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Ecophysiology of *Penicillium expansum* and patulin production in synthetic and olive-based media

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ABSTRACT

Olives and their derivatives, in particular olive oil, represent one of the most significant agricultural products in the Mediterranean basin. Storage under inadequate conditions poses serious problems concerning fungal contamination, with consequent defects and potential mycotoxin production in olives and olive oils. *Penicillium expansum* represents one of the most significant postharvest pathogens in several fruits, including olives. Not only it causes blue mold but also is one of the most relevant patulin producing species of the genus *Penicillium*.

The aim of this research was to evaluate the ecophysiological conditions governing growth and PAT production by *P. expansum* strains previously isolated from Tunisian olives. For this purpose, four *P. expansum* isolates were tested in a synthetic medium (Czapek Yeast Autolysate, CYA) and in olive-based medium (OM) for their ability to grow and produce PAT under different temperatures (4 °C, 15 °C and 25 °C) for 10 and 20 d. The mycotoxin was analysed by HPLC-UV.

Results showed that all isolates were able to grow on tested media at different temperatures. Different PAT production profiles were found, showing that at 25 °C *P. expansum* isolates were able to produce PAT on CYA and OM medium. At 15 °C the production of PAT was only detected on CYA medium, while no PAT production was detected at 4 °C for the two media.

1. Introduction

Olives and their derivatives, in particular olive oil, represent one of the most significant agricultural products in the Mediterranean basin. Tunisia is considered the fourth largest producer of olive oil in the world (Abdelhamid et al., 2013). Tunisian olive oil production has an important position in the olive oil market; it exports about 75 % of its production and is considered as the second largest exporter after the European Union with an average of 115,000 tons per y over the last five years (ONH, 2015). In Tunisia, the olive sector contributes directly or indirectly to more than one million people and provides 34 million d of work per y, which is equivalent to more than 20 % of agricultural employment (Gharbi et al., 2015).

Olives are often stored for a long time under conditions that promote the growth of molds, such as prolonged contact with the ground, in bags of jute and in little ventilated places with high relative humidity. Contamination of olive fruits by hazardous microorganisms may also occur through insect pest infestation (Al-Ameiri et al., 2015). Such conditions favor a moldy taste and appearance and thus reduce the acceptable quality of olives and olive oils (Heperkan et al., 2006). Inappropriate storage or fermentation conditions favor the proliferation of hazardous fungi such as *Penicillium* sp. (Heperkan et al., 2009, Heperkan, 2013).

According to Roussos et al. (2006), olives can support the growth of mold and mycotoxins production. Many fungal strains, in particular from *Aspergillus*, *Penicillium* and *Alternaria* species, are able to grow on olives and produce several mycotoxins, and these genera have been reported as the dominant fungi on olives and olive products (El Adlouni et al., 2006; Roussos et al., 2006; Bavaro et al., 2017). Mycotoxin contamination of olives has also been studied, mostly for aflatoxins, ochratoxin A and citrinin (Leontopoulos et al., 2003; Ghitakou et al., 2006; Heperkan et al., 2009). Still, compared with other agricultural commodities, studies concerning contamination of olives with toxigenic fungi and the ability of these fungi to produce mycotoxins in this matrix are scarce.

Several *Penicillium* species were found on olives, including *P. expansum*, *Penicillium citrinum* and *Penicillium crustosum* (Arici, 2000; Heperkan et al., 2006, 2009; Bavaro et al., 2017).

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P. expansum is a wound parasite fungus that enters fruits via injuries potentially caused by inadequate conditions before harvest or by rough handling, harvesting, and transport (Sanderson and Spotts, 1995). It is one of the best-known and most studied molds of the genus *Penicillium*, occurring frequently at fruit harvest or postharvest stages (Andersen et al., 2004). *P. expansum* is known to contaminate a wide range of foods including apples, pears, cherries, walnuts, pecans, olives, hazelnuts, and acorns (Filtenborg et al., 1996; Zouaoui et al., 2015). It's defined as an important producer of PAT and citrinin, and is the major mycotoxin menace in apples and apple-derived products such as apple juice, purée, jam and cider (Frisvad and Samson, 2004). Studies of this species have been generally devoted PAT contamination and production in apple, apple juice, pears and cereals. However, only few studies report the contamination of olives with this species (Arici, 2000; Bavaro et al., 2017). To our knowledge, no studies have been developed on the detection of PAT in olives or other olive-derived products.

