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Research Papers

Fungal pathogens associated with harvested table grapes in Lebanon, and characterization of the mycotoxigenic genera

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Summary. Table grapes are exposed to fungal infections before and after harvest. In particular, *Aspergillus*, *Penicillium*, and *Alternaria* can cause decays and contamination by mycotoxins. The main fungi affecting Lebanese table grapes after harvest were assessed as epiphytic populations, latent infections, and rots. Effects of storage with and without SO_2 generating pads were also evaluated. Representative isolates of toxigenic genera were characterised, and their genetic potential to produce ochratoxin A, fumonisins, and patulin was established. The epiphytic populations mainly included wound pathogens (*Aspergillus* spp. and *Penicillium* spp.), while latent infections and rots were mostly caused by *Botrytis* spp. The use of SO_2 generating pads reduced the epiphytic populations and rots, but was less effective against latent infections. Characterization of *Aspergillus*, *Penicillium,* and *Alternaria* isolates showed that *A. tubingensis*, *P. glabrum,* and *A. alternata* were the most common species. Strains of *A. welwitschiae* and *P. expansum* were also found to be genetically able to produce, respectively, ochratoxin A plus fumonisins and patulin. These data demonstrate the need for effective measures to prevent postharvest losses caused by toxigenic fungi.

Keywords. Postharvest, *Aspergillus*, *Penicillium*, *Alternaria*, mycotoxins, sulphur dioxide.

INTRODUCTION

Table grapes are among the most important fruit crops in Lebanon due to the favourable Mediterranean climatic conditions and long cultivation tradition. Table grapes are grown on 7,030 ha, with annual production of 62,014 t (FAOSTAT, 2019). Plantations for commercial production have long been made with the local cultivars Tfeifihi, Beitamouni, Maghdouchi, and Obeidi.

Recently, however, commercial cultivars have been introduced from Europe and the United States (Chalak *et al.*, 2016). Thirty-eight packinghouses and storage facilities exist in Lebanon, of which 22 process table grapes (Lebanese Chamber of Commerce, 2019). Most of these facilities are in the Bekaa region in Eastern Lebanon, where most table grape production occurs. The most common pre-cooling method, present in almost all these facilities, is the room cooling, where pre-cooling and storage are carried out in the same room at appropriate temperatures.

Grapes are susceptible to many fungal diseases in the field and during storage. Postharvest diseases, in favourable conditions and particularly in developing countries, can cause losses in total production of up to 55% (Sanzani *et al.*, 2016a). The most destructive postharvest disease of table grapes is grey mould caused by *Botrytis* spp. Infections often take place in the field, and the fungi remain latent until ripening (Sanzani *et al.*, 2012). Other diseases can occur during storage, including blue mould caused by *Penicillium* spp., some of which can grow even in refrigerated conditions (Sanzani *et al.*, 2013). The predominant *Penicillium* species isolated from grapes differ between vineyards and years, but the most common ones are *P. brevicompactum*, *P. citrinum*, *P. glabrum,* and *P. expansum* (Rousseaux *et al.*, 2014). Other diseases are more recurrent in presence of warm temperatures (*e.g.*, during transportation and marketing). These include rots caused by *Aspergillus* spp. (Droby and Lichter, 2007); the section *Nigri* is the most common on grapes, including the species *A. carbonarius*, *A. niger*, *A. tubingensis,* and *A. welwitschiae* (Perrone *et al.*, 2007). *Alternaria* spp. have also been reported as responsible for pre- and postharvest losses of agricultural commodities, including grapes (Lorenzini and Zapparoli, 2014; Garganese *et al.*, 2016), with *A. alternata* as most recurrent species (Stocco *et al.*, 2019).

These fungi have additional important detrimental roles, being able to produce mycotoxins. Mycotoxin production is highly influenced by climatic conditions (*e.g.*, temperature and humidity), berry characteristics (*e.g.*, pH and available water), fungicide applications, and harvesting and storage conditions (Covarelli *et al.*, 2012). The main mycotoxin produced by *Aspergillus* spp. on grapes and consequently grape-derived products is ochratoxin A (OTA), which is primarily produced by *A. carbonarius*, although *A. niger* and *A. welwitschiae* also contribute to contamination (de Souza Ferranti *et al.*, 2018). *Aspergillus* spp., including *A. niger* and *A. welwitschiae*, are also able to produce fumonisins (mostly FB_2) (Samson *et al.*, 2007; Hong *et al.*, 2013). OTA is immunotoxic, nephrotoxic and possibly carcinogenic (IARC, 1993), so that the European Commission set its maximum limits in foodstuffs deriving from grapes (EC, 2006). Fumonisins, although reported to be possibly carcinogenic (IARC, 1993), are regulated in the EU only on cereals and derived products (EC, 2007). The main mycotoxin produced by *Penicillium* spp. (particularly *P. expansum*) on fruits is patulin, which has been mainly reported on pome fruits, but is associated with several other crops including grapes (Sanzani *et al.*, 2013, 2016b). Patulin content is regulated in apple-derived products in the EU (EC, 2006); however, no official regulatory limits exist for grapes and derived products. Not less important is contamination by *Alternaria* and its related toxins, which have been reported on several commodities, including grapes, both in the field and during storage (Sanzani *et al.*, 2016a). The most relevant of these mycotoxins are tenuazonic acid (TeA), alternariol (AOH) and alternariol monomethyl ether (AME) (Wenderoth *et al.*, 2019). Although *Alternaria* toxins can cause adverse effects in human and animal systems (Schuchardt *et al.*, 2014), no regulatory limits exist for these compounds. Nevertheless, EFSA (2011) published a scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food.

Among the different practices for the control of postharvest rots during storage, given the lack or limited use of conventional fungicides, SO_2 generating pads in packages remain an important tool due to their practicality, efficiency, low cost, and low health risks compared to fungicides (Franck *et al.*, 2005).

