Archives of Microbiology
https://doi.org/10.1007/s00203-021-02551-9

ORIGINAL PAPER

An assessment of the air quality in apple warehouses: new records of *Aspergillus europaeus*, *Aspergillus pulverulentus*, *Penicillium allii* and *Penicillium sumatraense* as decay agents

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Received: 24 January 2021 / Revised: 19 August 2021 / Accepted: 22 August 2021

Abstract

Airborne fungi are one of the major components of aeromycobiota known to produce several fungal diseases in fruits. Their presence in indoor environment of warehouses may limit the storage period of apples. Qualitative and quantitative analyses of airborne fungal spores were conducted using gravity settling techniques to detect fungal airspora present in the atmosphere of two apple warehouses in Tunisia. In this study, 375 fungal isolates were obtained and purified. Phylogenetic analysis of calmodulin, beta-tubulin and ITS regions coupled with phenotypic characterization helped to identify 15 fungal species. *Penicillium* exhibited the highest diversity with ten species detected (*Penicillium allii*, *P. chrysogenum*, *P. citrinum*, *P. expansum*, *P. italicum*, *P. polonicum*, *P. solitum*, *P. steckii*, *P. sumatraense* and *P. viridicatum*), followed by four species of *Aspergillus* genus (*Aspergillus europaeus*, *A. flavus*, *A. niger* and *A. pulverulentus*) and *Alternaria alternata*. In vivo experiments confirmed the pathogenicity of 13 species at room temperature and under cold-storage conditions. Among them, *A. europaeus*, *A. pulverulentus*, *P. allii* and *P. sumatraense* were described for the first time as pathogens on apples. The present study identified the major airborne fungi associated with postharvest rot in apple storage facilities in Tunisia and may help in efficient control of postharvest and storage fruit diseases.

Keywords Postharvest disease · Cold rooms · Pathogenicity · Airspora · Fungi

Introduction

Postharvest fungal diseases are one of the significant factors that limit the shelf life of fruits (Iqbal et al. 2019; Zhang et al. 2019) by altering their organoleptic characteristics. Despite the use of improved storage technologies and plant protection products, fruit losses including apples are estimated to range up to 20–25% per year in developed countries (Nabi et al. 2017) but more severe in developing countries (Singh et al. 2017).

In Tunisia, apples are among the most produced and consumed fruits (ONAGRI 2018). Its production has increased from 98.300 tons in 2017 to 139.500 tons in 2018, which is an increase of 41% (GIFRUITS 2018). The governorate of Kasserine is considered as the most apple-producing region with 3 million apple trees followed by Siliana (30,150 tons) and greater Tunis (14,352 tons). The preservation of fruits in Tunisia depends heavily on the cold-storage industry. In fact, the agricultural sector holds more than 70% of the total refrigerated storage capacities, approximately 1100 m^3 are maintained to store fruits and vegetables.

Apple storage stations in Tunisia have reported significant losses in fruit quantity (Gara et al. 2019).

These losses are due to the occurrence of postharvest fungal diseases (Bahri et al. 2019). Postharvest diseases of apple are caused by a range of fungal pathogens (Sutton 2014). The most common and destructive postharvest

Communicated by Olaf Kniemeyer.

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Published online: 17 September 2021

disease of stored apples is the blue mold caused by *Penicillium expansum* (Spotts et al. 1999; Spadaro et al. 2010; Yu et al. 2014; da Rocha Neto et al. 2016; Darolt et al. 2016). Several other species such as *P. solitum* (Sholberg et al. 2005), *Penicillium griseofulvum* (Spadaro et al. 2011), *Penicillium carneum* (Peter et al. 2012), *P. crustosum* (Vico et al. 2014a,b), *Penicillium biourgeianum* (Azam et al. 2016) and *P. chrysogenum* (Rharmitt et al. 2016) have also been reported to be responsible for the rot.

Infected apples develop several other forms of rot such as Alternaria Rot caused by *Alternaria alternata* (Shtienberg 2012; Zhu et al. 2017), rot caused by *Aspergillus niger* and *A. flavus* (Hasan 2000a,b), grey mold by *Botrytis cinerea* (Romanazzi and Feliziani 2014), Bitter rot by *Colletotrichum acutatum* (Mari et al. 2012) and Bull's eye rot by *Neofabraea avagabunda* (Di Francesco et al. 2019). Wounds caused by insects and birds, as well as by physical damage occurring before or during harvest, are important entrance sites for pathogens (Wenneker and Thomma 2020). It has been reported that storage sites may represent a "reservoir" of airborne fungi (O'Gorman and Fuller 2008). These airborne fungi can cause devastating losses (Lucas et al. 1992). In addition, Fungi may produce mycotoxins such as patulin and trichothecenes in infected fruit and it may persist even if they are no longer present in food (Fernández-Cruz et al. 2010). Exposure to theses toxins pose health risk for consumers due to their properties to induce severe toxicity effects at low dose levels (Agriopoulou et al. 2020).

Since several fungal species can be responsible for apple decay, the detection and an accurate identification of pathogens represent a preliminary step to take to control postharvest disease. Conventional methods for fungal identification involve the examination of morphological characteristics on standardized media such as color and texture of colonies, growth rate and size of colonies and microscopic structures. Phenotypic characterization may be influenced by various environmental factors such as temperature, humidity and oxygen level (Tiwari et al. 2011) leading to misidentification of the isolates.

Nowadays, fungal pathogens are identified using molecular methods alongside morphological characterization. The internal transcribed spacer rDNA sequence (ITS) is the most widely sequenced marker for fungi. Universal primers are available, and it is the official sequence for barcoding (Schoch et al. 2012). Different gene regions have been also used and showed potential to make a clear distinction between closely related fungal species such as calmodulin (CaM) and beta-tubulin (benA) (Samson et al. 2014; Visagie et al. 2014).

A comprehensive research on the diversity and pathogenicity of airspora associated with stored apple is needed to develop efficient control of fungal disease in storage units. However, there is a lack of information about the airspora profile in indoor warehouses of apple in Tunisia.

Therefore, the aim of this study was to investigate the predominant of airborne fungi occupying the atmosphere of two cold-storage stations of apples in Tunisia, and to identify the obtained airspora using phenotypic and molecular tools and study their pathogenicity.

Materials and methods

Sample collection

Sample collection was undertaken from two cold-storage facilities of apples located in Ben Arous, Tunisia for 3 months (December 2016, February and April 2017). Station 1 (S1) and station 2 (S2) had three and seven refrigerated rooms, respectively. Airborne fungi were isolated using a calibrated impaction sampler MAS-100 Eco® (MBV, Switzerland). The MAS-100 Eco® was mounted in the center of each room, approximately 1 m from the floor. Two agar plates were taken from each room and the viable particles were impacted on the surface of potato dextrose agar plates, amended with chloramphenicol. Samples were carried to the laboratory and incubated at 25 °C for 7 days (Despot and Klaric 2014). Fungal colonies were counted and converted to CFU/m^3 using Feller's formula given by the manufacturer (MBV, Switzerland):

$$(\mathrm{Pr} = N\ 1/N\ +\ 1/N-1\ +\ 1/N-2\ +\ 1/N-r+1),$$

where Pr is the probable statistical total; r is the Number of CFU counted; N is the total number of holes in the sampling head.

Isolates grouping and selection of representative isolates

Since the number of isolates were large, fungi were grouped based upon their micro- and macro-morphological characteristics using standard taxonomic keys (Dugan 2006; Johnston 2008). Each group was assigned a number and a representative isolate was randomly chosen for molecular analysis.

Morphological identification

Spore suspension were made with a solution containing 0.2% agar and 0.05% tween 80 solution as recommended by Visagie et al. (2014) to obtain monosporic colonies. Macroscopic characteristics were studied on Czapek yeast autolysate agar (CYA), yeast extract sucrose agar (YES), malt extract agar (MEA) and potato dextrose agar (PDA) media (Rotem 1994; Samson et al. 2014; Visagie et al. 2014). The

studied characteristics were the colony size, colony color and texture. Microscopic observations were made from 7 to 10 days cultures grown on MEA media using light microscope (Leica).

DNA extraction

The genomic DNA was extracted from 7-day-old fungal cultures grown in culture plates. Using a sterilized scalpel, a piece of mycelia was transferred to a 1.5 mL Eppendorf tube containing 500 µL of lysis buffer (400 mM Tris–HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate). The tube was then maintained at room temperature for 10 min. After adding 150 µL of potassium acetate buffer (pH 4.8; 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of distilled water), the tube was vortexed for 10 min. and spun at 12,000 rpm for 2 min. and 11,000 rpm for 5 min. The supernatant was transferred to another Eppendorf tube and centrifuged again at 12,000 rpm for 2 min. An equal volume of isopropanol was added to the supernatant, recovered and mixed together by inversion. After centrifuging at 12,000 for 2 min, the DNA pellet was washed in 300 µL of 70% ethanol and spun at 12,000 rpm for 2 min. After removing the supernatant, the DNA pellet was air dried and dissolved in 50 µL of 1 × Tris–EDTA (Liu et al. 2000).

