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

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ABSTRACT

In the Mediterranean basin, a large number of olive varieties are cultivated. In Tunisia, oliviculture represents one of the most significant agricultural products that are produced annually. 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 the most relevant patulin (PAT) producing species of the genus *Penicillium.*

In this study, the first aim was to identify and characterize a selected group of fungi previously isolated from olives from Tunisian groves. For this purpose, 28 fungi were selected for identification by a polyphasic approach consisting of: i) morphological identification on Malt Extract Agar (MEA) and Czapek Yeast Autolysate (CYA); and ii) molecular identification by sequencing of the ITS region of the rRNA gene. Mycotoxigenic ability of fungi was screened on Coconut Agar Medium (CAM). Methanolic extracts of those fungi showing fluorescence on CAM were obtained from CYA cultures and analysed by HPLC. The second 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 (three from olives and one reference strain) were tested in a synthetic medium (CYA) and in olive-based medium (OM) for their ability to grow and produce PAT under different temperatures (4 ºC, 15 ºC and 25 ºC) for 10 and 20 days. Growth was measured through the colonies' diameter, and PAT mycotoxin was analyzed by HPLC-UV.

After morphological and molecular identification, eight species belonging to the genera *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, *Pleospora* and *Trichothecium* were identified. The dominant genus was *Penicillium* (82% of the isolates), and the species identified were *P. expansum*, *P. crustosum* and *P. polonicum*. Based on HPLC analysis three of the four *P. expansum* isolates were able to produce PAT.

In the ecophysiology study, all isolates were able to grow on tested media at different temperatures, with maximum growth at 25 ºC. Different PAT production profiles were

found. Maximum PAT production occurred at 15 ºC on CYA after 10 days of incubation, but a significant increase was observed on OM between 10 and 20 days of incubation. At 4 ºC, PAT was produced only on CYA after prolonged incubation.

In conclusion, contamination of olives with PAT must be considered in the safety and quality plans of production. The olive-based matrix does not seem to be highly adequate for growth and PAT production by *P. expansum* only if adequate temperatures throughout storage (refrigeration) are guaranteed. However, if there is an abuse on storage temperature and longevity, PAT becomes a real risk.

RESUMO

Na bacia mediterrânea é produzida uma grande variedade de cultivares de azeitona. Na Tunísia, a olivicultura representa um dos mais importantes produtos agrícolas produzidos anualmente. O armazenamento sob condições inadequadas constitui um sério problema no que diz respeito à contaminação fúngica, com os consequentes defeitos e potencial produção de micotoxinas em azeitonas e azeite.

Penicillium expansum representa um dos mais significativos fungos patogénicos de vários frutos, incluindo azeitonas. Não só causa a podridão azul, como é também o mais importante produtor de patulina (PAT) do género *Penicillium*.

O primeiro objetivo do presente estudo foi a identificação e caracterização de um grupo de fungos previamente isolados de azeitonas de olivais da Tunísia. Neste sentido, 28 fungos foram selecionados para identificação polifásica envolvendo identificação morfológica em Extrato de Malte Agar (MEA) e Czapek Yeast Autolysate (CYA) e identificação molecular por sequenciação da região ITS do gene rRNA. A capacidade micotoxigénica dos fungos foi avaliada em meio de coco (CAM) e, das culturas com fluorescência neste meio, através da análise por HPLC de extratos metanólicos das culturas correspondentes em CYA.

O segundo objetivo do estudo foi a avaliação das condições ecofisiológicas envolvidas no crescimento e produção de PAT pelas estirpes de *P. expansum* identificadas. Para este objetivo, três estirpes com origem em azeitonas da Tunísia e uma estirpe de referência (MUM 10.175) foram testadas num meio de cultura sintético (CYA) e num meio de cultura à base de puré de azeitona (OM) quanto à sua taxa de crescimento e produção de PAT sob temperaturas diferentes (4 ºC, 15 ºC e 25 ºC) ao longo de 20 dias de incubação. O crescimento foi avaliado através da medição do diâmetro das colónias, e a PAT foi quantificada por HPLC-UV.

Foram identificadas oito espécies pertencentes a seis géneros: *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, *Pleospora* e *Tricothecium*. O género prevalecente foi *Penicillium*, do qual foram identificadas as espécies *P. expansum*, *P. crustosum* e *P. polonicum*. Três dos quatro isolados de *P. expansum* identificados foram caracterizados por HPLC como produtores de PAT.