The table grape production chain in Lebanon encounters many of these phytosanitary problems. Most important are postharvest rots, which result from poor practices in the field, at harvesting, and during storage. The aims of the present study were: i) to assess the fungal populations associated with table grapes in Lebanese packinghouses; ii) to determine the effects of storage conditions on the fungal populations; iii) to characterize at the species level the populations of the most represented mycotoxigenic genera; and iv) to molecularly characterize the putative ability of fungi to produce relevant mycotoxins.

MATERIALS AND METHODS

Assessments of the fungal population on table grape bunches by the end of storage

Sampling

Table grapes samples were collected from five packinghouses located in Central Bekaa district (Bekaa

region, Lebanon), which apply room cooling systems, during February 2020. The grapes had been stored for three months at 1±2°C, 95% relative humidity (RH), and with SO_2 generating pads. In each facility, the two most abundant cultivars were selected for sampling. For each cultivar, a sample of 15 bunches was randomly taken from at least five boxes, the bunches were transported in refrigerated containers to the laboratory, and were processed within a maximum of 12 h.

Assessment of epiphytic populations

From each sample, three replicates of 20 berries each were randomly taken and placed separately in a sterile plastic bag containing 200 mL of 0.05% Tween 20 (Sigma Aldrich). Replicates were shaken for 30 min on an orbital shaker at 150 rpm. For each replicate, aliquots of 200 μL were then plated on three Petri plates (90 mm diam.) containing Dichloran Glycerol 18% Agar (DG18, Deben Diagnostics Ltd), and these were incubated for 3-5 d in the dark at 24±1°C (Aşkun *et al*., 2007). Fungal colonies were then counted, and the associated epiphytic population was expressed as Colony Forming Units per gram of fresh berry weight (CFU g^{-1} fbw).

Assessment of latent infections by fungal pathogens

For each sample, three replicates of 20 berries each were surface decontaminated by soaking in 2% NaOCl solution for 2 min, rinsed with sterile distilled water for 2 min, and then dried under a laminar flow hood. Each replicate was aseptically placed in a sterile plastic bag and kept at -20±1°C for 2 h to facilitate the collapse of the berry tissues (Sanzani *et al*., 2012). The bags were then placed in an incubator at 24±1°C in the dark for a maximum of 7 d. Berries showing signs of fungal infections were counted and the incidence of latent infections expressed as percentage (%) of symptomatic berries. The recovered fungal colonies were identified based on the morphological characteristics (Barnett and Hunter, 1999), and the frequency of each genus was calculated as percentage of the total recovered colonies.

Assessment of rots

In each packinghouse and for each cultivar, five boxes were inspected at the end of storage. Each bunch was visually checked for the presence of rot symptoms. Disease severity was assessed using an empirical scale of eight classes: 0 = sound cluster; 1 = one-two infected berries; 2 = three-five infected berries; 3 = six-ten infected berries; 4 = less than 25% infected cluster; 5 = 26–50% infected cluster; 6 = 51–75% infected cluster; and 7 = more than 76% infected cluster. The average severity scale was then calculated and the disease severity index (DSI) was determined using the following equation: DSI (%) = [sum (class frequency × score of rating class)] [(total number of clusters) × (maximal disease index)]$^{-1}$ × 100 (Chiang *et al*., 2017).

Effects of storage conditions on the fungal populations

To study the effects of storage conditions on the fungal populations, particularly the use of SO_2 generating pads, samples of 15 bunches of 'Red Globe' and 'Crimson' grapes were collected from one packinghouse in Ferzoul (Packinghouse 2) after packing and just before pre-cooling (T_0) and at the end of the storage period (T_f). Sampling was made from packages in which an SO_2 pad was present (+SO_2) and from packages that underwent the same storage conditions but without SO_2 pads (-SO_2). Epiphytic fungal populations, latent infections, and rots were evaluated as described above.

Characterization of toxigenic fungal pathogens

From each trial, a representative number of fungal isolates, belonging to the toxigenic genera *Aspergillus*, *Penicillium,* and *Alternaria,* was collected for subsequent characterization at species level, according to, respectively, Samson *et al*. (2014), Visagie *et al*. (2014), and Woudenberg *et al*. (2013; 2015). Monoconidial isolates were obtained by spreading conidium suspensions on 2.5% water agar and collecting single germinated spores using stereomicroscope observation. Isolates were stored at 4±1°C on slants of Potato Dextrose Agar (PDA, Himedia).

DNA extraction

For each fungal isolate, five mycelium plugs were collected from 7-d-old PDA colonies, and used to inoculate Malt Extract Agar (MEA, Fluka) in Petri plates with the agar surfaces overlapped by sterilized cellophane disks. After incubation at 24±1°C for 2–3 d, the layers of fresh hyphae were removed using a scraper, and were placed in 2 mL capacity microcentrifuge tubes and stored at -20±1°C. DNA extraction was carried out according to Murray and Thompson (1980) as modified by Rogers and Bendich (1989) with further slight

modifications. Briefly, two iron balls (5 mm diam.) were added to 100 mg of mycelium followed by liquid nitrogen. Once nitrogen evaporated, the tubes were placed in a tissue lyser (Qiagen) at maximum frequency (30 osc s^{-1}) for 45 s. For each isolate, 600 µL of CTAB buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.2% β-mercaptoethanol, 2% CTAB) (previously kept at 75±1°C for 30 min) were added to the sample and mixed gently. The samples were then frozen and defrozen three times, using liquid nitrogen and a water bath at 75±1°C. The samples were then kept in the water bath for 60 min at 75±1°C (inverted every 10 min). The tubes were then cooled, and 600 µL of chloroform were added to samples and vortexed. The tubes were then centrifuged at 14,000 rpm for 15 min, the liquid phase was transferred into new microcentrifuge tubes each containing 2 volumes of isopropanol, and the tubes were each inverted gently. The samples were maintained at -80±1°C for 30 min, and then centrifuged at 14,000 rpm and 4±1°C for 20 min. Each resulting pellet was washed with 200 µL 70% ethanol and centrifuged for 5 min in the same conditions. The pellets were then air-dried and re-suspended in 200 µL TE buffer (pH 8.0). The extracted DNA was quantified by a Nanodrop (Shimadzu) and diluted to 25 ng $µL^{-1}$.

