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Diversity of Pathogenic Fungi Associated with Apples in Cold Storage Facilities in Tunisia

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Abstract

Postharvest storage fungi are major limiting factors for the apple industry. In a study conducted in Tunisia in 2014, six refrigerated fruit storage facilities were surveyed in order to determine disease incidence, identify the pathogenic fungal species on apples and study mycelial growth *in vitro* and lesion diameters *in vivo* of sampled fungal isolates. Results showed that *Penicillium expansum* (42.3%) was the predominant fungus in apples, followed by *Alternaria* spp. (23%), *Botrytis* spp. (19.2%), *Aspergillus* spp. (13.5%) and *Fusarium* spp. (2%). Isolates collected from storage facilities with highly diverse fungal species had significantly greater mycelial growth at 24°C in *vitro* and larger lesion diameters on apples and oranges than isolates collected from storage facilities with less diversity. Moreover, a trade-off between the diversity in fungal species and the disease incidence in the storage facilities was suggested; facilities with a low or high disease incidence showed the lowest diversity in fungal species. Controlling disease incidence in cold storage facilities is recommended to limit the diversity in fungal species and the development of virulent isolates. Further studies are needed to determine the fruit storage conditions that influence fungal species diversity and their variability in pathogenicity, in order to implement efficient postharvest disease management.

Due to their organoleptic and nutritional qualities, including antioxidants, Apple (*Malus x domestica*) is among the fruits experiencing increasing international interest, with a world annual production of 84.6 million tons in 2014 (FAOSTAT, 2014). Countries in the northern hemisphere produce the highest quantities of apples. Asia accounts for more than 62% of global apple production, while the European Union countries produce 20.7% (FAOSTAT, 2014). In Tunisia, apples are a critically important local fresh fruit. Apple orchards cover 26,000 ha, 3 % of the total cultivated land. Approximately 90,000 tons of apples were produced in Tunisia in 2015. The crop is produced widely across the country, but the main production area is the governorate of Kasserine with 27.7% of the national apple production. The main cultivated local and introduced cultivars are 'Chahla', 'Anna', 'Aziza', 'Golden Delicious' and 'Gala' (GIFruits, 2016; Mlika et al., 2002).

The preservation of fruits and vegetables in Tunisia depends heavily on the cold storage industry. In fact, about 70% of the total refrigerated storage capacity in Tunisia is used for fruits and vegetables and is located at Beni Khaled area (API, 2006). These storage technologies keep fruit at < 4°C and 70% relative humidity using a sandwich panel structure as thermal insulation. Unfortunately they are precarious and poorly managed (Jammes, 2012; Jraidi, 2010). This contributes to development of postharvest fungal diseases, which are the major factor limiting the storage life of fruits and vegetables (Ogawa and English, 1991). In fact, while cold storage delays fruit maturation and senescence, it leads to numerous phytosanitary problems. Postharvest diseases can cause

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losses up to 50 % to the apple industry during storage, transport and marketing (ElGhaouth, 1997; Jurick et al., 2011). These diseases can originate from infection in the orchard, or are more commonly associated with post-harvest fruit handling practices. Leibinger et al. (1997) reported more than 90 fungal species infecting apples during storage. The most prevalent post-harvest fungal disease of apple is blue mold caused by *Penicillium expansum*. However, *Botrytis cinerea* (gray mold) and *Gloeoporus* spp. also are of economic concern in Europe (Dean et al., 2012; Droby and Lichter, 2004; Elad et al., 2015; Rupp et al., 2016). Traditionally, the application of synthetic fungicides is considered the most essential practice in a profitable post-harvest fruit storage (Conway et al., 1999; Eckert and Ogawa, 1988; Eckert and Sommer, 1967). However, the emergence of fungal strains that are resistant to fungicides, especially resistance of *P. expansum* to benzimidazoles (Holmes and Eckert, 1999; Rupp et al., 2016; Spotts and Cervantes, 1986), and regulations that restrict chemical application (Eckert et al., 1994; Gullino and Kuijpers, 1994; Ragsdale and Sisler, 1994) have stimulated a renewed interest in biocontrol strategies. Biocontrols have the potential to achieve sustainable post-harvest disease management while ensuring human health, environmental safety and consumer expectations of fruit quality (Janisiewicz and Korsten, 2002; Romanazzi et al., 2016; Wilson et al., 1993).

In Tunisia, the phytopathogenic fungi that occur in stored fruits have not been surveyed. Prior to exploring the feasibility of biocontrol and other non-chemical management strategies, it is critical to survey the types of fungi associated with cold apple storage in Tunisia, and the genetic diversity of these species. Hence, the goals of this research were 1) to identify the main phytopathogenic fungi infecting apples in the cold storage facilities in Tunisia, 2) to evaluate the relative aggressiveness of these fungal species *in vitro* and *in vivo* and 3) to study the effect of the storage diversities in fungal species on the potential pathogenicity of the phytopathogens.