Nos estudos de ecofisiologia, todos os fungos cresceram em todas as temperaturas testadas, com crescimento máximo entre 15 ºC e 25 ºC. Em relação à produção de PAT, foram detetados perfis de produção diferentes. A produção máxima ocorreu a 15 ºC em CYA após 10 dias de incubação, mas a produção de PAT em OM sofreu um aumento significativo de entre os 10 e os 20 dias. A 4 ºC, apenas foi detetada PAT em CYA após 20 dias de incubação.

Em conclusão, a matriz à base de azeitona parece não ser muito adequada à produção de PAT por *P. expansum* desde que sejam mantidas temperaturas de refrigeração. No caso de o armazenamento da azeitona ser feito a temperaturas mais elevadas e por longos períodos, a contaminação com PAT é um risco que deve ser considerado nos planos de segurança e qualidade.

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1. INTRODUCTION

1.1. Framework

Olive and its 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 country in the world **(Abdelhamid et al., 2013)**. Olive crops are spread over areas from the north to the south of the regions, from lower semiarid to arid conditions **(IOOC, 2003)**.

Olives are often stored for weeks in conditions that promote mold growth, and reports have shown that olives can support fungal growth and mycotoxin production (mostly aflatoxins and ochratoxin A) **(**e.g. **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.

Most species of molds are ubiquitous organisms that can grow almost anywhere if favorable conditions exist, and some are able to produce toxic secondary metabolites known as mycotoxins. Olives can be a suitable substrate for these toxigenic fungi and, due to olives characteristics, they are highly exposed to these fungi. The most commented fungi that contaminate olives during harvest and storage are *Aspergillus* **(Roussos et al., 2006)**, *Penicillium* **(El Adlouni et al., 2006)** and *Alternaria* **(Roussos et al., 2006)**.

Among *Penicillium* species, *Penicillium expansum* is a wound parasite fungus that enters fruits via injuries, which can be caused by inadequate conditions before harvest or by rough handling, harvesting, and transport **(Sanderson and Spotts 1995)**. This species is defined as an important patulin (PAT) producer **(Frisvad & Samson, 2004)**. PAT is one of the most widespread and studied mycotoxins in foods of vegetable origin, and is generally found in many moldy fruits, vegetables, cereals and other foods as a contaminant **(Zouaoui et al., 2015)**.

All studies of this species have been limited to the detection of PAT contamination and production in apple, apple juice, pears, cereals, etc and, to our knowledge, no studies have been developed on the detection of PAT in olives or other olive-derived products. 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 will be essential to make a risk assessment and to develop preventive measures especially during storage.

1.2. Objectives

Two main objectives were on the basis of this work:

i) To identify a set of twenty-eight fungi previously isolated from Tunisian olives by phenotypic and molecular methods, and to characterize the identified fungi in terms of their mycotoxin profile.

ii) To study the ecophysiology of *P. expansum* strains identified in the first part of the work in terms of growth and PAT production on a synthetic medium (CYA; as a positive control) and on olive based-medium (OM).

2. LITERATURE REVIEW

2.1. Olives and olive oil

The olive tree (*Olea europaea* L.) belongs to the *Oleaceae* family, and is defined as one of the oldest and the most important cultivated crops of the Mediterranean basin. It is one of the three core ingredients in the Mediterranean cuisine, and is characterized by considerable number of different olive cultivars **(Laaribi et al., 2017)**, especially in western and central Italy, Spain, Portugal, southern Morocco, Tunisia, eastern Turkey and Greece **(Loumou & Giourga, 2003).**

The domesticated olive tree, one of civilized man's first achievements, is of very ancient origin, probably arising at the dawn agriculture **(Kailis & Harris, 2007)**. Even though more attention has sometimes been given to their delicious oil than their whole food delights, olives are worldwide appreciated for their beneficial health effects and organoleptic characteristics **(Gharbi et al., 2015)**.

2.1.1. Olives and olive oil in Tunisia

For thousands of years, olives have been important in all the great civilizations that flourished in Tunisia. According to **FAO (2014)**, olives trees are the most important culture in Tunisia, where it occupies around 30% (1.68 million ha) of the cultivated land. The olive tree is cultivated practically in all regions of the country up to the border of the southern desert. Tunisia belongs to the Middle East and North Africa, which is known as one of the driest regions in the world **(World Bank, 1995)**.