High Resolution Melting assays to screen *Penicillium* and *Aspergillus* isolates

To screen the isolates belonging to *Penicillium* and *Aspergillus*, genus-specific primer pairs (Table 1), synthesized by Macrogen, were used in High Resolution Melting (HRM) reactions, run in a CFX96 Touch Real-time PCR Detection System (Bio-Rad) and analysed using CFX-Manager Software v1.6 (Bio-Rad), as reported by Mincuzzi *et al*. (2020). A cut-off genotype confidence percentage (GCP) ≥95% was set for assigning isolates to genotypes.

Molecular identification of fungi

All *Alternaria* (n = eight) isolates, and representative isolates of *Penicillium* (n = six) and *Aspergillus* (n = ten) selected based on the clusters obtained with HRM assays (at least one isolate per cluster) were sub-

Table 1. Primers for HRM screening, sequencing, and mycotoxin gene detection of *Penicillium*, *Aspergillus* and *Alternaria* isolates from grape samples.

Genus	Gene/ Region	Primer name	Sequence (5'- 3')	Amplicon size (bp)	Source
Penicillium	β-tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	330	Glass and Donaldson, 1995
		Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		
		PPF1	GAGCGYATGAACGTCTACTT	130	Mincuzzi *et al*., 2020
		PPR1	ACVAGGACGGCACGGGGAAC		
	msas	Pe 11F	CACTTATTGTGACCCGCAGA	288	Sanzani *et al*., 2009
		Pe 12R	CTCGAAGAGGATCCATGAGG		
Aspergillus	calmodulin	CMD5	CCGAGTACAAGGARGCCTTC	520	Hong *et al*., 2005
		CMD6	CCGATRGAGGTCATRACGTGG		
		HRM-CMDF	ATAGGACAAGGATGGCGATG	205	Mincuzzi *et al*., 2020
		HRM-CMDR	AGACTCGGAGGGGTTCTGGC		
	fum8	FUM8F	TTCGTTTGAGTGGTGGCA	651	Susca *et al*., 2014
		FUM8R	CAACTCCATASTTCWWGRRAGCCT		
	fum15	FUM15F	CGATTGGTAGCCCGAGGAA	701	
		FUM15R	CTTGATATTGCGGAGTGGTCC		
	ota1	OTA1F	CAATGCCGTCCAACCGTATG	776	Susca *et al*., 2016
		OTA1R	CCTTCGCCTCGCCCGTAG		
	ota3	OTA3F	TTAGACAAACTGCGCGAGGA	613	
		OTA3R	GCGTCGCTATGCCCAGATA		
Alternaria	OPA1-3	OPA1-3L	CAGGCCCTTCCAATCCAT	900	Peever *et al*., 2004
		OPA1-3R	AGGCCCTTCAAGCTCTCTTC		
	pksI	pksI-F	CCTCTCTATCCCAAACTCCACAC	249	Sanzani *et al*., 2021
		pksI-R	CACAGATTATGGCAAGGTTC		

Table 2. Reference strains used in phylogenetic analyses and their GenBank accession numbers.

Genus	Species	Strain	Accession No.
Alternaria	*A. alternata*	CBS 112249	MG063725
		CBS 116329	MF070417
		A214	MK204937
	A. arborescens	A43	KU933224
	A. solani	ASOL	KY561993
Aspergillus	*A. flavus*	CBS117733	EF202059
	A. nidulans	CBS 100522	KX423636
	A. porosus	CBS 375.75	LT671137
	A. tubingensis	AS5	MK919489
		AS18	MK919490
		DTO 178-B5	KP330146
	A. uvarum	AS13	MK919493
	A. welwitschiae	AS23	MK919491
		AS28	MK919492
		CBS 139.54	KC480196
		942	MH614648
Penicillium	*P. brevicompactum*	CMV006A8	MK451072
		G14	MK895703
	P. chrysogenum	CBS 109613	KJ866978
	P. expansum	CBS 48184	AY674399
	P. glabrum	DTO 057-A5	KM08875
	P. olsonii	CBS 38175	AY674444

jected to sequencing of portions of barcoding genes, using the primer pairs detailed in Table 1. PCR reactions were each carried out using 1× Ready Master Mix (Qiagen), 1.5 mM $MgCl_2$, 0.2 µM of each primer, and 25 ng of DNA template in a final volume of 25 µL. The amplifications were carried out in a T100 MyCycler thermal cycler (Bio-Rad) using the following conditions: 2 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55-58°C, 50 s at 72°C, and 5 min at 72°C. After this, 10 µL of each PCR product were loaded on a 1.5% agarose gel in 1× TAE buffer and visualized by the imager system Gel Doc 1000 (Bio-Rad). Purification of PCR products was then carried out using the QIAquick Gel Extraction Kit (Qiagen) following manufacturer's instructions. Purified PCR products were sequenced in both directions by the Medical Genetics Unit at Saint Joseph University (Beirut, Lebanon). For species identification, all sequences were aligned through Chromas software (https://chromas.software.informer.com/download/) and compared with the available sequences in NCBI BLAST database. Subsequently, using MEGA-X software (https://www.megasoftware.net/dload_win_gui), phylogenetic trees were constructed using the Maximum Likelihood method (Kumar *et al*., 2018), according to the Tamura-Nei model (1993) with 1000 bootstrap replications. Reference and CBS strains were included (Table 2).