PAT is considered to have potential mutagenic, carcinogenic and embryotoxic effects on humans (Puel et al., 2010). The World Health Organization and the Food and Agriculture Organization have set a Maximum Tolerable Daily Intake for PAT of 0.4 μg/kg body weight/day (Leggott and Shephard, 2001). The European Commission has set limits of allowable PAT content in various foodstuffs: 50 μg/L in fruit juices and spirit drinks, 25 μg/L in solid apple products and 10 μg/L in infant food (EC, 2006).

It is thus necessary to acknowledge the importance of *P. expansum* in olives and to determine its ability to produce PAT in olive-based substrates under different conditions, by determining the ecophysiological conditions governing PAT production by the fungus. This knowledge is of major importance to make a risk assessment and to develop preventive measures especially during storage.

The objectives of this work were to study the effect of temperature and matrix on *P. expansum* strains isolated from Tunisian olives in terms of growth and PAT production.

2. Materials and methods

2.1. Fungal isolates

Four PAT producing strains of *Penicilium expansum* were used in this study. From these, three were selected from a group of 28 isolates previously obtained from olives intended for oil production from Tunisian olive groves. The PAT producing strain *P. expansum* MUM 10.175 (further referred to as MUM), originating from a contaminated culture of *Botrytis cinerea* and obtained from Micoteca da Universidade do Minho (MUM), Braga, Portugal, was used as positive control.

All isolates were maintained in 20 % glycerol at −20 °C and grown on Malt Extract Agar (MEA: Malt 20 g/L, Glucose 20 g/L, Peptone 1 g/L, Agar 20 g/L; autoclaved for 15 min at 121 °C) in the dark for 7 d at 25 °C whenever needed for further studies.

2.2. Identification of fungal isolates

All fungal isolates were subjected to a preliminary morphological identification to the genus level following general taxonomic guides (Samson et al., 2004; Pitt and Hocking, 2009). From 7 d old cultures on MEA, a loop full of spores was suspended in 500 μL of 0.2 % agar with 0.05 % Tween 80, and this suspension was used for three-point inoculations on 9-cm diameter Petri dishes containing MEA and Czapek Yeast Extract Autolysate (CYA) Agar medium (sucrose 30 g/L; yeast extract 5 g/L; dipotassium hydrogen phosphate 1 g/L; sodium nitrate 0.3 g/L; potassium chloride 0.05 g/L; magnesium sulphate 0.05 g/L; ferrous sulphate 0.001 g/L; zinc sulphate 0.001 g/L; copper sulphate 0.0005 g/L; agar 15 g/L), as described in Rodrigues et al. (2013).

Cultures were incubated in the dark at 25 °C and were analyzed after 7 d of incubation for the following characters: colony growth and texture, obverse and reverse colony color, diffusible pigments and exudate production and microscopic characteristics, with the help of taxonomic guides of the genus *Penicillium* (Frisvad and Samson, 2004).

From the 28 olive-native isolates, four were morphologically identified as *P. expansum*. Morphological identification was confirmed by molecular analysis. Genomic DNA was obtained following the methodology described by Rodrigues et al. (2009). The universal primers ITS1-F (5′ CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990; Gardes and Bruns, 1993), which amplify a 600 bp segment of the ITS1/5.8S/ITS2 region of the rRNA gene, were used for sequencing purposes. PCR amplifications were performed as described by Rodrigues et al. (2011). PCR products were purified with the commercial GF-1 PCR cleanup kit (Vivantis), according to the instructions of the manufacturer.

Sequence analysis was performed in both directions, on an ABI 3730xl DNA Analyzer (Applied Biosystems). Sequence comparison was performed using the Basic Local Alignment Search Tool (BLAST) in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Pairwise Sequence Alignment in the MycoBank Database (http://www.mycobank.org/defaultinfo.aspx?Page=Home).

2.3. Screening of patulin producing ability by P. expansum isolates

The four *P. expansum* isolates were tested for PAT production on CYA. Fungi were inoculated by three-point inoculation on 9 cm Petri dishes containing 20 mL of CYA and incubated for 7 d in the dark at 25 °C. After incubation, the methodology of Bragulat et al. (2001) was employed by removing 3 agar plugs from one colony, placed into a 4 mL ambar vial, and 1 mL of methanol was added. After 60 min, the extract was filtered by 0.2 μm syringe filters into 1.8 mL HPLC vials.