Tunisian olive crop is spread over areas from the northern to the southern regions in which two specific varieties are cultivated: 'Chemleli' in the south and the center of the country, and "Chetoui" in the north **(Abdelhamid et al., 2013).** Other varieties, such as Oueslati, Chemchali, Zalmati, Zarrazi, Gerboui and Sayali, are grown in more restricted areas **(Gharbi et al., 2015)**. In Tunisia, the olive sector contributes directly or

indirectly to more than one million people and provides 34 million days of work per year, which is equivalent to more than 20% of agricultural employment **(Gharbi et al., 2015)**.

Healthy, nutritional, and sensorial properties of olive oil have been known for many centuries in the Mediterranean area. The importance attributed to olive oil is mainly due to its high content of monounsaturated fatty acids, specifically oleic acid (60–80%), and to its richness in some minor components, especially squalene, pigments, tocopherols and phenolic compounds, which contribute to the preservation of the oil chemical quality **(López Miranda et al., 2010)**. As it is considered one of the main sources of the Mediterranean diet, olive oil quality depends on several factors such as ripening, extraction method, soil type, climatic conditions, harvesting time, varieties and storage conditions **(Gharbi et al., 2015)**.

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 year over the last five years **(ONH, 2015)**. It plays an important role in the Tunisian agronomy and economy **(Dabbou et al., 2009)** by improving equilibrium of the commercial balance, providing employment, preservation of natural resources and limiting the rural exodus. In order to preserve its characteristic properties responsible for its nutritional, health benefits and pleasant flavor, only mechanical methods are used for the extraction of olive oil **(Gharbi et al., 2015)**.

2.2. Fungal contamination of olives

Olives and olive oil are currently consumed by many people for the benefits that they bring, but there are also unfavorable effects that can exist. If olives are subjected to inadequate conditions throughout production and storage, mold contamination can occur, with negative consequences in terms of olive and olive oil quality and safety.

Molds are widely distributed in nature and are considered as common contaminants of agricultural commodities, foods and feeds like cereals, olives, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fleshy fruits

(Gourama & Bullerman, 1995). More than 100.000 fungal species are generally found in a variety of substrates in warmer parts of the world especially in humid tropical areas.

Food is not commonly regarded as an ecosystem, perhaps on the basis that it is not a "natural" system. Nevertheless, as an ecosystem it is an important one, because food and plants, and the fungi that colonize their fruiting parts (seed and fruit) have been co-evolving for millennia. Food is by nature expected to be nutritious: Therefore, food represents a rich habitat for microorganisms, in contrary with the biggest natural systems, water, plants and soil. Given the right physical-chemical conditions, only the most fastidious microorganisms cannot grow in foods, so that factors other than nutrients usually select for particular types of microbial **(Pitt & Hocking, 2009)**.

Olives are often stored for a long time in 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. Such conditions favor a moldy taste and appearance and thus reduce the acceptable quality of olives **(Heperkan et al., 2006)**. In fact, molds associated with olive fruits infested with olive fruit fly (*Bactrocera oleae*) can cause some diseases and reduce fruit yields, adversely affecting quality **(Al-Ameiri et al., 2015).** According to **Pardo et al. (2005)**, inappropriate storage conditions also favor the proliferation of toxigenic fungi. In fact, most storage fungi belong to the genera *Aspergillus* and *Penicillum* **(Samane et al., 1991; El Adlouni et al., 2006),** which are adapted to low moisture conditions and can occur naturally on fresh and processed olives. In a study of **Roussos et al. (2006)**, 1,285 fungal strains from spoiled olives from Morroco were isolated belonging to ten genera: *Penicillium, Aspergillus, Geotrichum, Mucor, Rhizopus, Trichoderma, Alternaria, Acremonium, Humicola, and Ulocladium.*

Several *Penicillium* species were found on olives including *P. expansum* **(Arici, 2000)**, *Penicillium citrinum* **(Heperkan et al., 2006, 2009)** and *Penicillium crustosum* **(Heperkan et al., 2006)** from black olives. **Torbati et al. (2014)** reported that *P. expansum* was able to induce fruit rot symptoms after 7 days of inoculation, affecting oil quality. Besides *P. expansum*, other fungal species including *Alternaria alternata, Fusarium nygamai, Trichothecium roseum* and *Trichoderma harzianum* were also associated with olives.