Molecular characterization of putative ability to produce mycotoxins

According to genus and species, the strains were tested for the presence of genes involved in biosynthetic pathways of the most relevant mycotoxins. The assayed genes were: *pksI* for AOH/ AME biosynthesis, assayed for *Alternaria* strains; *ota1/ota3* for OTA and *fum8/fum15* for fumonisin biosynthesis, assayed for *Aspergillus* strains; and *msas* for patulin biosynthesis, assayed for *Penicillium* strains. Primers, reported in Table 1, were synthesized by Eurofins Genomics. PCR mixtures were each of 25 µL, containing 25 ng of DNA, 0.2 µM of each primer, and 1× Dream Taq Hot Start Green PCR Master Mix (Thermo Fischer Scientific); reactions were carried out according to authors' conditions (Table 1). The presence/absence of these genes was estimated by running an amplicon aliquot on 1.5% agarose gel and UV visualization.

Statistical analyses

Statistical analyses were carried out using IBM SPSS software (version 23). One-way Analysis of Variance (ANOVA) was performed to verify the significance of differences between means, and means were separated using Duncan's Multiple Range test (DMRT).

RESULTS

Epiphytic fungal populations, latent infections, and severity of grape berry decay

Table grapes from five table grape packinghouses in different Lebanese areas were assessed for epiphytic populations of filamentous fungi, latent infections, and rots at the end of storage. Two cultivars from each packinghouse were inspected. Assessment of the epiphytic populations (Table 3) showed low CFU g^{-1} fbw values. Particularly, from 'Autumn King' and 'Superior' samples from Packinghouse 1 (Zahlé area) and 'Crimson' from Packinghouse 4 (Chtaura area), no fungal colonies were recovered. In the other cases, the total fungal counts varied from 1 to 64 CFU g^{-1} fbw. Significant differences ($P \leq 0.05$) were determined between the cultivars in the different packinghouses.

Table 3. Mean epiphytic fungal populations, latent infections and disease severities on table grapes from different packinghouses in Bekaa region (Lebanon) at the end of storage.

Packinghouse	Locality	Cultivar	Epiphytic population (CFU g^{-1} fbw)*	Latent infections (%)*	Disease severity	
					Scale*	DSI** (%)
1	Zahlé	Autumn King	0 c	10.0 ± 8.2 a	1.7 ± 0.3 a	24.8
		Superior	0 c	11.7 ± 2.7 a	0.6 ± 0.2 bc	8.6
2	Ferzoul	Crimson	24 ± 9 a	10.0 ± 0.0 a	0.8 ± 0.2 bc	11.4
		Red Globe	4 ± 4 bc	10.0 ± 4.7 a	0.2 ± 0.1 c	2.9
3	Zahlé	Black Pearl	34 ± 24 a	6.7 ± 1.4 a	0.3 ± 0.1 c	4.8
		Red Globe	19 ± 16 ab	8.3 ± 6.9 a	1.2 ± 0.2 ab	17.1
4	Chtaura	Autumn King	64 ± 35 a	15.0 ± 7.1 a	0.8 ± 0.2 bc	11.4
		Crimson	0 c	6.7 ± 1.4 a	1.1 ± 0.3 ab	16.2
5	Zahlé	Chile	3 ± 3 bc	0 b	0.1 ± 0.1 c	1.0
		Crimson	1 ± 1 c	0 b	0.3 ± 0.1 c	3.8

* Means ±standard errors. In each column, values accompanied by different letters are significantly different ($P \le 0.05$).
** DSI = Disease severity index.

For example, samples of 'Autumn King' from two different packinghouses showed the opposite extreme values. Occurrence of latent infections at the end of the storage was evaluated (Table 3). Four packinghouses and related cultivars showed latent infection incidence varying from 6.7% on 'Black pearl' (Packinghouse 3) and 'Crimson' (Packinghouse 4) to 15% on 'Autumn King' (Packinghouse 4). The most frequent fungal genera causing latent infections were *Penicillium* (47.1%), followed by *Botrytis* (29.4%), *Alternaria* (7.8%), *Aspergillus* (5.9%), *Stemphylium* (4.0%), *Cladosporium* (3.9%), and other fungi (2.0%). Disease severity on bunches was measured at the end of storage in all packinghouses (Table 3). Significant differences were observed between the mean disease severities. The greatest severity (mean = 1.7, range 0-7) was recorded on 'Autumn King' samples from Packinghouse 1 (mean DSI = 24.8%), whereas the least severity (mean = 0.1) was detected on 'Chile' from Packinghouse 5 (mean DSI = 1%). In general, *Botrytis* was the predominant genus observed on stored bunches.

Effects of storage conditions on the fungal populations

Effects of the use of SO_2 generating pads were evaluated in Packinghouse 2, on 'Red Globe' and 'Crimson'. Large reductions in the epiphytic fungal populations on 'Red Globe' (97.9%) and 'Crimson' (99.3%) was detected between T_0 (beginning of storage) and T_f (end of storage) in presence of SO_2 pads (Figure 1). *Penicillium* growth was reduced by up to 99%. For *Aspergillus,* which was less than *Penicillium* at T_0, growth was completely prevented at T_f (Figure 1 a and b). However, the effect of SO_2 during storage on latent infections was variable, being effective on 'Red Globe' but not on 'Crimson'. Although SO_2 completely prevented *Botrytis* infections on both cultivars, it did not influence *Aspergillus* and *Penicillium* infections on 'Crimson' berries (Figure 1 c and d).

As confirmation, the effects of SO_2 were evaluated after three months of storage in the same packinghouse on boxes stored with or without SO_2 pads (Figures 2 and 3). Results confirmed the potential of the pads for reducing total latent infections (Figure 2). In the absence of pads, mean incidence of total latent infections at the end of storage was 31.7% on 'Red Globe' and 37.5% on 'Crimson', whereas in boxes stored with SO_2 pads, mean incidence was 10% on both cultivars. SO_2 generating pads completely prevented latent infections caused by *Botrytis* and *Alternaria*, whereas latent infections by *Penicillium* were observed in both types of packages, with mean incidence varying between 10 and 16.7%. Similarly, decay severity assessed after 3 months of storage on both cultivars was greater ($P \le 0.05$) in boxes stored without SO_2 pads than in boxes containing the pads (Figure 3).