Methods and Materials

Identification of post-harvest pathogenic fungi of apples in cold storage

Storage sites and sampling protocol. In 2014 apples were sampled from six cold fruit storage facilities distributed throughout the main post-harvest storage areas in Tunisia (Beni Khaled, Mornag, Khledia, Ras Jebel and Korba), to identify common phytopathogenic fungal species (Table 1). Apple samples (n = 30 fruits per storage unit) were collected in storage units from Feb. to April, and each storage unit was sampled once. At each site, 10 storage boxes and three apples per box were randomly selected based on box and fruit position in the storage unit and box, respectively. Selected apples did not have disease symptom and were kept separately in a bag to avoid cross-contaminations. For all storage units, fruits came from more than one orchard and were stored at 2-4°C; the number of fruit suppliers varied with the storage capacity. However, because traceability system is not implemented in the storage facilities, origin of fruits was unknown.

Isolation of fungi. A piece (~1 cm^2; 0.5 cm deep) of apple epicarp was taken from each apple collected from the different cold storage facilities and plated on potato dextrose agar (PDA), after surface wash in three different changes of distilled water. Each sample was processed separately to avoid cross-contamination. After incubation for seven days at 24°C in the dark, each fungal culture was purified by sub-culturing to new PDA plates. Subsequently, single-spore cultures were prepared from pure colonies; all isolates were stored as 7-day old single-spore cultures on PDA plates at 4°C and cultured in fresh PDA medium in darkness at 24°C for 7 days when needed.

Pathogenicity tests. Pathogenicity tests were performed with all isolated fungi in vivo by inoculating apple and pear fruits when Penicillium expansum was identified. Before

inoculation, fruits were surface-sterilized with 90% alcohol, washed with 1% sodium hypochlorite, rinsed with distilled water and air-dried at room temperature for 5 minutes. Surface sterilized fruits were then wounded twice at equal distance with a sterile tip by making an injury of ~0.5 cm diameter. Then, 20 µl of conidial suspension (prepared from 7 days old PDA cultures) of each isolate adjusted to 103 spores/ml were applied into each wound; two fruits were inoculated per isolate. Four fruits inoculated with sterilized distilled water were used as controls. Inoculated fruit were covered with plastic film to avoid contamination and to ensure 100% RH and incubated at 24°C in the dark. Fungi were re-isolated from infected apples and compared with the original isolates in order to confirm Koch's postulates. Isolates that induced rot symptoms on inoculated apples were considered pathogenic.

Identification of pathogenic fungi and diversity in fungal species associated with apple fruits in cold storage facilities. Fungal species isolated from stored apple fruit were identified based on macroscopic observations of the monosporic colonies on PDA, followed by microscopic observations of spores and pathogenicity tests on inoculated apple fruit. Only monosporic colonies that were pathogenic by inducing symptoms on inoculated apples were considered for identification. The macro and micro-morphological characteristics included colony growth and colour, presence or absence of wrinkles, presence or absence of septa, morphology and size of conidia, observation of the structures bearing spores, and rot symptoms on inoculated fruits. Fungal organisms were identified in accordance with (Domsch et al., 2007; Klich, 2002; Leslie and Summerell, 2006; Pitt and Hocking, 2009; Vico et al., 2014; Xiao and Kim, 2008). The incidence of disease was calculated for each cold storage unit as the number of apple tissue samples that yielded pathogenic fungal cultures divided by the total number of apple fruit. This disease incidence evaluated on 30 apples, represents the potential post-harvest loss in each storage. Furthermore, the frequency of each fungal species identified was assessed in each storage unit. The diversity in phytopathogenic fungal species of each storage unit was calculated according to the Shannon diversity index calculated as follows:

$$H' = \sum_{i=1}^{s} - (Pi * \ln Pi)$$

where S is the number of species observed and Pi is the fraction of the entire population made up of species i. The fruit storage facilities were grouped as having diverse and non-diverse phytopathogenic fungal populations when $H'>1$ and $H'\leq 1$, respectively.

Characterization of pathogenic fungi isolated from apples in cold storage

Mycelial growth. In vitro mycelial growth was measured for 14 isolates of the five phytopathogenic species isolated from apples in cold storage facilities. These isolates were selected arbitrarily and included six *Penicillium expansum*, three *Aspergillus* sp., two *Botrytis* sp., two *Alternaria* sp. and one *Fusarium* sp. isolates. Agar plugs (~5 mm diameter) were taken from each monosporic culture on PDA and plated onto new PDA plates. Each isolate was incubated at 24°C and 4°C and colony diameter measurements were recorded at 7 days post-incubation (DPI). There were three replicates per isolate x temperature treatment.

Lesion diameter. The diameter of the lesions 7 DPI of five isolates of four phytopathogenic species (two *Penicillium*, one *Aspergillus*, one *Botrytis*, one *Alternaria*) collected from stored apples was evaluated in vivo by inoculating mature 'Golden Delicious' fruits. Inoculation method was similar to that described above in the pathogenicity tests; however in this test five fruits were inoculated once at the equator with each isolate. Controls included fruits treated with sterilized distilled water. The pathogenicity of the five isolates was also assessed with a similar approach on mature orange fruits of

the cultivar 'Maltaise', the main orange cultivar grown and stored in Tunisia.