PCR amplifications

Calmodulin (Cam), beta-tubulin (β-tub) genes were targeted to design a specific primer set for isolates belonging to *Penicillium* (PCAM 1, 5′-AGGACAAGGATGGCGATGG-3′; PCAM 2, 5′-TCGTCGGTCAGCTTCTC-3′) and *Aspergillus* (ABEN 1, 5′-AGTCCGGTGCTGGTAACAACTGG-3′; ABEN 2, 5′-TCCTGGTACTGCTGGTACTCG-3′), respectively, using Vector NTI software.

These primers generated amplicon lengths of ≈500 and ≈ 900 bp. Specificity tests were conducted amplifying DNA from different fungal genera commonly associated with apple decay. All PCR amplifications were performed in a total reaction volume of 50 mL consisting of 1 µL DNA template, 5 µL of 10 × PCR buffer, 1.5 mL of 50 mM $MgCl_2$, 1 µL of dNTP (2.5 mM each), 3 µL of each primer, 1 µL of Taq DNA Polymerase and 34.5 µL of H_2O. The PCR cycle condition was performed in a thermal cycler (Biometra, Germany) with an initial denaturation step for 2 min at 92 °C, followed by 25 cycles (1 cycle consisting of denaturation for 30 s at 92 °C, annealing for 30 min at 57 °C and extension for 1 min at 72 °C), with a final extension of 10 min at 72 °C. An Internal Transcribed Spacer (ITS) gene was used to identify isolates belonging to *Alternaria* genus using universal primers ITS4 and ITS5 (White et al. 1990). The same PCR mix described previously was also used. Template DNA was initially denatured for 2 min at 94 °C followed by 25 cycles of amplification. Each cycle consisted of denaturation for 30 s at 94 °C, primer annealing for 30 s at 56 °C and extension for 1 min at 72 °C. The last cycle was followed by a final extension at 72 °C for 10 min. Amplicons were subjected to electrophoresis in 0.8% agarose gels.

Sequencing and phylogenetic analysis

The PCR products were purified using a PCR purification column (Macherey-Nagel) and sent for sequencing. The sequences of each gene (i.e., Cam, β-tub and ITS) were, firstly, compared using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and they were deposited in the GenBank. Then, phylogenetic trees topology was inferred by the maximum likelihood, neighbor-joining, and maximum-parsimony method using MEGA v7.0 (Kumar et al. 2016) with bootstrap values based on 1000 replications. *Aspergillus flavus* and *P. expansum* were used as outgroups.

Pathogenicity tests

To verify the pathogenicity potential of detected isolates, three mature healthy apples (cv. Golden delicious) with uniform size and without physical injuries were used to perform a pathogenicity test as described by Louw and Korsten (2014). All fungal isolates were grown on MEA for 7 days at 25 °C. Conidia were scraped from the culture and spore concentration was adjusted to 10^5 spores/mL. Apples were disinfected for two min in 2% sodium hypochloride solution, sterilized with 70% alcohol and rinsed three times with sterile distilled water. Next, two apples were wound-inoculated with 20 µL of conidial suspension on three sides per isolates and were placed on wet paper towels in plastic containers sealed with parafilm. Control fruit were inoculated with sterile distilled water and all the boxes were stored at 25 °C for 7 days in the darkness. All experiments were repeated three times.

Cold-storage trials

To describe pathogen behavior under storage conditions, mature healthy apples (cv. Golden delicious) were surface disinfested with 70° alcohol and then gently wounded with a sterile micropipette tips on three sides. A spore suspension (10^5 conidia/mL), from each isolate, was prepared from 10-day-old culture then inoculated to apples while, controls were inoculated with sterile distilled water. Three replicates were put in sealed container and incubated under refrigerated conditions (2 ± 1.7 °C) for 43 days in the dark. All the experiments were repeated three times. During the incubation period, the lesion diameters were recorded every second

day starting from the third day after inoculation as described by Louw and Korsten (2014).

Statistical analysis

SPSS program v21 was used for descriptive statistics. Concentrations of airborne fungi are represented as mean ± SD of CFU/m^3. The likelihood of statistically significant differences between the concentrations of airborne fungi measured in each sampling was assessed by one-way analysis of variance (ANOVA). Mean comparison of lesion diameter at storage conditions generated by 15 species was estimated according to Duncan test. Statistical significance was set at 0.001. Pathogenicity trials were conducted with two replications under a complete factorial design with two factors (species and incubation time).

Results

Fungal concentration

The study showed that fungal spores were present throughout the storage period in all sampled cold rooms in both stations. Concentrations of airborne fungi in CFU/m^3 found in both stations during three months of sampling are listed in Table 1.

In this study, the maximum measured concentration was 2228 CFU/m^3 and the lowest was 23.67 CFU/m^3.

In S1, the highest spore incidence (847.16 CFU/m^3) was recorded in N7 followed by N5 (643.5 CFU/m^3) and N4 (563.33 CFU/m^3). For S2, SF3 holds the lowest airborne fungal concentration with an average of 36.6 CFU/m^3 followed by SF1 with 121.33 CFU/m^3. As indicated in Fig. 1. Spore concentration in the atmosphere of all the sites peaked during the third month. Levels of airborne fungi in April were significantly higher than the first 2 months ($p < 0.001$) reaching 1071.14 and 177.33 CFU/m^3 for S1 and S2, respectively (Fig. 2).

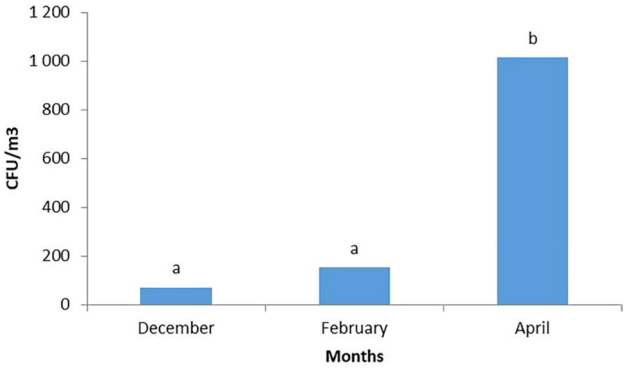

Fig. 1 Concentration of total airborne fungi in CFU/m^3 measured during 3 months of sampling in two apple storage facilities in Tunisia using MAS-100Eco® air monitoring system in S1. Letters that are dissimilar are significantly different (a, b)

Phenotypic characterization of collected airspora

Based on morphological features, 375 colonies were obtained from both locations. The obtained isolates were grouped into 21 morphologically similar groups. According to cultural and morphological characteristics, *Penicillium*, *Aspergillus* and *Alternaria* isolates presented 15, 4 and 2 different groups, respectively (Table 2).

The morphological characterization of representative isolates selected for identification is presented in Tables 3 and 4. In CYA, the diameter of the colonies ranged between 25 and 3 mm for *Penicillium* spp. and 35–65 mm for *Aspergillus* spp. Conidia of all examined penicillia were colored

Table 1 Mean total fungal spore concentration (CFU/m^3) measured at two apple's storage stations in Tunisia

Station	Room	December	February	April	Total (CFU/m^3)
Station 1	Room N1	60.5 ± 6.36	404 ± 67.9	287 ± 4.24	250
	Room N2	50.5 ± 7.78	78.5 ± 9.19	303 ± 18.38	144
	Room N3	117.5 ± 7.78	50.5 ± 0.7	906.5 ± 833.68	358
	Room N4	19.5 ± 6.36	280 ± 7.07	1390.5 ± 1184.4	563
	Room N5	66.5 ± 9.19	72 ± 9.9	1792 ± 616.6	643
	Room N6	44 ± 4.24	78.5 ± 12.02	583 ± 149.9	235
	Room N7	157 ± 9.9	156.5 ± 7.78	2228 ± 0	847
Station 2	Room SF1	32 ± 3.6	42.33 ± 2.51	289.7 ± 6.11	121
	Rom SF2	124.67 ± 5.69	166.67 ± 4.93	182.33 ± 12.22	157
	Room SF3	23.67 ± 1.15	26 ± 2.64	60 ± 6.56	36

[Mean±SD]; all numbers are positive-hole transformed to account for multiple depositions of particles at single impaction sites

N1–N7, 7 refrigerated rooms sampled belonging to station 1; SF1–SF3, 3 refrigerated rooms sampled belonging to station 2