Characterization of fungal strains and their putative abilities to produce mycotoxins

During the assessments of epiphytic fungal populations, latent infections, and rots, three genera among mycotoxigenic fungi were identified: *Alternaria*, *Aspergillus*, and *Penicillium*. A total of 44 isolates were col-

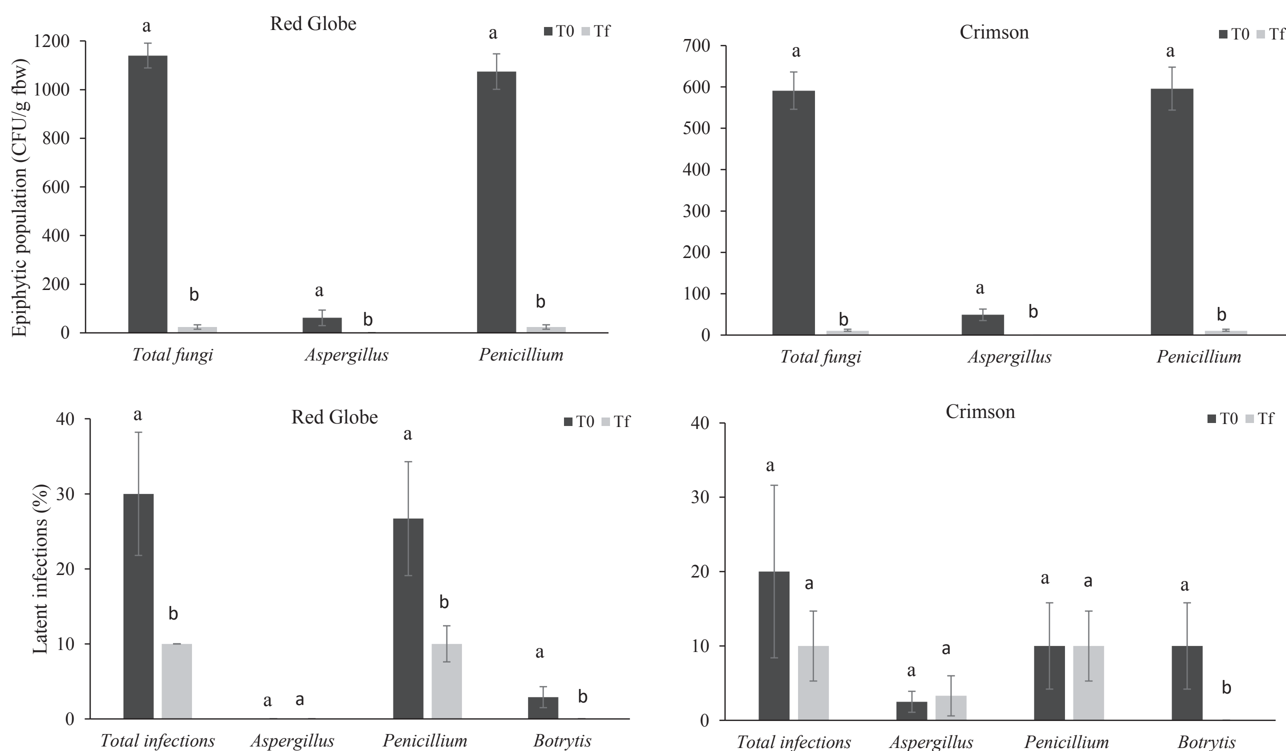

Figure 1. Mean populations of epihytic fungi, and proportions (%) of latent fungal infections, on 'Red Globe' and 'Crimson' table grapes, stored with SO_2 generating pads. For each variable, mean values at T_0 (black histogram, beginning of storage) and T_f (end of storage) accompanied by different letters are significantly different ($P \leq 0.05$). Bars represent standard errors of means, each from three replicates.

lected according to morphology and frequency of isolations. Twenty-four isolates were *Aspergillus*, 12 were *Penicillium*, and eight were *Alternaria*. All the isolates were assigned to a species according to morphological features on specific media and sequencing of barcoding genes/regions. *Aspergillus* and *Penicillium* isolates were the most abundant so these were initially screened by HRM to identify genetic clusters. For at least one isolate per cluster of *Aspergillus,* a portion of the calmodulin gene was sequenced, whereas for *Penicillium*, a portion of the β-tubulin gene was sequenced. Sequences were run against those in the GenBank database, and they showed 99-100% identity with relevant reference sequences. As further confirmation, a phylogenetic analysis was conducted including CBS and reference strains (Figures 4, 5 and 6).

For *Aspergillus*, all strains belonged to section *Nigri* and were divided into ten clusters (Table 4, Figure 4). Most of isolates belonged to series *Nigri*, being *A. tubingensis* (67%) and *A. welwitschiae* (12%), and the remaining were in series *Japonici*, as *A. uvarum* (21%). For *A. welwitschiae* strains (AS20, AS27, and AS31), the presence of key biosynthetic genes for OTA (*ota1* and *ota3*) and fumonisins (*fum8* and *fum15*) was assessed. Strains A27 and AS31, belonging to the same HRM cluster (9), possessed *ota3* and *fum15* genes, so were potential producers of OTA and fumonisins, while strain AS20 (HRM cluster 7) did not possess these genes (Table 4).

For *Penicillium* (Table 5, Figure 5), five HRM clusters were identified corresponding to three sections. The most abundant was sect. *Aspergilloides* with 50% of the strains identified as *P. glabrum*, followed by sect. *Brevicompacta* with *P. brevicompactum* (25%) and *P. olsonii* (17%), and sect. *Penicillium* with *P. expansum* (8%). *P expansum* strain P18 possessed *msas*, the key gene for patulin biosynthesis (Table 5).