Data analysis

Data were analysed as a completely randomized design with analysis of variance (ANOVA) using R 3.0.2 (R Core Team, 2013). Data for *in vitro* mycelial growth of fungal species at 24°C and at 4°C were analysed independently with 1-way ANOVAs. Similar analyses were performed for *in vitro* mycelial growth of fungal isolates within species at 24°C and 4°C. Following ANOVA, contrasts were used to compare mycelial growth means between isolates within species at 5% level of significance. In addition, the *in vitro* growth of fungal species and fungal isolates within species at 4°C and 24°C were analysed as 2-way factorials. Contrasts were performed to compare mycelial growth means between species and between isolates within species. The lesion diameter of fungal isolates on apple and orange fruits were analysed as 2-way factorial. Contrasts were also performed to compare mycelial growth and lesion diameter means between isolates sampled from storage units with index values H'>1 (high diversity in phytopathogenic species) and storage units with index H'≤ 1 (low diversity in phytopathogenic species). In addition, Pearson's correlation between lesion diameters on apple and orange, and between lesion diameters on apple, and mycelia growth on PDA media at 24°C were tested under R 3.0.2 (R Core Team, 2013) using the rcorr function in the Hmisc package.

Results

Identification of pathogenic fungi isolated from apples and diversity in fungal species in cold storage.

From the pathogenicity tests, 52 isolates from 180 apples tissue cultures were pathogenic on inoculated apples. Morphological analysis revealed the presence of five genera in these 52 pathogenic fungi. These included *Penicillium expansum*, *Aspergillus* sp., I sp., *Fusarium* sp. and *Botrytis* sp. (Table 1). These five pathogens had characteristic colony morphology and spores on PDA medium, and typical disease symptoms on inoculated apple fruits (Fig. 1).

Penicillium expansum was the most commonly observed fungus in stored apples with

Table 1. Number of fungal species and isolates recovered from apple fruits from six cold storage facilities in Tunisia between February and April 2014.

Storage ID	Region	# isolates	# fungal species	Pen [z]	Alt	Bot	Asp	Fus	H' [y]	Isolate ID [x]
1	Beni Khaled	10	4	4	3	2	0	1	1.84	Pen4, Bot1, Fus*
2	Korba	2	1	2	0	0	0	0	0.00	Pen1
3	Beni Khaled	14	3	6	7	0	1	0	1.29	Pen2, Alt3*, Asp3
4	Mornag	8	4	2	2	1	3	0	1.90	Pen6, Bot2, Alt2, Asp2*
5	Khelidia	5	2	2	0	0	3	0	0.97	Pen3*, Asp1
6	Ras Jebel	13	2	6	0	7	0	0	0.99	Pen5*
Total		52	5	22	12	10	7	1	1.75	

[z] Pen, Alt, Bot, Asp, Fus correspond to *Penicillium expansum, Alternaria* spp., *Botrytis* spp., *Aspergilus* spp., and *Fusarium* spp. isolates observed

[y] H': diversity index calculated according to the Shannon index

[x] Isolates ID corresponds to the isolates used for the *in vitro* tests on PDA media; asterisks indicate isolates used for pathogenicity assays on orange and apple fruits

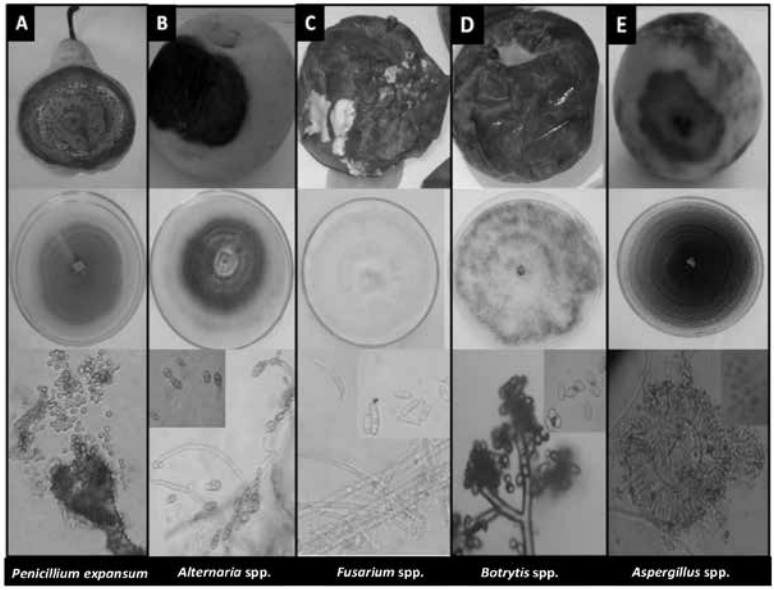

Figure 1. Mycelia cultures and spores characteristics on PDA media and typical disease symptoms on inoculated fruits for the 5 genera isolated *Penicillium expansum* (A), *Alternaria* spp. (B), *Fusarium* spp. (C), *Botrytis* spp. (D), and *Aspergilus* spp. (E).