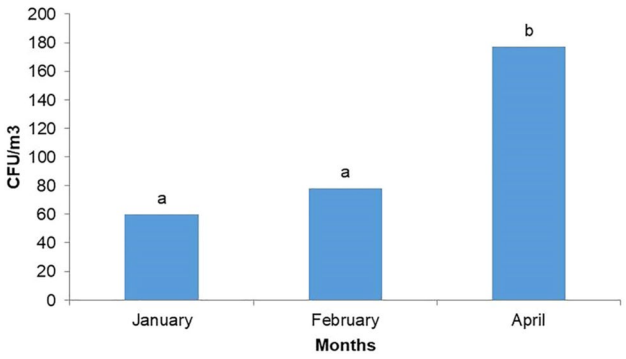

Fig. 2 Concentration of total airborne fungi in CFU/m^3 measured during 3 months of sampling in two apple storage facilities in Tunisia using MAS-100Eco® air monitoring system in S2. Letters that are dissimilar are significantly different (a, b)

Table 2 Frequency of fungal genera and groups selected for identification

Fugal genera	Number of isolates	Frequency (%)	Phenotypic groups
Penicillium spp.	288	76.8	15
Aspergillus spp.	54	14.4	4
Alternaria spp.	21	5.6	2
Other fungi	12	3.2	_
Total	375	100	21

in shades of grey–green, blue–green or dull green. In the other hand, *Aspergillus* isolates produced yellowish to grey, green or well-marked black conidia (Table 4). After 1 week of incubation at 25 °C, Sporulation of *Penicillium* isolates were observed on YES. Thirteen samples have a strong sporulation on more than 90% of the colony. C5B1 and C7A29 presented a moderate sporulation; only in the center of the colony. MEA provided the growth of isolates with different colony textures. In this survey, *Penicillium* sp. presented velutinous, fasciculate and floccose colony texture. Color of conidium varied between green, grey–green, blue–green or dull green. *Aspergillus* spp. presented only velutinous and floccose colonies with diameters ranged between 24 and 56 mm. Microscopic examination showed that the conidiophores of *Penicillium* isolates were one-stage branched (biverticillate) or two-stage branched (terverticillate) with metula length between 7.1 and 14 µm. Examined Conidia presented nearby diameters (2.3–3.7 µm) and were smooth walled except two isolates (S8 and D2) that were finely rough (Table 3). All of *Aspergillus* isolates had biseriate conidial heads. Conidia observed ranged between 3.5 and 4.7 µm. Ornamentation of spores was rough in C7A16 and C6B6 echinulate in C7A13 and verruculose in C5B26 (Table 4). On PDA, *Alternaria* isolates presented olivaceous black and black colonies. Conidiophores were branched and conidia were formed in long ovoid chains.

Molecular identification and phylogenetic analysis

Partial amplification of Cam and β-tub was successful in all tested isolates and yielded a single amplicon band of approximately 500 and 900 bp in gel electrophoresis, respectively. Each primer set (PCAM 1–PCAM 2) and (ABEN 1–ABEN 2) were only able to amplify isolates belonging to *Penicillium* and *Aspergillus* genus, respectively, thus confirming

Table 3 Morphological characterization of *Penicillium* groups detected in indoor air of storage facilities

Group	Isolate code	CYA	YES	MEA	Phialide	Conidia	Metula	Branching pattern
1	C7B20	38 mm, dull green	Strong	Fasiculate, grey–green	9.7 µm	3.7 µm, smooth	11.8 µm	Ter-verticillate
2	C5B1	40 mm, grey–green	Moderate	Velutinous, grey–green	10 µm	2.7 µm, smooth	8.1 µm	Bi-verticillate
3	C1A25	30 mm, dull green	Strong	Fasiculate, grey-green	6 µm	2.5 µm, smooth	11.5 µm	Ter-verticillate
4	C4A4	39 mm, blue–green	Strong	Floccose, green	7.7 µm	2.4 µm, smooth	10 µm	Ter-verticillate
5	C5A10	50 mm, grey–green	Strong	Velutinous, green	10.9 µm	4.9 µm, smooth	12.4 µm	Ter-verticillate
6	C6A1	30 mm, dark–green	Strong	Velutinous, blue–green	8.1 µm	2.9 µm, smooth	9.2 µm	Bi-verticillate
7	C2B12	52 mm, green	Strong	Floccose, green	7.7 µm	2.3 µm, smooth	14 µm	Ter-verticillate
8	D2	40 mm, Pure green	Strong	Velutinous, green	8.3 µm	2.9 µm, finely rough	12.8 µm	Ter-verticillate
9	C2A8	38 mm, blue–green	Strong	Velutinous, blue–green	8.9 µm	3.7 µm, smooth	11.9 µm	Ter-verticillate
10	C7A25	30 mm, dull green	Strong	Velutinous, blue-green	8 µm	3.3 µm, smooth	10.9 µm	Ter-verticillate
11	S7	38 mm, blue–green	Strong	Velutinous, blue–green	6.4 µm	2.2 µm, smooth	7.8 µm	Bi-verticillate
12	C7A29	27 mm, blue–green	Moderate	Velutinous, blue–green	9 µm	3.5 µm, smooth	11.6 µm	Ter-verticillate
13	C4A1	25 mm, grey–green	Strong	Velutinous, dull green	8.2 µm	2.4 µm, smooth	7.1 µm	Bi-verticillate
14	S8	18 mm, green	Strong	Velutinous, green	8.2 µm	2.8 µm, smooth	12.6 µm	Ter-verticillate
15	C7A1	24 mm, green	Strong	Fasiculate, green	7.8 µm	2.3 µm, smooth	14 µm	Ter-verticillate

CYA colony diameter, color of conidium; *YES* degree of sporulation; *MEA* colony texture, color of conidium

Table 4 Morphological characterization of *Aspergillus* groups detected in indoor air of apple storage facilities

Group	Isolate code	CYA	MEA	Conidia	Vesicles
1A	C7A16	35 mm, yellow–grey	24 mm, floccose	4.5 µm, rough	Biseriate
2B	C7A13	58 mm, yellow–green	56 mm, velutinous	3.5 µm, echinulate	Biseriate
3C	C6B6	65 mm, dark brown	50 mm, velutinous	4 µm, rough	Biseriate
4D	C5B26	63 mm, dark brown	49 mm, velutinous	4.7 µm, verruculose	Biseriate

CYA colony diameter, color; *MEA* colony diameter, texture

their specificity. Using (ITS4-ITS5) primers, PCR generated amplicons around 600 bp (data not shown). BLAST analysis of the calmodulin, the beta-tubulin and ITS sequences showed that there was 100% of sequence similarity of isolate C7A29 with *Penicillium solitum* CBS 424.89, isolate S7 with *Penicillium chrysogenum* CBS 109613, isolate C4A1 with *Penicillium steckii* CBS 139.45, isolate C6A1 with *Penicillium sumatraense* CBS 281.36, isolate C5A10 with *Penicillium italicum* CBS 339.48 and C5B1 with *Penicillium citrinum* CBS 139.45, isolate C7A16 with *Aspergillus europaeus* CCF 3365, isolate C7A13 with *Aspergillus flavus* NRRL3357, isolate C6B6 with *Aspergillus niger* CBS 513.88, isolate C5B26 with *Aspergillus pulverulentus* CBS 558.65 (Figs. 3, 4).

Phylogenetic analysis based on the maximum-likelihood and maximum-parsimony methods of calmodulin and beta-tubulin sequences revealed trees with similar topology as trees obtained in neighbor-joining analyses (Supplementary material Figure S1 and Figure S2). Isolate S7 was closely related with *P. chrysogenum*. However, the sequence from isolate S7 differed from the sequence of *P. chrysogenum* CBS: 109613 at two nucleotide positions. Moreover, isolate C6A1 and reference strain of *P. sumatraense* CBS 281.36 were clustered together in a group, although the sequence of C6A1 isolate differed from the sequence of *P. sumatraense* CBS 281.36 at two nucleotide position.