For *Alternaria*, *A. alternata* and the *A. arborescens* species complex were identified (Table 6). Isolate identity was supported by a phylogenetic analysis, including CBS and references strains (Figure 6). Eighty-eight percent of the strains were *A. alternata* morphotype *alternata*, and 12% were in the *A. arborescens* species complex. Strains were tested for the presence of the *pksI* gene for alternariol biosynthesis and all were potential AOH/AME producers (Table 6).

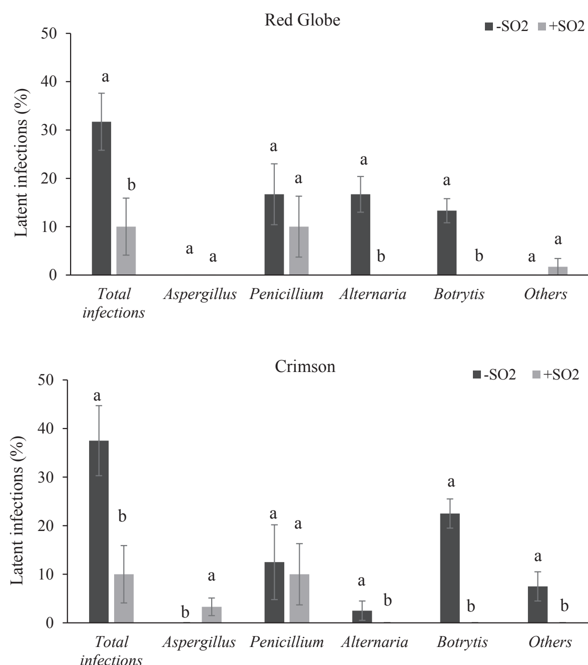

Figure 2. Mean proportions (%) of latent fungal infections on 'Red Globe' and 'Crimson' table grapes, stored either with (+SO_2) or without (black histograms, -SO_2) SO_2 pads, after three month of storage. For each variable, columns accompanied by different letters are significantly different ($P \leq 0.05$). Bars represent standard errors of mean, each from three replicates.

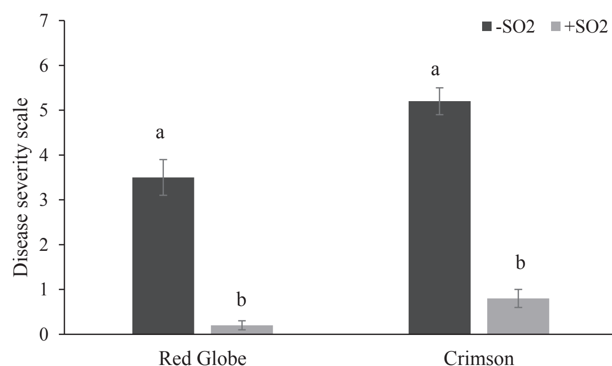

Figure 3. Disease severity (scale 0-7) on 'Red Globe' and 'Crimson' table grapes, stored either with (+SO_2) or without (black histograms, -SO_2) SO_2 pads, after three month of storage. Columns accompanied by different letters are significantly different ($P \leq 0.05$). Bars represent standard errors of means, each from three replicates.

DISCUSSION

Crops of table grapes are of increasing importance in Lebanon. However, there is little information available on the main fungal pathogens affecting table grape storability. The present study was conducted to collect information on the main threats to harvested table grapes, with particular attention on mycotoxigenic fungi. Five packinghouses were inspected in different areas of Lebanon for epiphytic fungal populations, latent infections, and rots. The study showed that *Botrytis*, *Penicillium*, *Aspergillus*, and *Alternaria* were the most abundant genera. These results are in line with other studies. Ding *et al*. (2019) reported epiphytic populations on grapes in subtropical China, and indicated that *Cladosporium, Penicillium, Aspergillus,* and *Alternaria* were among the most abundant fungi. Similarly, Oliveira *et al.* (2017) showed that these genera were the most frequently isolated from grape berries in Portugal. They also stressed the influence of atmospheric conditions on the composition of the fungal community detected. Abdelfattah *et al*. (2019) reported exchanges between grape plants and the surrounding environment, so that grape plants could be major sources of recruitment for the atmospheric microbiome.

Aspergillus and *Penicillium* species are considered to be the most important wound pathogens. They commonly enter host plants through wounds and natural openings. Wounds can be created at pre- and postharvest stages, especially if the products are subjected to improper handling either at harvesting or during packing and storage operations (Mincuzzi *et al*., 2020). In the present study, different packinghouses were assessed, each with two grape cultivars. Although the epiphytic fungal populations were generally low, some differences were observed. For example, despite the same storage procedures, including air cooling and the use of SO_2 generating pads in the two different packinghouses, the grapes of 'Autumn King' and 'Crimson' had different amounts of contamination. This could be due to different field inoculum loads and composition. The present study showed different susceptibilities to SO_2 among fungi, with *Botrytis* more susceptible to SO_2 than *Penicillium*. Furthermore, the different responses among cultivars could be related to features such as berry epidermis thickness or compaction, cell wall thickness, and/or epidermis microstructure (Fernández-Trujillo *et al*., 2012). Influences of vineyard management on epiphytic microbial composition could also be involved (Abdelfattah *et al*., 2019).

Latent fungal infections were present on most of the cultivars. These results are not surprising since *Botrytis* is well known for its ability to infect grapevine from the flowering stage, and can remain latent/dormant until reactivation following suitable conditions (*i.e.*, ripening and favourable environmental conditions; Sanzani *et al*., 2012). During packinghouse operations, berries may seem healthy, but eventually become rotted during

Table 4. *Aspergillus* HRM clusters, species, strains, GenBank accession numbers for the calmodulin gene, and presence of ochratoxin A (*ota1* and *ota3*) and fumonisins (*fum8* and *fum15*) biosynthetic genes.