a frequency of 42.3%, followed by *Alternaria* sp., *Botrytis* sp., *Aspergillus* sp. and *Fusarium* sp. with frequencies of 23% 19.2%, 13.5% and 2%, respectively. The six storage facilities surveyed had different levels of disease incidence and the diversity index H' in isolated fungal species. Storage facilities 3 and 6 had the highest overall disease incidences (46.6% and 43.3%, respectively), followed by storage facilities 1, 4, 5 and 2 with disease incidences of 33.3%, 26.6%, 16.6 % and 6.6%, respectively. Storage facilities 1, 3 and 4 had the highest mean diversity indices (H' = 1.68) with three to four species of fungi. Storage facilities 2, 5 and 6 had the least diverse fungal populations (from one to two species) with mean diversity indices ranging from 0 to 0.97 (Table 1).

Characterization of pathogenic fungi isolated from stored apples. In vitro mycelial growth of 14 fungal isolates was measured on PDA medium. Mycelial growth between the four fungal species tested at 24°C did not differ significantly (*Penicillium, Aspergillus, Alternaria* and *Botrytis*) (P = 0.858). However, temperature did significantly affect in vitro mycelial growth of 14 isolates on PDA medium. At 24°C, isolates Asp2, Asp3 grew to a mean diameter of 4.48 cm whereas the Pen5, Pen3 and Asp1 isolates had mean diameters of 1.96 cm. Fifty-seven percent of the fungal isolates showed intermediate mycelial growth ranging from 2.00 to 3.93 cm (Tables 2 and 3). Analysis by species showed that mycelial growth was significantly different among *Aspergillus* sp. (P = 0.0002) and among *Penicillium expansum* isolates (P = 0.020) at 24°C. Pen2 (Storage facility 3), Pen6 (Storage 4) and Pen4 (Storage 1) isolates had mycelial growth that was significantly greater than Pen5 (Storage 6) and Pen3 (Storage 5). The mycelial growth of isolates Asp2 (Storage 4) and Aps3 (Storage 3) was significantly greater than Asp1 (Storage 5). However, mycelial growth of the *Botrytis* sp. isolates, Bot1 (Storage 1) and

Bot2 (Storage 4) (P = 0.08) and the Alternaria sp. isolates Alt2 (Storage 4) and Alt3 (Storage 3) (P = 0.39) did not differ at 24°C on PDA. In addition, significant interaction effects of 'temperature x species' (P < 0.001) and 'temperature x isolate within species' (P < 0.001) were observed on mycelial growth. Mycelial growth at 4°C was greatest for Bot1, Pen2 and Pen1 isolates (average of 1.2 cm), which were intermediate at 24°C. In addition, isolates Fus, Asp3 and Asp2, the most fit at 24°C did not grow after 7 days of incubation at 4°C (Tables 2 and 3).

Lesion diameter of fungi isolated from stored apples. After 7 days of incubation at 24°C, lesion diameter of the five isolates tested were significantly different on both apple and orange fruit (P < 0.0001). The most virulent isolate on apple was Aps2 (Storage 4) (mean lesion diameter of ~ 1.2 cm), followed by Alt3 (Storage 3) (diameter = 0.72 cm). The isolates, Pen3 (Storage 1) and Pen5 (Storage 6) were the least virulent with barely visible lesions on apples after 7 days of incubation at 24°C. Mean lesion diameters were grew to 0.5 cm after 14 days of incubation. The most virulent isolates on apples were also the most virulent on oranges and lesion diameter on apples and oranges were correlated (Fig. 2; R^2 =0.982; P= 0.0011).

Correlations between mycelial growth, lesion diameter, disease incidence and H' index. Lesion diameter and in vitro mycelia growth for the tested isolates at 24°C were strongly correlated (Fig. 3; R^2 = 0.864 %; P= 0.0221). Asp2 isolate, with greatest mycelial growth *in vitro* at 24°C, had the highest mean lesion diameter. Isolates from storage facilities with a H' index > 1 (Storage facilities 1, 3 and 4) had significantly higher mycelial growth at 24°C than those from storage facilities with a H' index ≤ 1 (Storage facilities 2, 5 and 6) for all fungal species (P < 0.0001) and for *Aspergillus* sp. (P < 0.0001) and for *Penicillium expansum* (P = 0.0038) isolates tested separately. At 4°C, storage facilities with a H' index > 1 (Storage facilities 1, 3 and 4) and H' index ≤ 1(Storage facilities 2, 5 and 6) did not differ (P= 0.6383) for mycelial growth (Tables 2 and 3, Fig. 4). The lesion diameters were significantly higher for the isolates from storage facilities with a H' index > 1 (Storage facilities 1, 3 and 4) than for those from storage facilities with a H' index ≤ 1 on both apples and oranges (P < 0.0001) after 7 days of incubation at 24°C (Table 4, Fig. 5). The plot of the diversity H' index against the disease incidence of the six cold storage facilities showed that the H' reaches a maximum value for an intermediate disease incidence of 30 %. Storage facilities with low and high disease incidences had a lower diversity in fungal species (Fig. 6).