Species including *Penicillium expansum* (represented by three strains C4A4, C2B12 and C7A1), *Penicillium allii* (represented by two strains C7B20 and C1A25), *Penicillium viridicatum* (represented by two strains D2 and S8) and *Penicillium polonicum* (represented by two strains C2A8 and C7A25) were phylogenetically distinct from other species and orthologs to their reference strains (CBS 325.48, AS3.6669, SZMC 23186 and CBS 222.28, respectively) (Fig. 3). On the basis of DNA polymorphism analysis of calmodulin sequences, the latter species (*P. expansum*, *P. allii*, *Penicillium viridicatum* and *P. polonicum*) formed four different haplotypes when submitted to DnaSP software (Supplementary material Table S1). Haplotype 1 was the most frequent haplotype and consisted exclusively of 4

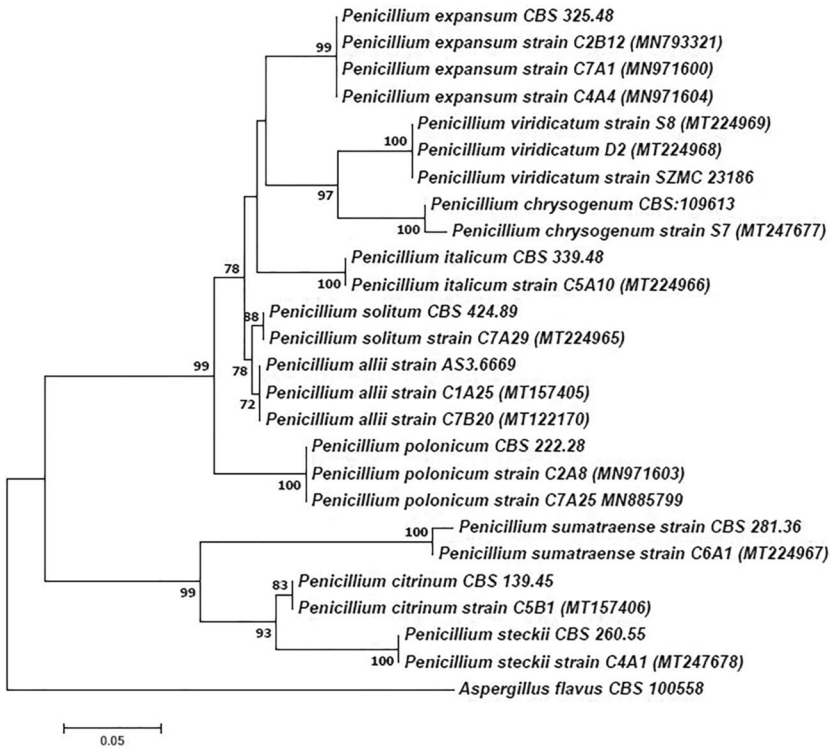

Fig. 3 Neighbor-joining tree based on calmodulin sequences showing the phylogenetic relationship between Penicillium strains. Bootstrap values (expressed as percentages of 1000 replications) > 70% are given at the nodes

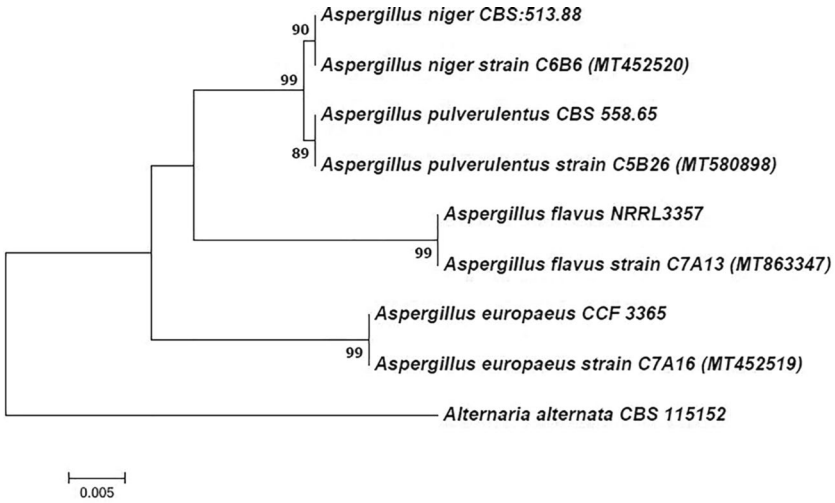

Fig. 4 Neighbor-joining tree based on beta-tubulin sequences showing the phylogenetic relationship between Aspergillus strains. Bootstrap values (expressed as percentages of 1000 replications) > 70% are given at the nodes

sequences of *P. expansum* (C4A4, C2B12, C7A25 and *P. expansum* type strain CBS 325.48). Haplotype 5, Haplotype 6 and Haplotype 10 corresponded to a set of 3 sequences of *P. polonicum* (C2A8, C7A25 and *P. polonicum* type strain CBS 222.28), *P. allii* (C7B20, C1A25 and *P. allii* type strain AS3.6669) and *P. viridicatum* (D2, S8 and *P. viridicatum* type strain SZMC 23186), respectively (Supplementary material Table S2). The genetic relationship of these haplotypes was graphically displayed by the program Network version 4.5.0.0 (Supplementary material Figure S3).

Based on sequence analysis of the ITS, SPS1 and SPS2 were identified as *Alternaria alternata* species complexes (MT126617 and MT126618). Phylogenetic analysis was not conducted for *Alternaria* isolates because only two strains were amplified by ITS4 and ITS5. The calmodulin, beta-tubulin and ITS sequences were deposited in the GenBank database and their accession numbers are listed in Table 5.

Table 5 Sequence identification results for phenotypic groups analyzed in this study

Groups	Sequence identification	Code isolate	Accession number	Frequency (%)
(1–3)	*Penicillium allii*	C7B20	MT122170	12.8
		C1A25	MT157405	
2	*Penicillium citrinum*	C5B1	MT157406	1
(4–7–15)	*Penicillium expansum*	C4A4	MN971604	42
		C2B12	MN79332	
		C7A1	MN971600	
5	*Penicillium italicum*	C5A10	MT224966	1.7
6	*Penicillium sumatraense*	C6A1	MT224967	3.5
(8–14)	*Penicillium viridicatum*	D2	MT224968	7.6
		S8	MT224969	
(9–10)	*Penicillium polonicum*	C2A8	MN971603	16.7
		C7A25	MN885799	
11	*Penicillium chrysogenum*	S7	MT247677	8.7
12	*Penicillium solitum*	C7A29	MT224965	4.9
13	*Penicillium steckii*	C4A1	MT247678	1
1A	*Aspergillus europaeus*	C7A16	MT452519	11.1
2B	*Aspergillus flavus*	C7A13	MT863347	20.4
1C	*Aspergillus niger*	C6B6	MT452520	53.7
3D	*Aspergillus pulverulentus*	C5B26	MT580898	14.8
(1a–2b)	*Alternaria alternata*	SPS1	MT126617	5.6
		SPS2	MT126618	

Biodiversity of aerospores in apple warehouses

The biodiversity of fungal species collected from the air of apple warehouses is listed in Table 5. Fifteen fungal species attributed to three genera were isolated. *Penicillium*, *Aspergillus* and *Alternaria* were the most common genera collected with different frequencies (Table 2). In which *Penicillium* was represented by ten species, followed by *Aspergillus* (four species), and one *Alternaria* species.

In this survey, *Penicillium* was by far the most common genus detected with a frequency of 76.8%. *P. expansum* was the most abundant specie with 42% followed by *P. polonicum* (16.7%), *P. allii* (12.8%), *P. chrysogenum* (8.7%), *P. viridicatum* (7.6%), *P. solitum* (4.9%), *P. sumatraense* (3.5%) and *P. italicum* (1.7%). *P. citrinum* and *P. steckii* occurred less frequently than the other species (1%).

Aspergillus was the second most common identified fungal genera with a frequency of (14.4%). *Aspergillus niger* was the most identified species with 53.7% followed by *A. flavus* (20.4%), *A. pulverulentus* (14.8%) and *A. europaeus* (11.1%).

Alternaria, represented by *Alternaria alternata*, ranked third in abundance in indoor atmosphere of apple warehouses with (5.6%).

In vivo experiments

Undescribed fungal pathogen to apple fruit

Ten species were found to be pathogenic at room temperature and under storage conditions. Among them, four undescribed fungal pathogen to apple fruit were observed and were able to cause decay in healthy apple fruit: *A. europaeus*, *A. pulverulentus*, *P. allii* and *P. sumatraense*. Lesions provoked by the later species varied in appearance and presented a light- to dark-brown color and wet to dry texture (Fig. 5).

Decayed area caused by *P. allii* and *P. sumatraense* began as water-soaked lesions and enlarged gradually in diameter and developed into brown and soft lesions. The pathogens invaded apples tissue slowly in the early stage and blue–green spores were formed on lesion surface in the late stage, starting at the infection sites.

Aspergillus europaeus and *A. pulverulentus* provoked brown and medium-soft lesions on infected apples. As the decayed area ages, they turned to dark brown slowly. Infected apples have an earthy odor and no apparent sporulation was observed along experiments in the inoculation spots.

The inner decayed tissue of inoculated apples appeared yellowish brown. Cross section of mold lesion on apples showed a sharp margin between decayed and healthy tissue.

