Trouillas *et al*.2010). *Fomitiporia* spp., which was also identified in the present study associated with Esca, has been reported by many authors as a cause of Esca in association with *Phaeoacremonium* spp. (Fischer *et al.,* 2005; Cobos and Martin 2007; Luque *et al.,* 2009; Mohammadi *et al* 2013b; Cloete *et al.,* 2015).

Ilyonectria liriodendri was identified in this study as one of the GTD pathogens in Jordan. This fungus was reported as a causal agent of black foot of grapevines in California (Petit and Gubler 2007), Australia (Whitelaw-Weckert *et al*., 2007) Spain (Alaniz *et al*., 2009), Iran (Mohammadi *et al.,* 2013a), and Uruguay (Abreo *et al*., 2010). Similarly, the *Ilyonectria* sp., which was isolated at low frequency in the present study, was also previously reported from grapevines affected by black foot in British Columbia (Urbez-Torres *et al*., 2014), China (Parkinson *et al*., 2017), Uruguay (Abreo *et al*., 2010), and Turkey (Savas *et al*., 2015).

Relationships were observed between grapevine vascular symptoms and particular fungal infections. Species of *Botryosphaeriaceae* (*D. seriata, L. theobromae, N. parvum*) were mostly isolated from wedge-shaped cankers, while *P. parasiticum, P. aleophilum,* and *P. rubrigenum,* were almost exclusively isolated from dark streaked and soft-yellowish wood. These observations agree with those in previous studies, where these fungi were elsewhere isolated from the same types of vascular symptoms (Mugnai *et al.,* 1999; Úrbez-Torres *et al.,* 2006; Luque *et al.,* 2009, Úrbez-Torres *et al.,* 2009; Trouillas *et al*., 2010). Some samples collected in the present study were hypothesized to have *Eutypa* infections, because they showed symptoms of Eutypa dieback, mainly wedge-shaped wood cankers. However, *Eutypa lata* was not isolated from collected samples which had wood discolouration symptoms resembling those caused by *Eutypa lata*.

Results of the pathogenicity assessments of different isolates on different grapevine cultivars did not provide insights into cultivar susceptibility, since no significant differences in disease development or final assessments of stem cross-sections were observed among the different cultivars examined. More research is required, probably using different methodology for inoculum delivery, to accurately assess susceptibility in host cultivars to different fungal GTD pathogens.

Most of the fungal pathogens identified in this study, except for those causing vascular wilt and root rots, are reported here from Jordan for the first time. It is likely that these pathogens have recently been introduced into this country on infected transplants or on rootstocks imported from different countries, especially from Europe. This hypothesis is based on questions directed to vineyard owners about the sources of their transplants, which showed that they imported wine grapevine and some table grape cultivars from different European countries during the last 10 years. This hypothesis conclusion is supported by results from the present study, which have demonstrated the presence of many GTD pathogens previously reported from European countries.

Jordanian grape producers in the Al Mafraq area and other locations are currently experiencing severe losses due to GTDs. Currently, there are no estimates of these losses, but future studies should investigate their extent and magnitude. Results of the present study suggest that GTDs are widespread in Jordan. Most grapevine nurseries are located in Al Mafraq region, and these enterprises distribute transplants to most grape growing areas in Jordan.

Effective management of GTDs is difficult, and there are no single and simple control methods for grape trunk diseases (Mondello *et al*., 2018). The present results confirm the urgency for adopting a "national strategy" for the management of GTDs that is applied in all Jordanian grapevine production areas. Strategies using different methods can be applied by nurserymen and grape growers to limit the economic impacts of GTDs in Jordan.

ACKNOWLEDGMENTS

The authors thank Agr. Eng. Botros Al-Kawalit and Agri. Eng. Yousef Al-Kawalit for their invaluable assistance carrying out the field surveys of this study. The authors also extend appreciation to the Deanship of Scientific Research at Jordan University of Science and Technology for supporting this research through grant no. 41/2022.

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