HRM Cluster	Species	Strain	Accession no.*	Detection of biosynthetic genes**			
				ota1	ota3	fum8	fum15
1	*A. tubingensis*	AS17	n.a.	n.a.	n.a.	n.a.	n.a.
		AS21	MZ241120	n.a.	n.a.	n.a.	n.a.
		AS24	n.a.	n.a.	n.a.	n.a.	n.a.
2	*A. tubingensis*	AS15	MZ241118	n.a.	n.a.	n.a.	n.a.
		AS18	n.a.	n.a.	n.a.	n.a.	n.a.
		AS22	n.a.	n.a.	n.a.	n.a.	n.a.
3	*A. tubingensis*	AS2	MZ241114	n.a.	n.a.	n.a.	n.a.
		AS19	n.a.	n.a.	n.a.	n.a.	n.a.
		AS25	n.a.	n.a.	n.a.	n.a.	n.a.
4	*A. tubingensis*	AS14	MZ241117	n.a.	n.a.	n.a.	n.a.
		AS16	MZ241119	n.a.	n.a.	n.a.	n.a.
		AS23	n.a.	n.a.	n.a.	n.a.	n.a.
5	*A. tubingensis*	AS9	n.a.	n.a.	n.a.	n.a.	n.a.
		AS29	MZ241121	n.a.	n.a.	n.a.	n.a.
6	*A. uvarum*	AS1	n.a.	n.a.	n.a.	n.a.	n.a.
		AS10	MZ241125	n.a.	n.a.	n.a.	n.a.
		AS11	MZ241126	n.a.	n.a.	n.a.	n.a.
		AS26	MZ241127	n.a.	n.a.	n.a.	n.a.
		AS28	MZ241128	n.a.	n.a.	n.a.	n.a.
7	*A. welwitschiae*	AS20	MZ241122	-	-	-	-
8	*A. tubingensis*	AS13	MZ241116	n.a.	n.a.	n.a.	n.a.
9	*A. welwitschiae*	AS27	MZ241123	+	-	+	-
		AS31	MZ241124	+	-	+	-
10	*A. tubingensis*	AS12	MZ241115	n.a.	n.a.	n.a.	n.a.

* n.a. = not analyzed.
** The presence of OTA and fumonisin biosynthetic genes was checked in *A. welwitschiae* strains. + = Present; - = Absent.

storage. Being situated within berry grape tissues, *Botrytis* rots might not be prevented by surface treatments such as the SO_2 generated by the preservation pads. SO_2 does not penetrate deeply into berry tissues/skins (Smilanick *et al.*, 1990), especially the skins are particularly impenetrable. Thus, in the present study, rots, mostly caused by *B. cinerea*, were significantly reduced by SO_2 pads, although not prevented. The reduced sensitivity of 'Crimson' to SO_2 compared to 'Red Globe' may be a cultivar effect, or due to the extent or composition of pathogen contamination (*i.e.*, presence of *Penicillium* and *Aspergillus*). However, Youssef *et al.* (2020) found that SO_2 generating pads, even at different concentrations and release rates, could completely inhibit grape decay caused by *B. cinerea*, if combined with a field control strategy to reduce infections rate during grapevine growth. These field treatments should be scheduled from flowering, to reduce rots during cold storage of harvested grapes. Alternative treatments with little to no potential harmful environmental effects have raised public interest. For example, protein hydrolysates (*e.g.*, from soybean or casein) were tested with good results (Lachhab *et al.*, 2016).

The toxigenic fungi contaminating harvested Lebanese table grapes included *Aspergilli* of section *Nigri*. *A. tubingensis* is known as a non-producer of OTA (Storari *et al.*, 2012); *A. uvarum* is a relatively newly discovered species, mostly occurring on grapes and is not toxigenic (Somma *et al.*, 2012); and *A. welwitschiae* produces OTA and fumonisins (Perrone and Gallo, 2016), as observed for 2 of the 3 strains in the present study. For *Penicillium*, strains of *P. glabrum* and *P. brevicompactum* were found, which, despite being reported to possess genes for patulin biosynthesis (Bokhari and Aly, 2009; Diaz *et al.*, 2011), have recently been questioned for their ability to produce patulin (Frisvad, 2018). *P. olsonii*, quite common in confined environments but reported as a non-producer of patulin (Frisvad, 2018), was also present. In the pre-

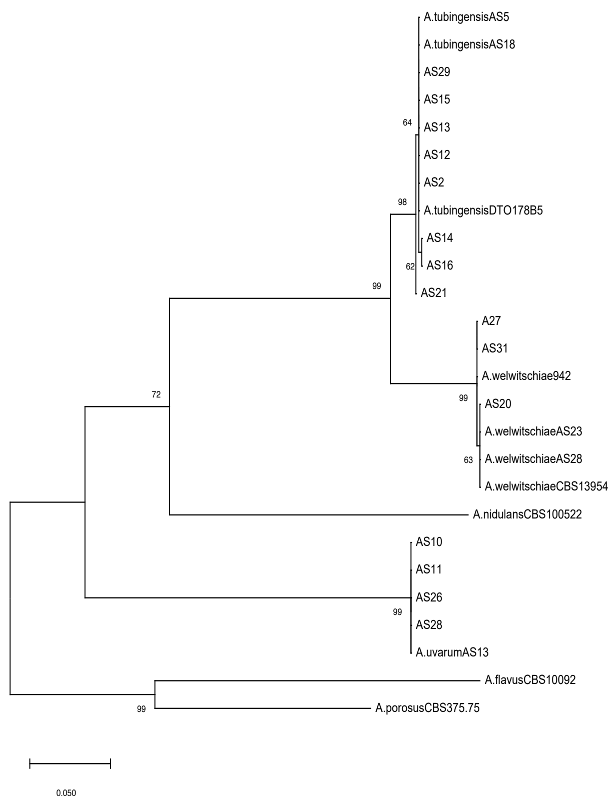

Figure 4. Phylogenetic tree for *Aspergillus* strains based on a portion of the calmodulin gene. Numbers on nodes represent the Maximum Likelihood bootstrap percentages. Branch lengths are proportional to the numbers of nucleotide substitutions and can be measured using the bar scale (0.05).