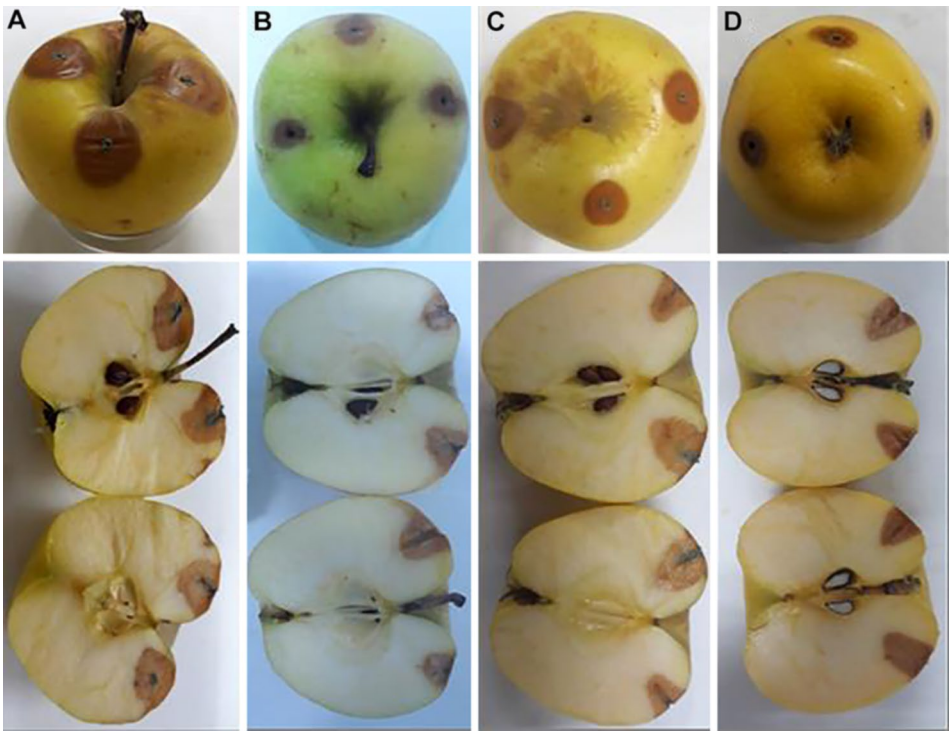

Fig. 5 Golden delicious apples inoculated with **A** *P. allii*; **B** *A. pulverulentus*; **C** *P. sumatraense* and **D** *A. europaeus*. Line 1 showing symptom of mold of the four corresponding pathogens on apple, Line 2 showing cross-section of mold lesion on apple for each pathogen

Aspergillus europaeus presented a V-shaped lesions expanding towards the core of the apple.

Pathogenicity of *Penicillium*, *Aspergillus* and *Alternaria* isolates on apples

In ambient conditions, all three repeats produced similar pathogenicity results. The most devastating species were *P. expansum* (C4A4) and *P. polonicum* (C2A8), generating a decay area of 37 mm after 7 days post-inoculation, followed by *P. allii* (C7B20) and *A. flavus* (C5B26) with 16 mm. *A. alternata* (SP1) and *P. solitum* (C7A29) had a moderate pathogenicity and presented diameters of 12 mm. Slightly brown lesions of 8 and 7 mm were produced by *A. pulverulentus* (C5B26) and by *P. viridicatum* (D2), respectively. *Penicillium italicum* (C5A10) and *A. niger* (C6B6) were weak pathogens and they generated the smallest decay area among all tested species (4 mm).

Differences in terms of days to significant infection and diameters of lesions were observed among species, during cold-storage trials. Decay began to develop 5 days after inoculation for some isolates. None of the inoculated apples presented sporulation or mycelia growth on injected wounds excluding *P. expansum*, which started to develop white mycelium on day 37. Pathogenicity varied within the investigated strains and five levels were described according to days where isolates presented a significant infection. *P. expansum*, *P. polonicum*, *P. solitum*, *P. italicum* and *A. flavus* showed the first significant lesions on the fifth and the seventh day, respectively, and presented a severe pathogenicity. *P. viridicatum* and *P. pulverulentus* started to provoke lesions that were classified as moderately severe on the 9th and 11th day, respectively. After 13 and 15 days post-inoculation, pathogenicity was described as moderate for *A. europaeus*, *P. sumatraense* and *P. chrysogenum.* Since the 17th day, pathogenicity was considered mild for the four following species: *A. niger*, *Alternaria alternata* and *P. allii* (Table 6). *P. citrinum* and *P. steckii* were not pathogens to apples and did not present any lesions throughout the test period. According to the Duncan test, values of mean lesions diameters of tested species were significantly different ($p < 0.001$) and presented 11 different groups of fungi over the storage period (Table 6). *Penicillium expansum* generated the highest mean lesion size (18.31 mm) followed by *P. solitum* (9.42 mm). The lowest decay area was recorded in *A. niger* and *P. chrysogenum* (2.77 and 2.82 mm, respectively). The experiment was stopped on the 43rd day due to total decay of fruit by *P. expansum*. Among tested isolates, *P. expansum* was the only species able to provoke large lesions reaching 35 mm on apples. *Penicillium solitum* was able to generate lesions; however, they were smaller than *P. expansum* (15.6 mm) followed by *P. sumatraense* (11 mm). All three repeats produced similar pathogenicity results during cold trials.

Table 6 Pathogenicity trials at room temperature storage conditions

Species	Days to significant infection	Pathogenicity	Mean lesion size at day 43 (mm)	Mean lesion size during incubation at 4 °C (mm)	Mean lesion size during incubation at 25 °C (mm)
Penicillium citrinum	–	–	0	0 a	0 a
Penicillium steckii	–	–	0	0 a	0 a
Aspergillus niger	17	+	6.33	2.77 b	4 b
Penicillium chrysogenum	15	+ +	5.5	2.82 b	0 a
Penicillium viridicatum	9	+ + +	4.67	3.28 cd	7 c
Alternaria alternata	17	+	8.83	3.33 cd	12 d
Penicillium allii	17	+	6.3	3.46 d	16 e
Aspergillus pulverulentus	11	+ + +	7.17	3.78 e	8 c
Aspergillus europaeus	13	+ +	9.17	4.14 f	0 a
Penicillium sumatraense	13	+ +	11.00	5.14 g	0 a
Penicillium italicum	7	+ + + +	7.33	5.44 h	4 b
Penicillium polonicum	5	+ + + +	9.00	5.87 i	37 f
Aspergillus flavus	7	+ + + +	11.00	6.83 j	16 e
Penicillium solitum	5	+ + + +	15.6	9.42 k	12 d
Penicillium expansum	5	+ + + +	35.33	18.31 l	37 f

*Values with different letter are significantly different

– No lesion observed along test period

Scale for pathogenicity: severe (++++), moderately severe (+++), moderate (++), mild (+), (−) none pathogen

Taxonomic description

Taxonomic description, photos of colonies, and fungal structures of *P. allii*, *P. sumatraense*, *A. pulverulentus* and *A. europaeus* which are undescribed pathogen to apple fruit and novel to Tunisia are given below and presented in Figs. 6, 7, 8 and 9.

Aspergillus europaeus Hubka, A. Nováková, Samson, Houbraken, Frisvad& M. Kolařík (Fig. 6)

Pl. Syst. Evol.:645 (2016)

Isolate that was examined was C7A16.

Colonies on MEA at 25 °C, after 7 days: 28 mm, floccose, with raised colony center, colony surface delicately granular, mycelium yellowish grey, sporulation on almost entire surface, reverse brilliant yellow with greyish yellow tint in the colony center. Colonies on CYA at 25 °C, after 7 days: 38 mm, floccose, yellowish white, sporulation yellow, poorly sporulate after 7 days, no exudate, no soluble pigment and reverse yellowish white colony.

Stipes are colorless, smooth, occasionally longer; vesicle biseriate; metulae broadening toward the top, 15 µm long, phialides ampulliform, 9.1 × 5 µm; conidia colorless, globose, coarsely roughened, 4.2 µm.

Notes: Colony characteristics and micromorphology of MT452519 agreed well with the description of *A. europaeus* (Hubka et al. 2016). *Aspergillus europaeus* bears some resemblance to *A. wentii* and *A. dimorphicus*. But the later species have globose or subglobose vesicles in contrast to pyriform vesicles of *A. europaeus*.

Aspergillus pulverulentus (McAlpine) Wehmer (Fig. 7)

Centralbl Bacteriol 2. Abth.18: 394 (1907).

≡ *Sterigmatocystis pulverulenta* McAlpine, AgricGaz New South Wales 7: 302 (1897).

Isolate that was examined was C5B26.

This strain develops quickly on CYA, produces colonies 35–40 mm in diameter after 7 days at 25 °C. Texture is velvety, surface black, reverse white-cream. Basal mycelium is white, exudates are small and colorless. Conidial heads are blackish grey, characteristically radiate. Colonies on MEA developed rapidly and reached 50 mm in diameter at 25 °C in 7 days. Texture is velvety, surface brown and black, reverse color white; colorless exudates.