PAT detection was performed on an HPLC system equipped with: an autosampler (HTA, HT800L); a pump (Varian 9010); a reverse phase C18 column (Supelco Kromasil, 4.6 × 150 mm, 5 μm), fitted with a precolumn with the same stationary phase; and an ultra-violet detector with Diode-Array Detection (PDA) set to 276 nm wavelength (Varian, Prostar; 330). Oven temperature was set to 30 °C. Star chromatography Workstation version 6.41 software was used for instrument control and data processing. The mobile phase was composed of methanol and water at 10:90 (v/v) with a flow rate of 0.8 mL/min. The injection volume was 10 μL and the run was 20 min. The detection of patulin was done by comparison with the standard.

Among the four isolates tested, three were detected as PAT producers. The three PAT producing isolates of *P. expansum* TUN20, TUN22 and TUN23 (further referred to as TUN strains) were selected for further ecophysiological studies in terms of growth and PAT production.

2.4. Ecophysiology of Penicillium expansum growth and PAT production

2.4.1. Media preparation

For the ecophysiological studies, two different culture media were used: CYA, used as control; and olive-based medium (OM), used as a model to reflect the olive matrix. OM was prepared as follows: Tunisian olives previously collected were rinsed with bleach for 5 min, washed twice with distilled water, deboned and ground with a blender at low speed for 5 s to obtain a homogeneous

paste. The paste pH was 5.3, and water activity was 0.98 (measured with a water activity meter 4 TE, AQUA LAB). Olive based-medium was prepared by mixing one part of olive paste with 6 parts of water (p:v). 1.5 % agar were added and the medium was autoclaved for 15 min at 121 °C. Both media were plated in 9 cm Petri dishes.

2.4.2. Inoculation, incubation and measurement of growth

Spore suspensions of each *P. expansum* strain were obtained by adding 1 mL of sterilized water with 0.05 % Tween 80 to a 7 d-old culture and by scrubbing the spores. Spore suspensions were adjusted to 10^6 spores/mL by counting cells with the aid of a Neubauer counting chamber. 10 μL of each suspension were transferred by central–point inoculation to Petri dishes containing 20 mL of each medium (CYA and OM). Sets of three Petri dishes were incubated at 3 different temperatures: 25 °C, 15 °C and 4 °C for 20 d. Petri dishes without fungi (medium only) were used as negative control.

Fungal growth was observed daily and measurement of fungal colonies diameter (in cm) was made after 3, 5, 8, 10, 15 and 20 d of incubation. The procedure was done in triplicate.

2.4.3. Quantification of PAT production

2.4.3.1. Preparation of PAT standard solutions. The stock standard solution of PAT was dissolved in ethyl acetate at a concentration of 1 mg/mL. This stock solution was diluted with methanol to obtain working solutions as needed. Concentration of PAT standards was confirmed by measuring the UV absorbance at 276 nm and calculated by using the molar extinction coefficient ε of 14,600 L/mol/cm (AOAC, 2000). The prepared solutions were stored at 4 °C.

2.4.3.2. Method validation. HPLC analysis using methanol as solvent was selected as the analytical method for PAT detection and quantification. Linearity, limit of detection (LOD) and limit of quantification (LOQ) were determined following the analytical method validation guidelines described by Taverniers et al. (2004). Linearity was demonstrated by using 7 standard solutions in methanol with concentrations ranging from 0.2 μg/mL to 125 μg/mL. LOD and LOQ were calculated according to the following equations: $LOD = y_0 + 3 \times S_{y/x}$ and $LOQ = y_0 + 10 \times S_{y/x}$, where Y_0 is the intercept of the regression line obtained from the calibration curve, and $S_{y/x}$ is the standard error of the slope. OTA was quantified by gram of agar, taking into consideration the weight of the agar plugs used for extraction.

Recovery from the two culture media was determined as follows: 50 μg/g of PAT were spiked into 1 g of each medium (CYA and OM) and kept for 30 min in the dark. Then 1 mL of methanol was added and PAT extraction occurred for 60 min, with vortexing every 15 min. The extract was filtered through PVDF 0.2 μm syringe filters and stored at 4 °C until HPLC analysis. The procedure was done in triplicate. Recovery rate (%) was calculated by the ratio of recovered PAT concentration relative to the known spiked concentration. Precision was calculated in terms of intra-day repeatability (RSDr; n = 3), and is given by the corresponding relative standard deviation.

2.4.3.3. PAT extraction from fungal cultures. After 10 d and 20 d of incubation, all cultures were submitted to PAT extraction. Three agar plugs were removed from the inner, middle and outer areas of the colony, weighted and extracted with methanol as previously described. Quantification of PAT was performed by measuring area's peak at PAT retention time and plotting against the calibration curve. PAT was quantified by gram of agar, taking into consideration the weight of the agar plugs used for extraction.