2.3. Mycotoxins and mycotoxigenic fungi of olives

Among the food safety issues, the occurrence of fungal species able to produce mycotoxins on the agro-food products has acquired a general attention. Mycotoxins are generally defined as low-molecular-weight natural products produced as secondary metabolites under suitable environmental conditions (mostly temperature and humidity) by filamentous fungi. These metabolites are characterized by having toxic effects in human beings and other vertebrates even when present at low amounts **(Zain, 2010).**

Mycotoxins are produced by molds belonging to an array of diverse fungal species that are generally saprophytic or opportunistic weak pathogens. The most common mycotoxins which are considered as important food contaminants are secreted by three genera of fungi, namely *Aspergillus, Penicillium* and *Fusarium* **(CAST, 2003; Filtenborg et al., 2004; Venâncio & Paterson, 2007; Reddy et al., 2009; Paterson & Lima, 2010)**. Several fungal species are able to produce more than one mycotoxin, and a given mycotoxin can also be produced by species that belong to different genera.

Olive postharvest storage conditions that promote mold growth can result in high risk of contamination by mycotoxins that are unsafe for consumption **(Munimbazi & Bullerman 1996).** According to **Roussos et al. (2006)**, olives can support the growth of mold and mycotoxins production. Many fungal strains, in particular from *Aspergillus* and *Penicillium* 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 **(Gracian et al., 1962; Roussos et al., 2006)**. Studies on mycotoxin contamination of olives and olive oils are mostly devoted to aflatoxin B1, ochratoxin A and citrinin. **Ghitakou et al. (2006)** and **Roussos et al. (2006)** have reported that strains from genus *Aspergillus* were capable of producing aflatoxins and ochratoxin A in Moroccan olives and olive cake. **Ferracane et al. (2007)** and **Papachristou & Markaki (2004**) detected low amounts of ochratoxin A and aflatoxin B1 in olive oils from North Africa, but not from Italy. The occurrence of ochratoxin A, citrinin and/or aflatoxins has also been reported in black table olives ”Greek style” of Moroccan origin by **El Adlouni et al. (2006)**, and **Yassa et al. (1994)** reported that *A. flavus* and *A. parasiticus* were able to produce aflatoxins in black table olives produced in Egypt. In addition, **Daradimos et al. (2000)** and **Gracian & Arevalo (1980)** proved that aflatoxin presence in oils is originated from olives. **Heperkan et al. (2009)** reported that *Penicillium citrinum* was

able to produce citrinin in olives. Further, in molded olive samples collected in Apulia (Italy), 30% (4/13) of samples were contaminated with *Alternaria* mycotoxins, namely alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altertoxin (ATX I) or tenuazonic acid (TA) **(Logrieco et al., 2003)**.

2.4. *Penicillium expansum* and patulin contamination of olives

P. expansum is one of the best-known and most studied molds of the genus *Penicillium*, as it is one of the most common foodborne fungi on fruits occurring at harvest or postharvest **(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, 1996).** Like most other species of *Penicillium*, *P. expansum* is one of mycotoxigenic fungi that can affect food and produce mycotoxins under unfavorable conditions. 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 & Samson, 2004a)**. However, only few studies report the contamination of olives with this species **(Arici, 2000)** and, 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)**. Chemically, PAT [4-hydroxy-4-H-furo(3,2-c)pyran-2(6H)-one], is defined as an unsaturated heterocyclic lactone with an empirical formula of $C_7H_6O_4$ (Figure 2.1) and with a molecular weight of 154 g/mol **(Stott & Bullerman, 1975).**

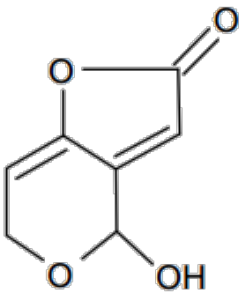


Figure 2.1. Chemical structure of patulin **(de Souza Sant'Ana et al., 2008).**

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 & 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)**.