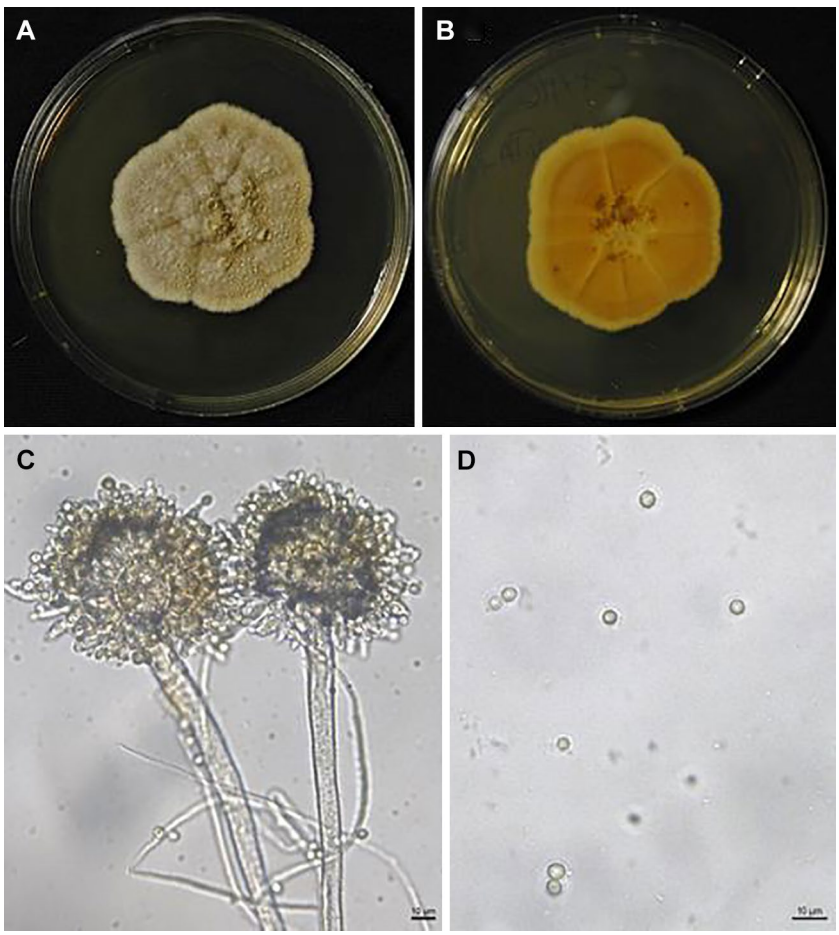

Fig. 6 Macromorphology and micromorphology of *Aspergillus europaeus* after 7 days of incubation at CYA, **A** obverse, **B** reverse, **C** Conidiophores, **D** Conidia. Scale bar = 10 µm

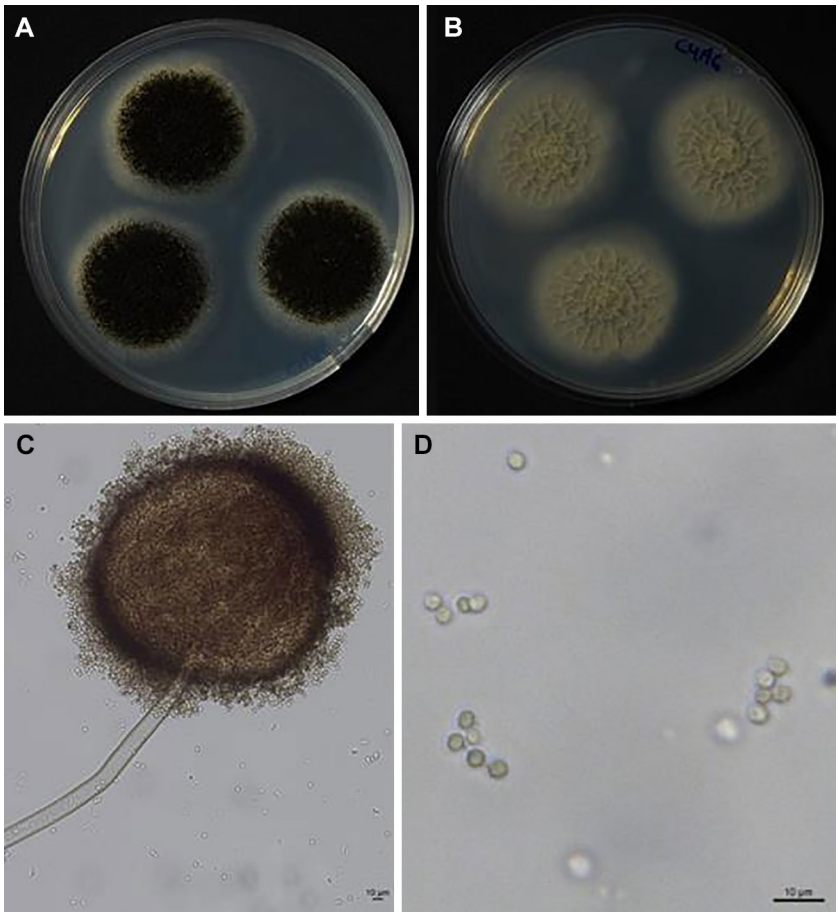

Fig. 7 Macromorphology and micromorphology of *Aspergillus pulverulentus* after 7 days of incubation at CYA, **A** obverse, **B** reverse, **C** Conidiophores, **D** Conidia. Scale bar = 10 µm

Stipes are smooth, colorless, upper parts are light brown, 15–20 µm in width, 1000–3000 µm sometimes 5000 µm in length. Vesicles are globose, 60–75 µm in diameter. Phialide biseriate, metulae 20–30 × 3.0–3.5 µm. Conidia are globose and 4.5–5.0 µm in diameter.

Notes: The strain MT580898 agreed with description of *A. pulverulentus* given by Thom (1926). *Aspergillus pulverulentus* is similar to *A. niger* in colony morphology and coloration. However, *A. pulverulentus* has globose to subglobose conidia, smooth to verruculose.

Penicillium allii Vincent and Pitt (Fig. 8)

Mycologia 81: 300, 1989.

= *Penicillium hirsutum var. allii* (Vincent & Pitt) Frisvad, Mycologia 81: 856, 1989.

Isolate that was examined was C7B20.

Penicillium allii produces colonies 38 mm in diameter on CYA after 1 week of incubation at 25 °C. Colony texture is Granular to weakly fasciculate and only sulcate in center of colony. Conidium is Dull green. Exudate droplets are present, small and clear. Ehrlich reaction is Pink. On MEA, colonies are 36 mm in diameter with grey–green conidium.

Conidiophores are Ter-verticillate, conidia 3.7 µm in diameter, smooth-walled and globose; phialides 9.7 × 3 µm; Metulae: 11.8 × 2.2–3.8 µm; stipes are short, rough-walled, 75–400 × 3.5–5 µm.

Notes: The strain C7B20 reported under the accession number MT122170 agreed with the description of *P. allii* (Vincent and Pitt 1989; Frisvad and Samson 2004). *P. allii* Vincent & Pitt is a synonymous denomination of "*Penicillium hirsutum* Dierckx" and "*Penicillium corymbiferum* Westling" as used in earlier publications (Cavagnaro et al. 2005; Dugan et al. 2007, 2011; Valdez et al. 2009). This fungus differs from *P. hirsutum* by fewer and more lightly colored exudates droplets and from *Penicillium crustosum* by not becoming crustose on MEA.

Penicillium sumatraense Szilvinyi (Fig. 9)

Archiv. Hydrobiol. 14, Suppl. 6: 535. 1936.

Isolate that was examined was C6A1.

Colonies on CYA 33–42 mm in diameter after 7 days, velvety, conidia dull-green or dark-green, exudate absent or present as small or large (pale)-yellow droplets, occasionally clear or light brown, soluble pigments in most strains