2.5. Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics v.22.0 software (Armonk, NY: IBM Corp.). The variables under study (growth and PAT concentration) did not show a normal distribution, therefore the non-parametric statistical Kruskal–Wallis test was used for comparison of means, and post-hoc analyses were performed with corresponding U-Mann Whitney test. Correlation between fungal growth and PAT production was studied by analyzing Spearman correlation index. In all cases, statistical significance was established at $p \leq 0.05$.

3. Results and discussion

This study was carried out on three strains of *P. expansum* previously isolated from Tunisian groves (TUN strains), using a culture collection strain (MUM 10.175) as non-native control.

P. expansum has frequently been defined as an important contaminant and producer of PAT and citrinin in several fruits, mostly apples and pears (Gimeno and Martins, 1983; Laidou et al., 2001; Martins et al., 2002; Frisvad and Samson, 2004; Baert et al., 2007a; Morales et al., 2007a, 2007b; 2007c, 2008; 2010; Abramson et al., 2009). In our study, among a group of 28 fungal isolates, four (14 %) were identified as *P. expansum,* of which three were able to produce patulin. These results are in agreement with those from another recent study on molds associated with fermented black table olives from Italy and Turkey (Bavaro et al., 2017), where 10 % of the isolated strains belonged to this species. According to these results, *P. expansum* seems to be an important contaminant of olives.

Few studies of *P. expansum* have been conducted to characterize the growth and the toxigenic conditions of this species, despite its large implication in foodstuff contamination. The understanding of ecophysiology of *P. expansum* under controlled experimental conditions may help to understand its behavior under natural storage conditions and forecast its potential risks on the fruit and consumer's health.

For this, *P. expansum* strains were tested on a synthetic medium (CYA) and on olive based-medium (OM) and were submitted to adequate (4 °C) and abusive (15 °C and 25 °C) storage temperatures. Water activity was not included as a variable since olives don't suffer significant aw variations throughout storage. Atmosphere composition was also not included since previous research on apples has shown that atmosphere composition had no effect on *P. expansum* growth and patulin production (Baert et al., 2007b).

3.1. Influence of matrix and temperature on fungal growth

In terms of fungal growth (colony diameter), the Kruskal–Wallis test did not reveal significant differences between the three olive-native strains (TUN) analyzed ($p > 0.05$), but significant differences were observed between the MUM strain and the TUN strains ($p < 0.05$). Therefore, results for growth are further represented separately for MUM (three replicas) and for TUN (three strains x three replicas).

The results of fungal growth throughout 20 d of incubation in both media for all temperatures tested are shown in Fig. 1. Fig. 2 compares the fungal growth, in mm/day, of MUM and TUN strains. For all the tested conditions, the growth curves based on colony diameters were typical of a linear fungal growth until the moment when fungal growth was limited by the Petri dish dimension (single colony in 9 cm). In such cases, growth curves lose their linear appearance just after reaching the limiting diameter of 9 cm.

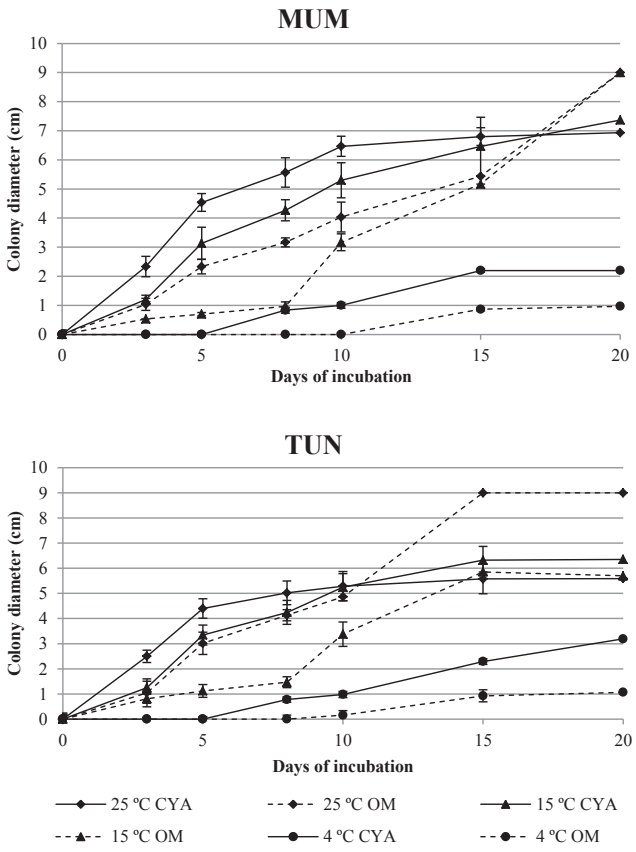

Fig. 1. Growth curves of Penicillium expansum strains. Top: MUM (n = 3); Bottom: TUN (n = 9), at 4 °C, 15 °C and 25 °C in CYA and OM.