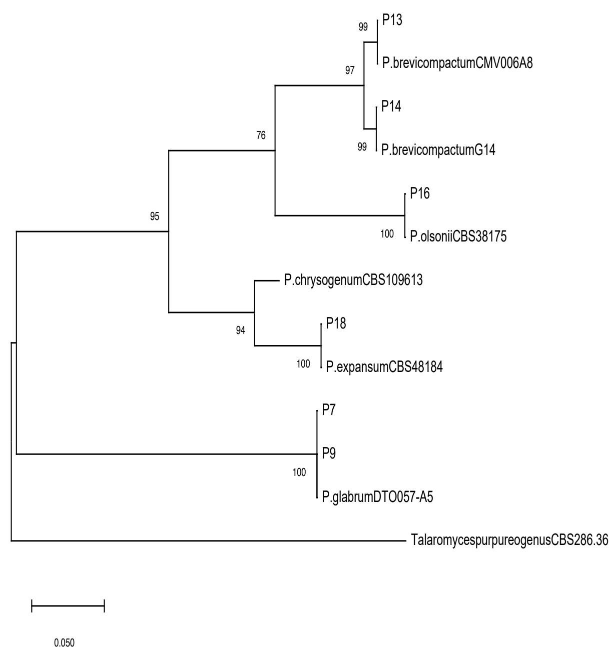

Figure 5. Phylogenetic tree for *Penicillium* strains based on a portion of the β-tubulin gene. Numbers on nodes represent the Maximum Likelihood bootstrap percentages. Branch lengths are proportional to the numbers of nucleotide substitutions and can be measured using the bar scale (0.05).

Table 5. *Penicillium* HRM clusters, species, strains, GenBank accession numbers for the β-tubulin gene, and presence of a patulin biosynthetic gene (*msas*).

HRM cluster	Species	Strain	Accession No.*	Detection of *msas***
1	*P. glabrum*	P6	n.a.	n.a.
		P7	MZ241137	n.a.
		P8	n.a.	n.a.
		P9	MZ241138	n.a.
		P10	n.a.	n.a.
		P11	n.a.	n.a.
2	*P. brevicompactum*	P14	MZ241140	n.a.
		P17	n.a.	n.a.
3	*P. brevicompactum*	P13	MZ241139	n.a.
4	*P. olsonii*	P12	n.a.	n.a.
		P16	MZ241141	n.a.
5	*P. expansum*	P18	MZ241142	+

* n.a.= not analyzed.
** The presence of patulin biosynthetic genes was checked in *P. expansum* strains. + = Present; - = Absent.

Table 6. *Alternaria* species/species complexes, morphotypes, strains, GenBank accession numbers for the OPA1-3 region, and presence of an alternariol biosynthetic gene (*pksI*).

Species	Morphotype	Strain	Accession No.	Detection of *pksI**
A. arborescens		Al1	MZ241129	+
A. alternata	*alternata*	Al2	MZ241130	+
		Al4	MZ241131	+
		Al5	MZ241132	+
		Al7	MZ241133	+
		Al11	MZ241134	+
		Al12	MZ241135	+
		Al16	MZ241136	+

* + = Present; - = Absent.

sent study, a single strain of *P. expansum* able to produce patulin was detected, belonging to the species known as the most potent patulin producer on fruit crops (Sanzani *et al.,* 2013). For *Alternaria*, *A. alternata* and the *A. arborescens* species complex were detected, and the strains proved to be AOH/AME producers. Similar results were found for grape bunch rot during withering (Lorenzini and Zapparoli, 2014). In general, the presence of fun-

Figure 6. Phylogenetic tree for *Alternaria* strains based on a portion of the SCAR Marker OPA1-3. Numbers on nodes represent the Maximum Likelihood bootstrap percentages. Branch lengths are proportional to the numbers of nucleotide substitutions and can be measured using the bar scale (0.02).

gal pathogens was in line with other studies (Diaz *et al.*, 2011; Oliveira *et al.,* 2017; Ding *et al*., 2019).

Fungi, including *Aspergillus, Penicillium,* and *Alternaria*, can switch lifestyle between saprophytic and plant pathogenic forms. Mycotoxin production by these fungi may even affect their positions in ecological niches and their interactions with plants, animals, and the environment (Pfliegler *et al*., 2019), causing major effects on the severity of the diseases caused (Sanzani *et al*., 2012; Zaccaria *et al*., 2015; Wenderoth *et al*., 2019).

Harvested Lebanese table grapes were shown to harbour relevant fungal pathogens from spoilage and food safety perspectives, both as epiphytic agents and latent infections. *Alternaria*, *Aspergillus*, *Penicillium,* and *Botrytis* were the most commonly identified fungi. Furthermore, SO_2 generating pads were found to be more effective for reducing epiphytic fungal populations during grape storage than for reducing latent infections, confirming the need of an effective field control of these infections. The mycotoxin producing genera *Aspergillus*, *Penicillium,* and *Alternaria* were characterized at species level. Strains of *A. welwitschiae* and *P. expansum* were also found, which are reported in grapes to be important producers of mycotoxins, the contents of which in food commodities is regulated by international legislation. As such, pre- and postharvest management should consider to control grape spoilage and to prevent mycotoxin production. Particularly, studies are recommended to assess conditions that favour fungal secretion of toxic secondary metabolites, and their fates during storage of table grapes.

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