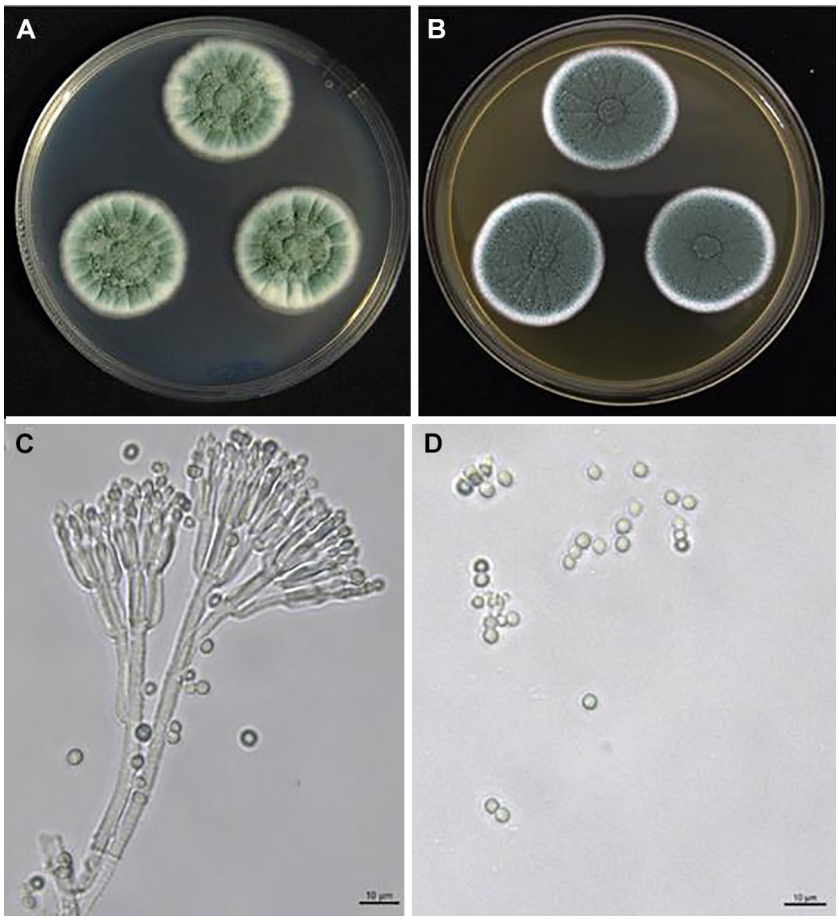

Fig. 8 Macromorphology and micromorphology of *Penicillium allii* after 7 days of incubation at **A** CYA and **B** MEA, **C** Conidiophores, **D** Conidia. Scale bar = 10 µm

absent, in some isolates weakly produced and light-brown colored, margin entire.

Colonies on MEA 27–36 mm in diameter after 7 days, conidia blue–green, light green or grayish-green, floccose colony texture in fresh isolate. Ehrlich reaction is negative.

Conidiophores predominantly biverticillate, occasionally with an additional branch, stipes smooth or finely rough walls 2.0–3.0 µm wide. Metulae vesiculate (10–) 12–16 × 2.0–3.0 µm. Phialides ampulliform, 8.0–10 × 2–3.5 µm. Conidia subglobose or broadly ellipsoidal, finely roughened, occasionally smooth, 2.0–2.5 µm diam.

Notes: The examined isolate MT224967 agreed well with the description of *P. sumatraense* (Houbraken et al. 2011). *P. sumatraense* was formally considered a synonym of *Penicillium corylophilum* (Pitt 1980), but Peterson (2000) and Houbraken and Samson (2011) showed that these two species are phylogenetically unrelated. *Penicillium meleagrinum* var. *viridiflavum* was described without a Latin diagnosis, making the description invalid. Therefore, Pitt et al. (2000) synonymized this species with *Penicillium janthinellum*; however, Serra et al. (2008) showed that *P. meleagrinum* var. *viridiflavum* is genetically close to the type strain of *P. sumatraense*. Therefore, they were maintained as one species.

Discussion

To our knowledge, this is the first aeromycological survey that provides an overview of fungi profile in the atmosphere of apple warehouses in Tunisia and a description of four previously unrecorded fungal pathogens of apple.

A good deal of the literature on airborne fungal spores of work places and industrial environments has been published (Şimşekli et al. 1999; Sawane and Saoji 2005; Kakde and Kakde 2012; Barontini et al. 2014; Nova et al. 2015; Kozdrój et al. 2019). However, there are only a few papers which deal with airspora collected from cold-storage rooms and which have the potential to affect the quality of stored apple (Giraud and Fauré 2000; Amiri and Bompeix 2005).

Quantitative analyses of fungal spores revealed that both warehouses had more spore concentration in April than the other 2 months (1016.24 and 177.33 CFU/m^3 in S1 and S2, respectively). The occurrence of fungal spores in cold chambers found in this study since the first month of sampling could be related to different factors such poor hygiene conditions. It has been suggested that lack of sophisticated storage rooms, poor handling practices by workers and poor sanitary conditions (Sharma et al. 2009; Kibret and Abera 2012) may highly contribute to spore dispersal, thus causing fruit decay.

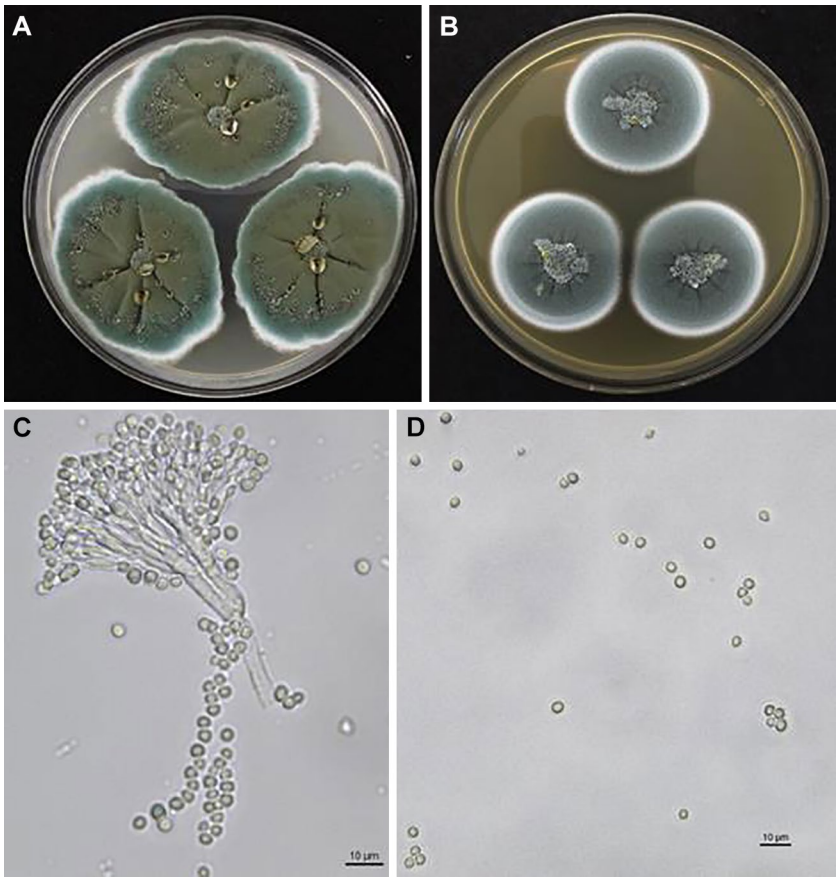

Fig. 9 Macromorphology and micromorphology of *Penicillium sumatraense* after 7 days of incubation at **A** CYA and **B** MEA, **C** Conidiophores, **D** Conidia. Scale bar = 10 µm

In addition, it has been reported that high spore counts in the air can be attributed to spore discharge mechanisms of the fungi, availability of substrates, temperature and humidity (Money 2015). Sufficient light and oxygen may also favor fungal growth in indoor environments (Khan and Karuppayil 2012). In addition, moving and handling fruit crates have been suggested to be factors responsible for the increase of airborne fungal spore dispersal in refrigerated rooms (Lehtonen and Reponen 1993; Grisoli et al. 2009). Moreover, unclean fruit crates, rotten fruit and discarded leaves and stems could act as substrates for fungal growth and enhance spore dispersal in air (Arya and Arya 2007; Vermani et al. 2014). In addition, the outdoor air is often reported as the dominant source of indoor fungi. Spores can usually enter a building through doors and ventilation systems, hence fungal growth and sporulation may occur (Shelton et al. 2002).

Three different fungal genera, *Penicillium*, *Aspergillus*, and *Alternaria* were the most occurring fungi. In several aerobiological studies, the airborne spores belonging to these three genera have been demonstrated as the most widespread fungi detected (Dhruv et al. 2010; Khan and Karuppayil 2012; Jean Phellipe et al. 2019). Furthermore, these fungi were found to be responsible for considerable postharvest disease in a similar storage unit of fruit (Oliveri et al. 2007; Barontini et al. 2014). Our results revealed that among the total counts of viable fungi identified, *Penicillium* (76.8%) was the dominant genera followed by *Aspergillus* (14.4%) and *Alternaria* (5.6%). An abundance of *Penicillium* compared to the other fungi may be due to the amount of decay caused by this fungi found in these storage facilities. Amiri and Bompeix (2005) have confirmed that there is a correlation between the level of airborne *Penicillium* within the atmosphere of warehouses and the concentration of Penicillium spp. spores on apple surfaces. In such a manner, the decay occurring on apples in storage enhances the release of spores, thus increasing the level of airborne *Penicillium*. Among obtained isolates, we have determined 21 groups with distinct phenotypes. *Penicillium* and *Aspergillus* isolates were phenotypically diverse compared to *Alternaria* isolates. They were grouped into 15 and 5 morphologically different groups, respectively. Relying on sequencing of markers calmodulin and beta-tubulin allowed for accurate fungal identification of ten *Penicillium* species and four *Aspergillus* species. Using ITS4 and ITS5 primers, two *Alternaria* isolates were identified as *A. alternata*.