The influence of the matrix on *P. expansum* growth was studied by inoculating the strains on two different media: CYA, a synthetic standardized medium generally used to study fungal growth, was used as positive control; and OM, an olive-based medium, was used as a model system to substitute the use of integral olives. Culture media prepared from selected food products have been generally considered a good approximation to the growth and toxin production patterns contained in natural substrates (Pardo et al., 2005), and have been frequently used as model systems in similar ecophysiological studies (e.g. Marín et al., 2006; Gil-Serna et al., 2014; Rodríguez et al., 2015; Vipotnik et al., 2017).

In the present study, the matrix had a significant influence on the growth profile throughout time. Fungal growth was better on CYA for 25 °C and 15 °C until the 10th d (Fig. 1), but between 10 and 20 d of incubation growth trend suffered an inflection, and fungi started to grow faster on OM. When analyzing growth at the end of incubation, this parameter was generally significantly higher ($p < 0.05$) on OM for both fungi.

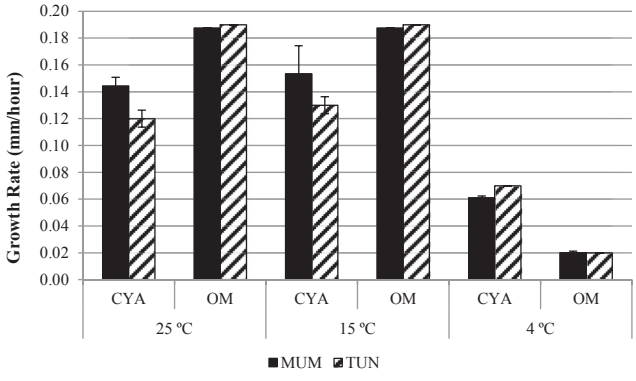

Fig. 2. Growth (mm/day) of MUM and TUN strains at 4 °C, 15 °C and 25 °C, on CYA and OM.

Interestingly, the strains displayed very different colony morphologies on the two media (Fig. 3). On CYA, growth was abundant (Fig. 3A–C), whereas on OM growth was poor on mycelium and was mostly based on highly spread (hence the colony diameter) and strongly sporulating synnemata (Fig. 3D–F), i.e. large, erect reproductive structures bearing compact conidiophores, which fuse together to form a strand with conidia at the end. The atypical growth on OM is probably a stress response to the less nutritive and more complex medium, while optimal growth conditions are offered by CYA.

At 4 °C, growth followed a similar trend throughout time on both media, but with a growth significantly higher on CYA than on OM ($p < 0.05$) for MUM and TUN strains. Growth rate of TUN was not significantly higher than that of MUM on both CYA ($p = 0.25$) and on OM ($p = 0.508$).

Several studies have reported the growth conditions of *P. expansum* on fruit–based media, usually on apple or apple-based media. However, to our knowledge, this is the first study considering the growth of this fungus on olives and olive-based matrices. Radial growth of *P. expansum* growing at 25 °C in OM was 0.190 mm h^{-1}, comparable to that reported on apples by Lahlali et al. (2005; 0.160 mm h^{-1}), for the same temperature, but lower than that reported by Baert et al. (2007a; between 0.340 and 0.212 mm h^{-1}) and Marín et al. (2006; 0.396 mm h^{-1}). The lag time reported on apples by Baert et al. (2007a) was of 1 and 10 d at 25 °C and 4 °C, respectively. In our study, growth on OM for the same temperatures was observed at 1 and 9 d of incubation, respectively. This might indicate that, independently of the temperature, olives are not necessarily an inadequate matrix for *P. expansum*, as suggested for other fungi.

In fact, several studies have devoted to growth of other fungi on olives. Leontopoulos et al. (2003) studied the growth of *Aspergillus parasiticus* on intact black olives, and concluded that olives are not adequate for the growth of this aflatoxigenic fungus. However, previous reports (Mahjoub and Bullerman, 1987; Eltem, 1996) had indicated that damaged olives exhibited fast and extensive mycelial growth. It is probable that the outer pellicle of olives impedes fungal growth, and that olives are only open to fungal growth if injured. It must be noted that olives intended for oil extraction tend to be stored under inadequate conditions for long periods, and usually become highly injured and unprotected against fungal development.