Table 2. Mycelia growth (cm) of 14 isolates recovered from apple fruits in cold storage facilities in Tunisia after 7 days of incubation on potato dextrose agar media at 25°C and at 4°C.

Isolate	4°C[z]	24°C[z]	Mean[z]
Alt3	0.45^{A}	3.17^{A}	1.81^{A}
Alt2	0.74^{B}	3.53^{A}	2.14^{A}
Asp1	0.00^{A}	2.03^{A}	1.02^{A}
Asp3	0.00^{A}	4.30^{B}	2.15^{B}
Asp2	0.00^{A}	4.67^{B}	2.33^{B}
Bot2	0.13^{A}	3.30^{A}	1.71^{A}
Bot1	1.25^{B}	2.30^{A}	1.78^{A}
F	0.00	3.93	1.97
Pen5	0.60^{A}	1.87^{A}	1.23^{A}
Pen3	0.73^{A}	2.00^{AB}	1.36^{AB}
Pen4	0.70^{A}	3.13^{C}	1.92^{BC}
Pen6	0.68^{A}	3.20^{C}	1.94^{BC}
Pen1	1.11^{B}	2.93^{BC}	2.02^{C}
Pen2	1.19^{B}	3.27^{C}	2.23^{C}

[z] Contrasts were performed between isolates within species. Isolate with common letters do not differ at the 5% level of significance.

Discussion

Phytopathogens can cause significant economic losses in post-harvest fruit storage. This study focused on identifying and characterizing phytopathogenic fungi common in

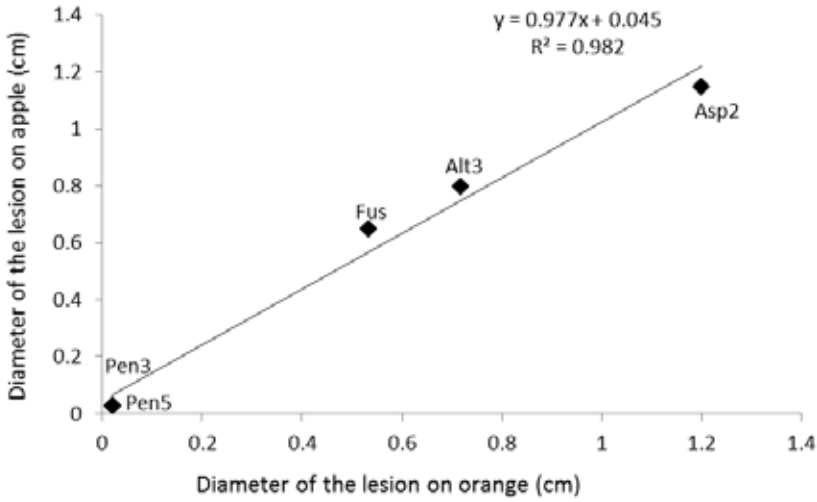

Figure 2. Correlation between mean lesion diameter on orange and apple fruits after 7 days of incubation at 24°C for five fungal isolates recovered from apple fruits stored in cold storage facilities in Tunisia in 2014. Each value represents the mean of four observations.

Table 3. Analysis of variance of the mycelia growth (cm) of five fungal species and 14 isolates within species recovered from apple fruits in cold storage facilities in Tunisia after 7 days of incubation on potato dextrose agar media at 24°C and at 4°C. Contrasts were shown between H'≤1 and H'>1 overall and, at 24°C independently.

Source of variation	Df	Sum Sq	Mean Sq	F value	Pr>F
Temperature	1	139.4	139.4	351.399	< 2e-16 ***
Species	4	0.52	0.13	0.328	0.858
Temperature x Species	4	12.97	3.24	8.174	1.63e-05 ***
Residuals	74	29.36	0.4		

Source of variation	Df	Sum Sq	Mean Sq	F value	Pr>F
Temperature	1	139.4	139.4	1198.184	< 2e-16 ***
Isolate within species	9	11.09	1.23	10.59	2.28e-09 ***
Temperature x Isolate within species	13	24.72	1.9	16.346	1.45e-14 ***
Residuals	56	6.52	0.12		

Contrasts (Pr>F):	
H'≤1 vs. H'> 1	<.0001
H'≤1 vs. H'> 1 at 4°C	0.6383
H'≤1 vs. H'> 1 at 24°C	<.0001

Df: Degrees of freedom; Sum Sq: Sum of squares; Mean Sq: Mean squares.

Table 4. Analysis of variance of the lesion diameter of five isolates recovered from apple fruits in cold storage facilities in Tunisia after 7 days of incubation at 24°C on apple and orange fruits (Host). Contrasts were shown between H'≤1 and H'>1 overall and, at 24°C independently.