2.5. Ecophysiology of *Penicillium expansum* and patulin production in olives

Like all eukaryotic organisms, fungi require a diverse array of minerals to sustain growth and development. Growth and mycotoxin production of fungi require an adequate substrate and toxin producing strains. Intrinsic and extrinsic factors in food are responsible for the contaminating and dominating mycobiota. This phenomenon results from physiology of fungi and they behave differently in different matrices and environmental conditions. Temperature, substrate water activity (aw), relative humidity, gas composition, substrate composition, hydrogen ion concentration are considered as important factors that influence growth and mycotoxin production **(Pitt & Hocking, 2009).** Among the environmental conditions, temperature is one of the key parameters influencing growth and evolution of filamentous fungi **(Grigoryan & Hakobyan, 2015).** The optimum temperature for mold growth is usually between 20 and 30 °C, however they can grow in a wide range of temperature from 4 to 40 °C. The lowest temperature that can provide fungal growth has been reported by **Pitt & Hocking (2009)** in the range of -7 to 0 °C.

P. expansum growth and PAT production are affected by environmental and endogenous factors typical of the substrate, being the most important ones temperature and the matrix composition, water activity and pH **(Tannous et al., 2016).** *P. expansum* is considered a psychrotrophic fungus **(Pitt & Hocking, 2009).** Ecophysiological studies of *P. expansum* have been developed almost exclusively on synthetic media and on apple and apple-based matrices **(Baert et al., 2007a; Morales et al., 2007b, 2008, 2010; Pitt & Hocking, 2009; Tannous et al., 2016)**. When tested under in vitro conditions in synthetic or apple-based media, the optimum temperature for this species in terms of growth and PAT production ranged between 16 and 25 °C, but growth

occurred from 0 ºC to 30 ºC **(Baert et al., 2007b; Pitt & Hocking, 2009; Tannous et al., 2016)**.

P. expansum infects fruit primarily through wounds caused by stem punctures or bruises occurring at harvest or during postharvest handling **(Vico et al., 2014)**. The fungus can also enter the fruit through natural openings, i.e. lenticels, stem ends and the calyx end **(Rosenberger et al., 2006)**. During harvest, olives can be left on the soil for a long time. Surface physical damage of the olive fruit due to unfavorable conditions, e.g. low temperature and insects, etc., is an enhance precondition for fungal penetration into the fruit pulp and for mycelial proliferation **(Logrieco et al., 2003)**. These conditions can promote the growth of *P. expansum* in olives wherever there is enough surface moisture to keep them alive. Infection of olives by *P. expansum* can lead to an unfavorable effect in which can affect their quality and can present a high risk for human health.

2.6. Fungal identification and Mycotoxin detection

2.6.1. Methods of fungal identification

The identification of fungi in routine relies essentially on the analysis of macroscopic and microscopic morphological characters. These identification methods can be supplemented by molecular analysis. In food spoiled by fungi, it is possible to observe the responsible fungal growth by naked eye followed by observation in an optical microscope **(Samson et al., 2000)**.

2.6.1.1. Phenotypic identification

Fungi require nutritive elements for their growth which are usually present in ordinary media or they can be added to the medium. For isolation of pathogenic and saprobic fungi several media are used. The most common ones are malt extract agar (MEA; **Raper & Thom, 1949**), Czapek Yeast Autolysate agar (CYA; **Pitt, 1973**) and

Potato Dextrose Agar (PDA; **Crous et al., 2009**). Colony characters and diameters on specific media are important for macromorphological identification of *Penicillium*. For *P. expansum*, two standard media are recommended, CYA and MEA **(Visagie et al., 2014)** (Figure 2.2). On CYA, *P. expansum* is known to have a diameter of 26-50 mm in which it presents a blue green to moderate bluish green colony with surface typically tufted and reversed color cream to yellow with brown center, orange brown or dark brown after 7 days of incubation at 25 ºC . On MEA, colonies achieve 16-34 mm, they present the same colony color as on CYA or slightly greyer and pale to orange brown on the reverse **(Frisvad & Samson, 2004)**. As described by **Frisvad & Samson (2004)**, *P. expansum* presents terverticillate conidiophores, finely to roughly walled stipes, ellipsoidal smooth-walled conidia, and occasionally fasciculate synnemata. In terms of secondary metabolites, it produces PAT, roquefortin C, communesins, chaetoglobosins, expansolid, geosmin and citrinin **(Frisvad & Samson, 2004)**.