The effect of temperature on fungal growth was performed at 4 °C, 15 °C and 25 °C for both media. These temperature conditions intend to reflect adequate conditions (4 °C; refrigerated) and abusive storage conditions (15 °C and 25 °C; plastic or jute bags piled outdoors) to which olives can be submitted during the storage period.

All the investigated strains of *P. expansum* were able to grow in the studied temperature range. Growth on CYA at the end of incubation was higher (but not significantly; $p = 0.822$) at 15 °C than at 25 °C for both MUM and TUN strains, being that MUM grew significantly faster than TUN at 25 °C ($p = 0.019$), but not at 15 °C ($p = 0.073$). Growth on OM was maximum for both temperatures, with 9 cm being achieved at the end of incubation. On the other hand, growth at 4 °C was significantly reduced when compared with higher temperatures ($p = 0.037$). However, growth was not constant throughout this period. In general, the optimal growth temperature for MUM and TUN strains was 25 °C, at which the fungus exhibited the faster initial growth and the most significant

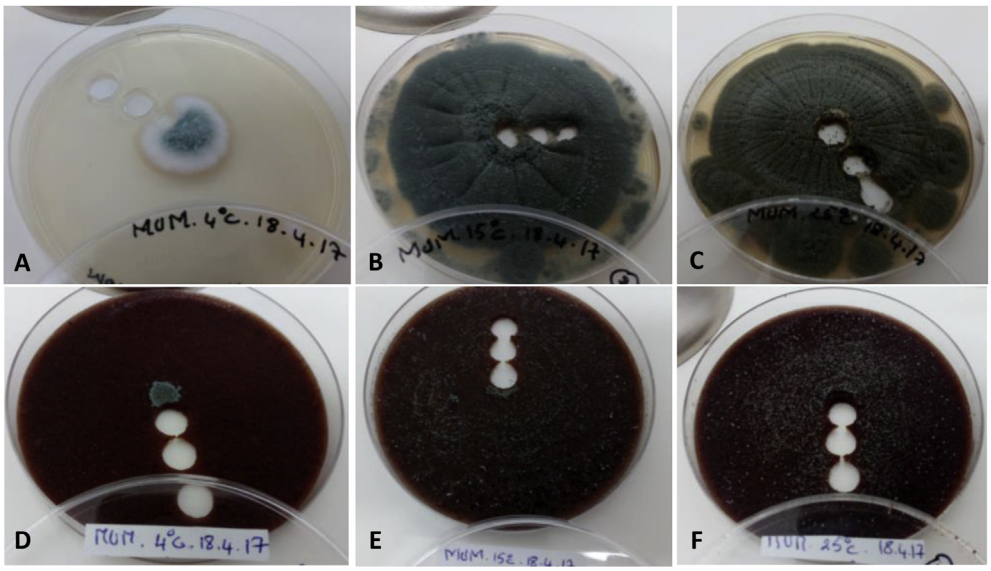

Fig. 3. Colonies of Penicillium expansum MUM 10.175 after 20 d of incubation: A, B and C: growth on CYA at 4 °C, 15 °C and 25 °C, respectively; D, E and F: growth on OM at 4 °C, 15 °C and 25 °C, respectively.

colony growth. The exception goes to growth of TUN strains, which was higher at 15 °C on CYA than at 25 °C on OM. This difference was, however, not significant ($p > 0.05$). The observation of optimal growth temperature is in accordance with that of other studies which describe also an optimum growth temperature for this species between 15 °C and 25 °C (Lahlali et al., 2005; Baert et al., 2007b; Pitt and Hocking, 2009; Tannous et al., 2016).

At 4 °C, growth was only observed after 5 d (on CYA) or 10 d (on OM) for both MUM and TUN fungi. A lag phase of 6 d was reported by Tannous et al. (2016) at 4 °C on CYA, with this period being reduced with increased temperatures. This result supports the conclusion of Baert et al. (2007a) that cold storage does not prevent fruit deterioration by *P. expansum*, just delays it. Also, although decay proceeds slowly at cold storage temperatures, rapid development occurs when the fruit is transferred to a warm environment (Fallik et al., 2001).

P. expansum is a psychrotrophic: optimal growth temperatures range from 15 °C to 25 °C, but minimum temperatures for growth have been reported between −2 and −6 °C, with growth still quite strong at 0 °C (Pitt and Hocking, 2009). *P. expansum* grew better at 25 °C than at 4 °C in OM, although with longer incubation periods under refrigerated conditions, fungal growth could eventually catch up and result in significant contamination of olives.