Source of variation	Df	Sum Sq	Mean Sq	F value	Pr>F
Host	1	0.01	0.006	0.074	0.788
Isolate	4	42.14	10.535	136.319	< 2e-16 ***
Host x Isolate	4	0.01	0.002	0.021	0.999
Residuals	40	3.09	0.077		
Contrasts (Pr>F):					
H'≤1 vs. H'> 1	<.0001				
H'≤1 vs. H'> 1 on Apple	<.0001				
H'≤1 vs. H'> 1 on Orange	<.0001				

Df: Degrees of freedom; Sum Sq: Sum of squares; Mean Sq: Mean squares.

stored apples in Tunisia. In 2014, five fungal species associated with apples in cold storage conditions were identified (*Penicillium expansum, Alternaria* spp., *Botrytis* spp., *Aspergillus* spp. and *Fusarium* spp.). These fungi are also among the most widely reported pathogens in cold storage facilities around the world (Leibinger et al., 1997; Louw and Korsten, 2014; Pepejnjak et al., 2002; Romanazzi et al., 2016; Rosenberger, 1990). Because of their ubiquitous nature, broad host range in post-harvest conditions and necrotrophic lifestyle, they represent a significant economic threat (Rosenberger, 1990; Rosenberger et al., 2006; Sommer et al., 2002). According to our survey, Penicillium expansum was the most prevalent post-harvest fungus on apple in cold storage facilities in Tunisia, followed by *Alternaria* spp., *Botrytis* spp., *Aspergillus* spp. and *Fusarium* spp. The detection frequencies that we observed for these fungi reflect previous reports worldwide. Blue mold caused by P. expansum is the most common disease of post-harvest apples (Janisiewicz and Korsten, 2002; Leibinger et al., 1997; Louw and Korsten, 2014; Pepejnjak et al., 2002; Rosenberger, 1990). This decay impacts also fruit processing by the production of the carcinogenic mycotoxin patulin (Barkai-Golan, 2008). In the United States, gray mold caused by *Botrytis cinerea* is the main cause of post-harvest losses on pears, and the second most common disease on apples after blue mold (Rosenberger, 1990). The lower economic importance of *Fusarium* infections in storage facilities has also been reported (Snowdon, 1990). The low prevalence of *Fusarium* spp. in storage facilities suggests it is opportunistic on apples. A second survey performed in 2015 across eight cold storage facilities in Tunisia similarly revealed the prevalence of *Penicillium expansum* and *Alternaria* spp. on over 137 isolates sampled, with respective frequencies of 53% and 46% (Bahri et al., Unpublished results). In addition, *P. expansum* is a necrotrophic pathogen that has a saprotrophic phase on the fruit surface before initiating infections (Blakeman and Brodie, 1977). This saprotrophic phase gives *P. expansum* a postharvest survival advantage and explains its high prevalence under storage conditions. It is likely that primary infection by these fungi begins in the orchard, and presence or absence of a fungal species in a storage unit could be due to differences in orchard origin of fruits. Because the origin of fruits is unknown, we don't know the effect of apple growing conditions and protective stray practices in the different orchards on pathogen population variabilities in the storage facilities. However, out of 308 iso-

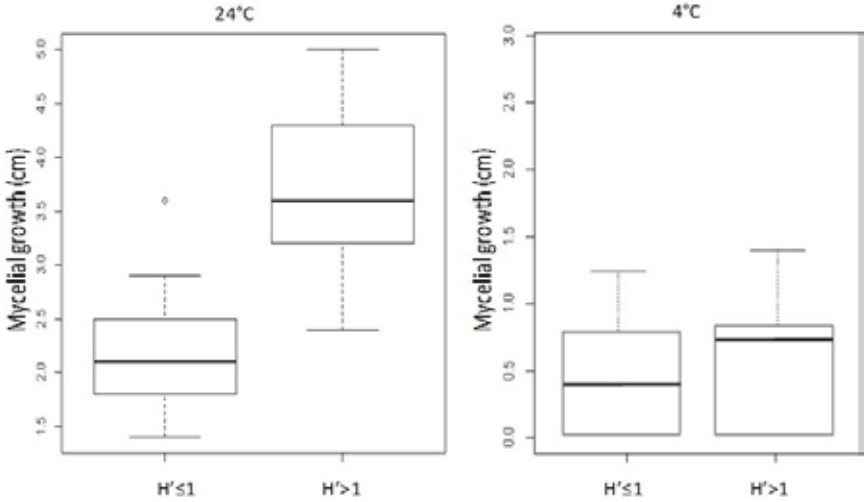

Figure 4. Box plots for mycelial growth by the diversity index H' for fungal isolates recovered from apple fruits in cold storage facilities in Tunisia in 2014. Isolates were grown at 24°C (left) and 4°C (right) for 7 days on potato dextrose agar media. The bottom and top of each box are the first and third quartiles of the data set, respectively. The band inside the box is the median. The ends of the dashed lines (whiskers) are the minimum and maximum observations.