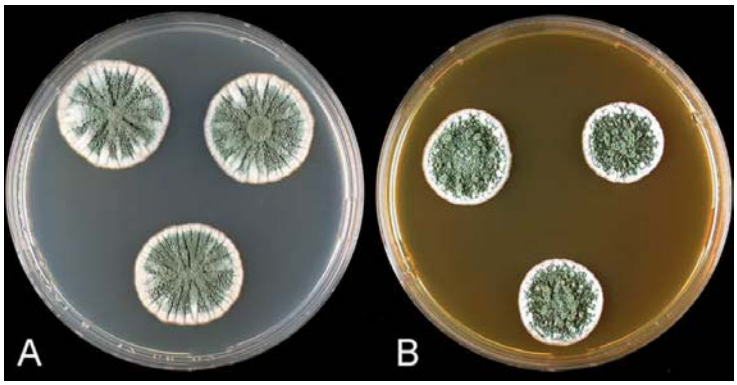


Figure 2.2. *Penicillium expansum* colonies after 7 days of incubation at 25 ºC;
A: on CYA; **B:** on MEA **(Frisvad & Samson, 2004)**

2.6.1.2. Genotypic identification

Fungal species are hard to identify, and morphological identification only is not enough to distinguish species. Many molecular identification approaches have been evaluated to date for fungi **(Makimura et al., 1994; Sandhu et al., 1995; Borman et al., 2006).** Of these, polymerase chain reaction (PCR) amplification of genomic DNA followed by sequencing of resulting amplicons has shown to be the most promising. For this, PCR is performed after isolation and DNA extraction of fungi.

The ITS region, which is situated between small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes, is the most frequently amplified and sequenced genetic marker of fungi and it is used as a barcode for phylogenetic studies and identification of fungal strains **(Begerow et al., 2010).** Universal ITS primers should be able to detect and amplify target DNA regions, even in the presence of high concentrations of non-target DNA co-extracted during DNA extraction **(Kosh & Summers, 2013).** Several additional gene regions other that ITS including β-tubulin (benA) and calmodulin (CaM) have been used to distinguish specifically *Penicillium* species **(Seifert & Louis-Seize, 2000; Samson et al., 2004; Visagie et al., 2014; Houbarken et al., 2014).**

2.6.2. Detection of Mycotoxins

To determine the contamination of food by mycotoxin, one must test for them. Clear and clean sampling procedures are desired to have reliable results **(Whitaker & Dickens, 1979).** The most conventional analytical methods for mycotoxin are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). Generally, those techniques require solid phase column cleanup of extracts and immunoaffinity techniques to remove interferences to improve the measurement of mycotoxins **(Zheng et al., 2006).** Furthermore, several analytic methods have been also used in order to detect PAT including Micro Extraction in Packed Syringe (MEPS) methodology for the determination of PAT **(Pires et al., 2012)**.

Nowadays, HPLC analysis for mycotoxins is the most utilized process for quantitative purposes. Generally, almost 80% of organic compounds in the world are determined using HPLC **(Li et al., 2011).** Usually, HPLC technique make use of a stationary and a mobile phase comprising aqueous/organic solvents in which extracted samples can run through and compounds of interest are separated.

2.6.2.1. Patulin extraction and detection

Several methodologies have been adapted to extract PAT before its analysis from foodstuffs such as fruits, fruit juices, purees or others. PAT analysis includes several

important steps: sampling, sample preparation, extraction, identification, detection, quantification and in some case statistical evaluation **(Pires et al., 2012)**.

One of the most commonly used protocols for PAT extraction is based on solvent extraction. PAT extraction and detection can occur by using different extraction solutions coupled with a detection system. Several extraction solutions, including ethyl acetate and methanol with water (10:90) (v/v), have been used in order to provide better results to fulfill the analytical purpose. Ethyl acetate has been the universal extraction solvent and almost all PAT extraction studies were performed by extracting with ethyl acetate **(**e.g. **Morales et al., 2008; Sargenti et al., 2010; Elhariry et al., 2011)**. PAT was extracted by **Tannous et al. (2016)** in which ethyl acetate were add to a plugs already removed from culture medium and were evaporated under liquid nitrogen. Less frequently, methanol extraction has also been successfully employed **(Pires et al., 2012)**. In order to obtain a profile of mycotoxins produced by fungi in vitro, a method was describes by **Bragulat et al. (2001)** which consists of the removal of three plugs from a culture medium into a volume of methanol that can cover the plugs, maintained for 60 min. According to **Aktas et al. (2004)**, several mobile phases were used in different column systems for PAT extraction. In this study, results showed that acetonitrile in mobile phase (at 5% (v/v)) provided better separation and detection by HPLC. Retention time of PAT extracted differ from samples and with the nature of the column used. Among chromatographic methods, HPLC coupled with UV detection has been considered well suited for the determination of patulin, because it is relatively polar and exhibits a strong absorption spectrum **(Shephard & Leggott, 2000).**

3. MATERIALS AND METHODS

3.1. Fungal isolates

Twenty-eight fungi were isolated from olives intended for oil production from Tunisian olive groves. All isolates were maintained in 20% glycerol at -20 ºC 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 ºC) in the dark for 7 days at 25 ºC whenever needed for further studies.