Our results lead to the assumption that olives are adequate for *P. expansum* growth, and that olive matrix can be a limiting factor for fungal spread on olives only when low temperatures are maintained throughout storage. At abusive temperatures, *P. expansum* seems to induce a stress response and increase sporulation and dissemination, leading to potentially increased contamination by the fungus.

Table 1
Calibration parameters for patulin detection and quantification.

Parameter	Patulin
R_t (retention time)	7.2
Calibration curve	y = 4E−06x + 0.9422
Correlation coefficient (R^2)	0.9994
Linearity range (μg/mL)	0.2 to 125
LOD (μg/mL)	4.5
LOQ (μg/mL)	12.9

3.2. Influence of matrix and temperature on patulin production

The calibration parameters including equations of the linear regression, correlation coefficient (R^2), LOD and LOQ for patulin are shown in Table 1.

The recovery rate for each of the two media was determined by spiking 1 g of CYA and OM medium with a known concentration of 50 μg/g. The recovery rate and relative standard deviation (RSD) are presented in Table 2.

Patulin recovery from OM (93.7 %) is higher than from CYA (77.3 %), but the RSD is also higher (21.4 %), meaning that extraction from OM can introduce more bias in the result than from CYA. Indeed, patulin recovery obtained in our study is within the range of that observed in other studies, for example 92.9 %–100.5 % from commercial baby foods from Turkey (Karakose et al., 2015), 74 %–105 % in apple juice produced in Turkey (Aktas et al., 2004) and 86.5 % in various fruit juices marketed in Tunisia (Zouaoui et al., 2015).

Patulin concentration on each medium (CYA and OM) and each temperature (4 °C, 15 °C, 25 °C) is shown in Fig. 4. A significant difference in patulin production was generally observed between MUM and TUN fungi ($p < 0.033$) after 10 d of incubation. The amount of patulin after 20 d was significantly different between fungi at 25 °C ($p = 0.024$), at 15 °C on OM ($p = 0.033$) and at 4 °C on CYA ($p = 0.013$), with TUN fungi producing higher amounts of patulin than MUM.

The differential production of patulin between MUM and TUN fungi might be due to the fact that the host of origin of the MUM strain was a contaminant of a *B. cinerea* culture (potentially originating from grapes) instead of olives. Sanzani et al. (2013) investigated the influence of the origin on *P. expansum* pathogenicity/virulence and found that the *P. expansum* strains originating from a

Table 2
Recovery rate and relative standard deviation (RSD; n = 3) of patulin on CYA and OM medium.

	Patulin (50 μg/g)	
	CYA	OM
Recovery (%)	77.3	93.7
RSD (%)	4.1	21.4

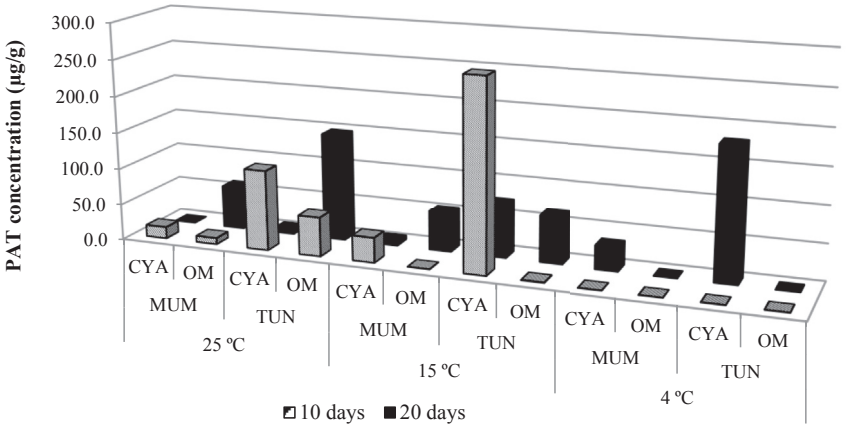

Fig. 4. Patulin production by MUM and TUN fungi at 25 °C, 15 °C and 4 °C after 10 and 20 d of incubation on CYA and OM.

given matrix produced more patulin in that host than strains originating from other hosts.