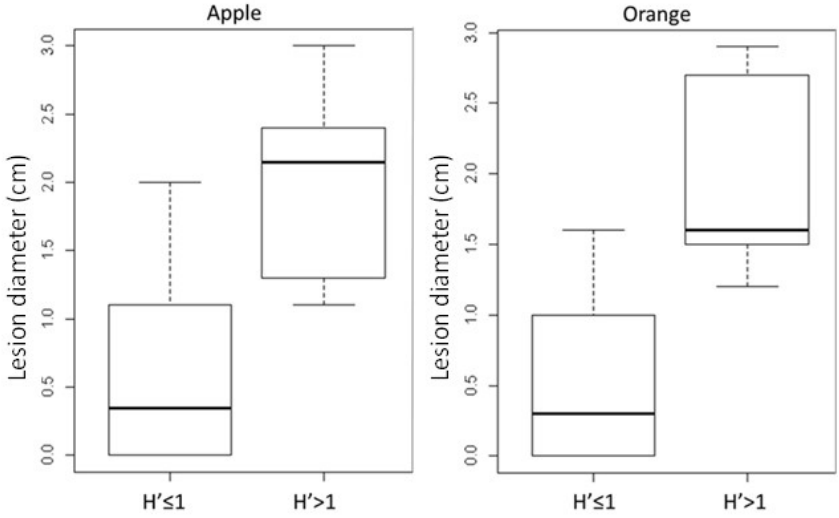

Figure 5. Box plots for lesion diameter influenced by the fungal species diversity index H' for apple (left) and orange (right) fruits after 7 days of incubation at 24°C with fungal isolates recovered from apples in cold storage facilities in Tunisia. The bottom and top of each box are the first and third quartiles of the data set, respectively. The band inside the box is the median. The ends of the dashed lines (whiskers) are the minimum and maximum observations.

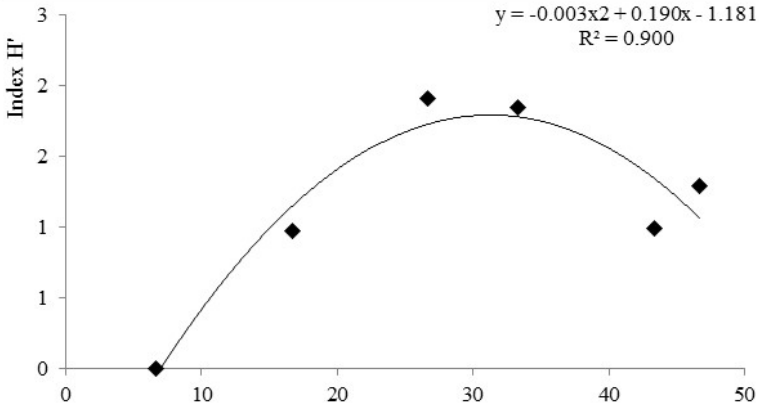

Figure 6. Relationship between the fungal species diversity index H' and disease incidence on apple fruits from six cold storages facilities in Tunisia in 2014.

lates sampled in 29 Tunisian apple orchards distributed over nine governorates, no variability in fungal species frequencies were observed across regions, and *Alternaria* sp. was the most prevalent fungus (96%) on symptomatic leaves although orchards differed in disease severity and fungicide application practices. Furthermore, fungicide treated orchards had similar levels of disease severity to untreated orchards (Bahri *et al., Unpublished results*). This study, based on 180 apples sampled from six storage units, provides the first pathogenic fungal assessment in cold storage facilities in Tunisia. However, a more exhaustive sampling could allow us to draw clearer conclusions on the relative proportions of these fungi in storage facilities.

In the current study, significant differences were observed between the isolates for mycelial growth on PDA and lesion diameter on apple and orange fruits incubated at 24°C. This variability could be explained by differences in storage conditions as well as differences in orchard origins. The growth traits of the isolates correlated significantly with the H' Shannon diversity index for fungi isolated from fruits in the different storage facilities. The only significant differences in mycelial growth at 24°C on PDA and in lesion diameters on apple and orange fruits were observed among isolates collected from storage facilities with H'> 1 compared to those from storage facilities with H'≤ 1. *P. expansum* isolates, Pen2, Pen6 and Pen4 and *Aspergillus* spp. isolates, Asp2 and Aps3, all derived from H'> 1 storage facilities, had mycelial growth that on average was 2 times greater than Pen5 and Aps1, respectively. Pen5 and Aps1 recovered from storage units with H'≤ 1. In addition, *Botrytis* spp. and *Alternaria* spp. isolates that were all recovered from H'> 1 storage units did not significant differ in mycelial growth at 24°C. Similarly, isolates Aps2, Aps3 and Fus, derived from H'> 1 storage units had a mean lesion diameter that was 28 times greater than that produced by Pen3 and Pen5. Pen3 and Pen5 both originated from storage units with H'≤ 1. Additionally, growth characteristics of the isolates were positively correlated. Specifically, there was a strong positive correlation between lesion diameter on apples and oranges for the tested isolates. Because lesion diameters on oranges were similar on apples, we think that the fungal species isolated from stored apple fruits are not opportunistic pathogens of orange in Tunisian storage conditions. Citrus, mainly orange, is usually processed with apples in the storage facilities. Hence, cross-contaminations are likely to occur exposing