3.2. Identification of fungal isolates

3.2.1. Morphological identification

All fungal isolates were subjected to a preliminary morphological identification to the genus level following general taxonomic guides **(Samson et al. 2004; Pitt & Hocking, 2009)**.

For those isolates identified as *Penicillium* sp., further morphological identification was done. From a 7 days old culture on MEA, a loop full of spores was suspended in 500 µL of sterilized water 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 days 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 & Samson, 2004).** *Aspergillus oryzae* was not isolated from Tunisian olive groves, and was used for quality control of the molecular identification procedures.

3.2.2. Molecular identification

3.2.2.1. DNA extraction and amplification

Two different protocols were tested for the extraction of genomic DNA from fungal isolates and for the amplification of the ITS region: the commercial *REDExtract-N-Amp Plant PCR kit* (SIGMA) and the traditional SDS extraction protocol **(Rodrigues et al., 2009**). Both protocols are described below.

REDExtract-N-Amp Plant PCR kit

For genomic DNA extraction, a loop full of spores from each fungal culture was immersed into 2 mL collection tubes containing 100 µL of Extraction Solution. After vortexing, tubes were incubated at 95 °C for 10 minutes. 100 µL of Dilution Solution were then added and vortexed. This solution was used directly for PCR amplification following the conditions described in Table 3.1.

Table 3.1. PCR mix for the ITS region amplification using the REDExtract-N-Amp Plant PCR kit.

REDExtract PCR Mix	**Volume**	
Water	8 µL	
RED Extract-N-Amp PCR Ready Mix (Red)	20 µL	
Forward primer	2 µL	Vf 40 µL
Reverse primer	2 µL	
gDNA	8 µL	

The region between the genes for 18S rRNA and 28S rRNA was amplified using ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), as reported by **White et al. (1990) and Gardes & Bruns (1993).** The PCR reactions were carried out in a thermal cycler BioRad Mycycler. The PCR program is described in Table 3.2.

Table 3.2. PCR program used for the amplification of the ITS region.

Amplification Program	**Conditions**	
Initial denaturation	94 ºC, 3 min	
Denaturation	94 ºC, 30 sec	35x
Annealing	55 ºC, 30 sec	
Extension	72 ºC, 2 min	
Final extension	72 ºC, 10 min	

SDS extraction protocol

A loop full of spores from 7 day-old cultures was transferred from MEA into a 1.5 mL eppendorf containing 300 µL of Lysis Buffer 0.5% SDS (200 mM Tris-HCl pH 8.5; 250 mM NaCl; 25 mM EDTA; 0.5% [w/v] SDS) and approximately 0.5 g of sterile 0.4- to 0.6-mm diameter glass beads (Sigma, St. Louis, MO, USA), previously washed with nitric acid and vortexed 5 min at maximum speed. 150 µL of cold 3 M sodium acetate at pH 5.5 was added and mixed gently by inversion and stored at -20 ºC for 10 min. This solution was separated by centrifugation at 10,000 rpm for 10 min (4 ºC) in centrifuge. The cleaned supernatant was transferred to a new tube and was centrifuged again at 10,000 rpm for 10 min (4 ºC). After centrifugation, the clean supernatant was transferred to a new tube and an equal volume of isopropanol (-20 ºC) was added to precipitate DNA. The supernatant was gently mixed by inversion for a few minutes and incubated at -20 ºC for one hour and centrifuged at 10,000 rpm for 10 min (4 ºC). DNA pellet was washed twice with 500µL of cold 70% ethanol and centrifuged at 10,000 rpm for 7 min (4 ºC) and air dried. DNA was dissolved in 30 to 50 µL of ultra-pure water and stored at -20 ºC.