A weak correlation between fungal origin and patulin production on OM and CYA was detected in our study after 10 d (Pearson correlation = 0.309; p = 0.008) and after 20 d (Pearson correlation = 0.328; p = 0.005) of incubation, but this correlation was not consistent, since in most cases the amount of patulin produced by TUN fungi was higher on CYA than on OM. De Clercq et al. (2016) found that patulin production was more dependent on the temperature/atmosphere conditions or strain than on strain's origin.

Patulin production by *P. expansum* MUM and TUN exhibited a marked temperature- and matrix-dependent variability. The highest amount of patulin detected corresponded to TUN fungi growing on CYA, at 15 °C, after 10 d of incubation. In fact, after this period patulin was always produced at higher amounts on CYA than on OM (significantly different for 15 °C and 25 °C; $p < 0.05$), regardless of the fungus, except at 4 °C, where patulin was not detected. However, after 20 d of incubation, patulin was detected at significantly higher amounts on OM than on CYA at 25 °C for both fungi and at 15 °C for MUM ($p < 0.033$). The amount of patulin was similar on both media at 15 °C for TUN fungi. At 4 °C, patulin amounts increased significantly from 10 to 20 d, and were significantly higher on CYA than on OM ($p < 0.037$). In fact, fungi were unable to produce detectable amounts of patulin at 4 °C on the olive-based matrix.

Our results match those of other studies, including that of Baert et al. (2007b), where patulin production was reduced when the temperature decreased from 20 to 10 or 4 °C. Also it was reported by Tannous et al. (2016) that the highest patulin concentrations were attained at around 16–17 °C. In a study of patulin production by *P. expansum* in apple puree medium, De Clercq et al. (2016) indicated that low temperatures (4 °C) caused a delay of fungal metabolite production, but not its total inhibition.

Our results show two interesting aspects. One is that temperature, time of incubation and matrix affect significantly the ability of fungi to produce patulin. After 10 d of incubation, more patulin was detected on CYA than on OM, but an inflection of this trend was observed when the longer incubation period was analyzed. For 20 d of incubation, patulin was generally found in higher amounts on OM, except at 4 °C. Even though fungi were able to produce patulin on CYA at 4 °C, no patulin was produced on OM. Considering our results, refrigeration throughout storage seems to be safe for olives, at least for a storage period of up to 20 d. On the contrary, storage temperatures of 15 °C and 25 °C are abusive conditions for long periods of storage. On this respect, fungi seem to have a long lag phase of patulin production on OM, but once this phase is overcome, high amounts of patulin are produced. Other authors have also reported that the length of time apples are stored at ambient temperature (20 °C) is critical for patulin accumulation (Morales et al., 2007c; Welke et al., 2011; Baert et al., 2012).

The other interesting aspect of these results is that amounts of patulin suffered a significant reduction from the 10th to 20th d of incubation on CYA at 25 °C and 15 °C ($p < 0.05$). This effect was not observed at 4 °C and also not on OM at any temperature. Although this could be interpreted as a procedural error, this outcome was observed consistently in all fungi and in all replicas tested. The mechanism behind degradation of PAT and other mycotoxins has been reported by others, but is not well understood. Suggestions have been made that reduction in PAT contamination can be due to metabolic destruction caused by the fungus itself (Sommer et al., 1974). Furthermore, this metabolite decrease has been detected in olive matrices for other mycotoxins. It has been reported by Ghitakou et al. (2006) that the amount of aflatoxin B1 detected in black olives (produced by *A. parasiticus*) decreased after 9 d at 30 °C. In a study by Leontopoulos et al. (2003), aflatoxin B1 produced by *A. parasiticus* increased significantly after the 12th d of incubation, while on olives incubated at 20 °C the toxin suffered a massive decrease after the same period of incubation. Citrinin amounts also decreased after 40 d of incubation at temperatures ranging from 20 to 30 °C on black table olives (Heperkan et al., 2009).

It must be noted that these studies have occurred using pure cultures of the mycotoxigenic fungi, so mycotoxin degradation by competing microorganisms should not be considered. To our knowledge, no studies on mycotoxin degradation by the self-producing fungi have been developed, and for that reason no explanation to this occurrence is available.

4. Conclusions

In conclusion, contamination of olives with patulin must be considered a potential risk in the safety and quality plans of the olive producing chain. The olive-based matrix seems to be adequate for growth and patulin production by *P. expansum* at storage abusive temperatures, so adequate temperatures throughout storage (refrigeration) are required to guarantee the safety of the product. If there is an abuse on storage temperature and longevity, patulin can turn into a real risk.

To our knowledge, this is the first report on the risk of *P. expansum* growth and patulin production on olives and

olive-based products. More studies are needed to reinforce the results obtained in the present study.

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