citrus to typical post-harvest fungi such as *P. expansum* and *Alternaria* spp. (Louw and Korsten, 2014, 2015). In addition, *in vitro* mycelial growth of the isolates were correlated with their lesion diameters *in vivo*. Isolates collected from storage facilities with H'> 1 grew abundantly on PDA medium and had the highest pathogenicity in vivo on apples and oranges. Further analyses are needed to test the current hypothesis where the diversity in fungal species could determine potential pathogen pathogenicity in storages. In addition, sporulation rate is also an important life-trait component of pathogen fitness (Bruns et al., 2012; Pariaud et al., 2009). In storage units, infected fruits can produce inoculum and spores can be disseminated by cooling fans and contaminate neighbouring fruits (Janisiewicz and Korsten, 2002; Nair et al., 1995). Thus, the contribution of fungal sporulation to variation in frequency of disease infection in storage units should also be considered in future works. Because trade-off between virulence and transmission fungal traits was previously shown in several pathosystems (Ebert and Bull, 2003; Ewald and De Leo, 2002; Frank, 1996; Koella and Agnew, 1999), investigation on sporulation capacity could lead to contrasting findings compared to mycelial growth results shown in this study.

Our results suggest a trade-off between the fungal species diversity and disease incidence in the storage facilities for post-harvest apple pathogens. Storage units with a low and high disease incidence had low H' indices. The diversity in fungi in the storage facilities was highest at an average disease incidence of 30%. A high disease incidence in storage facilities can lead to strong competition between isolates that could select for a low diversity in phytopathogenic species. Our results may suggest that a competitive equilibrium between isolates in storage conditions must be achieved for the selection of isolates with high pathogenicity. This study highlights the importance of limiting the development of virulent isolates by reducing disease incidence in storage. The level of competition between isolates may be an important factor in phytopathogenic population structure (species diversity and virulence) in storage conditions. The classical theory of pathogen evolution predicts that within-host competition increases pathogen virulence (Ebert and Mangin, 1997). In fact, resource competition among strains was suggested to favor more virulent strains that transmit at faster rates (May and Anderson, 1983; May and Nowak, 1994). However, as in the current study, empirical studies do not always support the classical theory of pathogen evolution. Recent observations suggested that pathogen evolution in multiple infections differ from what the theory predicts and does not lead to an increase in virulence (Gower and Webster, 2005; Staves and Knell, 2010).

Although we observed significant differences among fungal isolates based on the parameters measured, most isolates from stored apple fruits showed intermediate levels of mycelial growth and pathogenicity. Virulence during the infectious phase is influenced by several evolutionary processes, and may be counter-balanced at other stages in the pathogen's life cycle to avoid an unlimited increase and stabilize around an intermediate value. Some studies showed that the most virulent phytopathogenic fungal isolates are not necessarily the fittest over one or several seasons (Flier et al., 1998). Thus, highly virulent isolates favored during infection conditions at 24°C in our study could be under negative selection pressure during fruit storage conditions. In fact, our results showed significant 'temperature x isolate' interaction and isolates with the highest mycelia growth at 24°C had the lowest mycelia growth at 4°C. In addition, the most fit isolates at 4°C had intermediate mycelial growth at 24°C. This can be explained by a trade-off between virulence in the field and survival in cold storage that would maintain the diversity in fungi isolates on stored apples in Tunisia. In natural systems, trade-offs explain the diversity in pathogen populations, the mainte-

nance of pathogenicity polymorphisms, and the counter-selection of highly virulent isolates (Ebert and Bull, 2003; Ewald and De Leo, 2002; Frank, 1996; Koella and Agnew, 1999). Pathogen adaptation to their host and to their environment also depends on genetic factors affecting their fitness components (Pariaud et al., 2009); these genetic factors could also explain the mycelial growth variations observed in this study. The diversity in pathogenicity of phytopathogenic fungi could also be driven by conditions in storage. In our study, storage facilities 1, 3 and 4 had H'> 1 and were characterized by the highest fruit storage capacity, the highest number of fruit suppliers and were probably the most diversified in fruit species stored. Some studies have also shown the influence of different storage conditions, e.i. pH and temperature of controlled atmosphere storages, on the decay caused by *P. expansum* in apples and the ability of the fungus to growth and produce patulin (Baert et al., 2007; Morales et al., 2007; Sydenham et al., 1995). Further analysis is needed to understand the diversity in fungal species and to determine the main factors explaining the maintenance of the variability in pathogen pathogenicity in Tunisian storages.

Conclusions

Results indicate that *Penicillium expansum* was the predominant pathogenic fungal species isolated in apples from cold storage facilities in Tunisia, followed by *Alternaria* spp., *Botrytis* spp., *Aspergilus* spp. and *Fusarium* spp. In addition, fungal diversity in a storage may impact the evolution of virulence in fungal pathogens. Isolates collected from storage facilities with highly diverse fungal species had significantly more mycelial growth at 24°C *in vitro* and larger lesion diameters on apples and oranges than isolates collected from storage facilities with less diversity. Storage facilities with a low or high disease incidence had the lowest diversity in fungal species. Thus, we recommend controlling disease incidence in cold storage facilities to limit the diversity in fungal species and the development of virulent isolates.

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