In this study, a rich diversity of airborne *Penicillium* spp. existed in the atmosphere of apple warehouses. These results were similar to that reported by Amiri and Bompeix (2005) in a survey conducted in cold rooms in France. However, we reported the presence of *P. polonicum*, *P. allii*, *P.*

viridicatum, *P. sumatraense*, *P. italicum*, *P. citrinum* and *P. steckii*, which were not identified in the later study. Similar Penicillium species were also reported in the pear fruit chain (Scholtz and Korsten 2016) except for *P. allii*, *P. viridicatum*, *P. sumatraense* and *P. steckii.* No other similar study could be found for comparative purposes.

Penicillium expansum, *P. polonicum*, *P. allii* and *P. chrysogenum* represented approximately 80% of the total *Penicillium* species isolated. According to literature, these species were commonly associated with apple during the postharvest stage except for *P. allii*. Other *Penicillium* species identified in this study accounted for 20% of the total *Penicillium* population. *Penicillium expansum* scored the highest frequency and was the most dominant specie detected in the atmosphere. This is in agreement with that study that investigated airborne *Penicillium* from the atmosphere of cold rooms in France (Amiri et al. 2005). Several studies reported that *P. expansum* is considered among the most economically important postharvest spoilage fungal species that causes decay in cold-stored pome fruits and found mainly in warehouses (Ye et al. 2012; Vico et al. 2014a,b; Víctor et al. 2014; Grantina-Ievina 2015; Gong et al. 2019). *Penicillium polonicum* was the second most dominant *Penicillium* specie found in this study. This specie was isolated by Scholtz and Korsten (2016) from the pear export chain. In addition, *P. polonicum* is common to cause decay in postharvest environment on stored onions (Duduk et al. 2014), stored yam (Kim et al. 2008) and stored cactus pear (Faedda et al. 2015). In this study, *P. polonicum* recorded a severe pathogenicity similar to *P. expansum.* This can be explained by apple cultivar sensitivity towards pathogens which plays an important role in pathogen invasion (Louw and Korsten 2014). *Penicillium chrysogenum* was also detected from the atmosphere of sampled cold rooms. This specie was isolated from the air of different environment and it was also detected on stored apple (Alwakeel 2013).

Penicillium chrysogenum is regarded as one of the most common representatives of its genus in indoor environments because of its ability to grow at low water activities. It was reported as an important fungal pathogen known to be found in temperate regions (Filtenborg et al. 1996) and to be associated with saprophytic colonization of wounded or decaying fruit.

Penicillium viridicatum, *P. solitum*, *P. sumatraense*, *P. italicum*, *P. citrinum* and *P. steckii* have been isolated at a frequency of less than 8%. Most of these species are ubiquitous in the food environment and are typical indoor environmental organisms. They were reported in several aeromycological studies (Johnston 2008; Altunatmaz et al. 2012; Scholtz and Korsten 2016).

Aspergillus was the second most common identified fungal genera from the atmosphere of cold rooms. A*spergillus niger* and *A. flavus* represented approximately 75% of the total Aspergillus species isolated. Conidia of Aspergillus spp are commonly recovered from both indoors environments and outside (Gautam et al. 2011). It has been suggested that *Aspergillus* conidia released in the air may remain airborne for prolonged periods. Therefore, spores founded in the atmosphere could contaminate anything in contact with air (Richardson 1998). *Aspergillus niger* is most commonly found on decaying vegetation or soil and plants (Schuster et al. 2002).It causes rot in many fruits including apples (Liu et al. 2017; Zhang et al. 2018). *Aspergillus flavus* is a common constituent of airborne mycoflora. It could infect cottonseed, feed crops and apples under specific environmental conditions (Hasan 2000a,b; Bock et al. 2004). The presence of *A. flavus* in indoor environment should be of concern because it has been reported that 65% of airborne *A. flavus* produced aflatoxin (Holtmeyer and Wailin 1980), a carcinogenic toxin that cause liver cancer. Then, Aflatoxin synthesis starts simultaneously with spore emergence, and culminates with maximum spore yield.

In this study, *Alternaria*, represented by *Alternaria alternata*, ranked third in abundance in indoor atmosphere of apple warehouses. It has been reported that the indoor level of *Alternaria* spores in the air is influenced by the activity in the room, changes in temperature and relative humidity and the ventilation rate (Woudenberg et al. 2015). *Alternaria alternata* is known to cause decay on apple fruit during storage (Jurick et al. 2014) and Alternaria blotch in the orchard (Li et al. 2012).

The cold-storage trial was performed to determine pathogenicity potential of collected airborne fungi on apples during storage. Thirteen of the 15 species produced lesions on apples at cold-storage temperature.

The ability of these species to cause disease symptoms under storage conditions makes them important pathogens to consider in apple warehouses. Among them, we found four undescribed fungal pathogen to apple; *A. europaeus*, *A. pulverulentus*, *P. allii* and *P. sumatraense*. This could be explained by cross-contamination phenome.

In this study, apart from apples, other fruits were also stored in sampled cold rooms. Scholtz and Korsten (2016) reported that environmental isolates that originate from different areas (e.g., surfaces, air, and other fruit sources) may cross-infect different fruit types. The risk of cross-contamination therefore exists in areas where various fruit types from diverse origins are received and stored.

A. europaeus was described for the first by Hubka et al. (2016) by re-identifying some Aspergillus isolates collected during surveys of the soil in Romania, Spain and Czech Republic, France and Tunisia. It has been reported from corn grain in Korea (Nguyen et al. 2020). To our knowledge, this is the first report of *A. europaeus* pathogenic to apple.

The strain MT580898 agreed with description of *A. pulverulentus* given by Thom (1926). It has been isolated

from soil of Wadi El Natrun, Egypt (Moubasher et al. 2015), from vineyards in Turkey (Eltem et al. 2004). In our study, it is the first record in Tunisia and pathogenic to apple.

The strain C7B20 reported under the accession number MT122170 agreed with the description of *P. allii* (Frisvad and Samson 2004; Vincent and Pitt 1989).*P. allii* is the main postharvest pathogen of garlic (Allen 2009). The fungus has been reported in Argentina (Valdez et al. 2006) and many other countries (Frisvad and Samson 2004). This is the first record of *P. allii* in Tunisia and virulent to apple.

The examined isolate MT224967 agreed well with the description of *P. sumatraense* (Houbraken et al. 2011). This fungus has been reported from marine environments (Malmstrøm et al. 2000), from cork (Serra et al. 2008), packaging material imported into the Netherlands, pomegranates and bromeliad leaf tissue, from grapes in Iran (Mahdian and Zafari, 2016). *P. sumatraense* has also been isolated from soil from Ras Rajel, in Tunisia (Houbraken et al. 2011). In our study, *P. sumatraense* was newly described as pathogenic to apple.

This finding confirms first that elucidating which microorganisms are present in indoor air is fundamentally important to assess decay incidence in storage rooms. Second, airborne fungi in apple warehouses could persist, invade and infect apples during storage at low temperature. An increase of the number of storage facilities sampled, extending the sampling period and studying pathogenicity of the isolates on a large range of apple cultivars are required. Further studies must be undertaken on mycotoxin content and effect on apple, related to the four discovered species: *A. europaeus*, *A. pulverulentus*, *P. allii* and *P. sumatraense*.

Conclusion

Quantitative and qualitative analysis of air samples showed that two investigated apple warehouses in Tunisia were loaded with large quantities of airborne fungi and were contaminated by three major fungal spore type *Penicillium*, *Aspergillus* and *Alternaria*. Molecular identification using calmodulin, b-tubulin and ITS in conjunction with phenotypic characterization helped to identify 15 species of fungi. Thirteen species were confirmed to be associated with fruit decay at ambient temperature and cold-storage trials. Among them, *P. allii*, *A. pulverulentus*, *A. europaeus* and *P. sumatraense* were described for the first time as pathogenic on apples. The presence of these pathogens affects the quality of indoor air and poses a potential threat to stored apple shelf life. Vigilant fruit handling, cold room sanitation and correct temperature management during storage are, therefore, essential in reducing fruit losses during storage.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02551-9.

Acknowledgements This work was supported by funds from the VRR Project (no. 0310) and by the Ministry of Higher Education and Scientific Research of Tunisia (LR16ES05).

Author contributions Marwa SMIRI carried out the experiment and wrote the manuscript with support from Najla SADFI, Eduardo Antonio Espeso and Mustapha Rouissi and Mohamed Zouaoui. Najla SADFI supervised the Project. Amina Kheireddine helped in sampling. Eduardo Antonio Espeso contributed to molecular analysis. All the authors read and approved the manuscript.

Funding This study was funded by Ministry of Higher Education and Scientific Research of Tunisia (LR16ES05) and by funds from the Project of Valorization of Results (VRR no. 0310).

Availability of data and material All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent All the authors consent to this submission.

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