Quality and concentration of the obtained genomic DNA was determined by horizontal gel electrophoresis. Electrophoretic analysis was done on 1% agarose gels

with Tris-Acetate-EDTA buffer (TAE: 40 mM Tris-HCl; 40 mM acetic acid; 1.0 mM EDTA, pH 8.0) stained with EZ-vision (Amresco). Runs were made in TAE buffer, at constant voltage of 5 V/cm for approximately one hour. Three μL of genomic DNA and one μL of Blue Loading Buffer were loaded on the gel. DNA was visualized under UV light and images were obtained by the image analysis system Gel DocTM XR+ System (Biorad).

PCR products obtained from both methods were separated on a 1.2% agarose/TAE gel stained with EZ-vision, and compared to a DNA size marker 100 bp DNA Ladder (BIORON). Electrophoretic runs and image acquisition were as previously described.

3.2.2.2. PCR product purification (clean up)

Before sequencing, PCR products were purified from excessive dNTPs and primers with the commercial kit GF-1 PCR CleanUp Kit (Vivantis), containing special buffers to provide the correct salt concentration and pH for efficient recovery (80 - 90%) of DNA. PCR was carried out as described in the instructions of manufacturer, the volume of the PCR product was adjusted to 100 μL by adding sterile water, and 500 μL of Buffer PCR were then added. The samples were transferred after being vortexed into a column assembled in a clean collection tube and were centrifuged at 10.500 g for 1 min. The flow through was discarded and 750 μL of Wash Buffer were added. The elimination of Wash Buffer was carried out by two centrifugations at 10.500 g for 1 min. The columns were transferred to a clean eppendorf for DNA elution and 35 μL of sterile water were added The PCR product was stored at -20 °C. The concentration of the purified PCR product was determined by electrophoresis as previously described and sent for sequencing.

3.2.2.3. Sequencing and Sequence analysis

Sequence analyses were carried on an ABI 3730xl DNA Analyzer (Applied Biosystems), by outsourcing. PCR products were sequenced in both directions, and a consensus sequence was created from the assembly of the forward and backward sequences using the package Sequencher 4.9 (Gene Codes, Ann Arbor Michigan). Sequence process was performed by sequencing of the internal transcribed spacer (ITS) region of the rRNA gene. The consensus sequences were manually adjusted by chromatogram comparison and then aligned with the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm.

3.3. Mycotoxin production by fungal isolates

3.3.1. Screening of mycotoxin production in Coconut Agar Medium

After identification, all fungi were screened for mycotoxin production in 6 cm Petri dishes containing 10 mL of Coconut Agar Medium (CAM) (Figure 3.1). CAM was prepared by mixing 200 mL of coconut milk, 200 mL of water and 8 g of agar. For each fungus, a loop full of spores was transferred into 300 µL of water and 0.05% Tween 80, homogenized, and inoculated by central-point inoculation on CAM. Fungi were incubated for 7 days at 25 °C. Cultures were observed for fluorescence under long-wave UV light (365 nm) after 3, 5 and 7 days. Isolates were scored by presence/absence of fluorescence and by fluorescence color.

Figure 3.1. Coconut Agar Medium. **A:** preparation; **B:** plating.

3.3.2. Patulin detection by HPLC analysis

All *Penicillium* 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 days 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 minutes, 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 a fluorescence Photo Diode Array (PDA) detector 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 PAT was done by comparison with the standard.

3.4. Ecophysiology of *Penicillium expansum* growth and PAT production

3.4.1. Fungal isolates

From the isolates identified in section 3.2, four were identified as *P. expansum*, and three were screened as PAT producers (section 3.3). The three PAT producing isolates of *P. expansum* TUN20, TUN22 and TUN23 (further referred to as TUN strains) were selected for ecophysiological studies in terms of growth and PAT production. 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.

3.4.2. 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. For this paste, pH was determined to be 5.3, and water activity was 0.98 (measured with a water activity meter 4TE, 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 (20 mL; Figure 3.2).

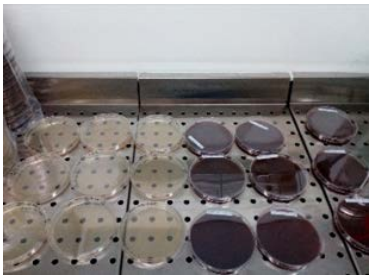


Figure 3.2. CYA (cream) and OM (dark) media.

3.4.3. 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 day-old culture and by scrubbing the spores (Figure 3.3). 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 10 and 20 days. Petri dishes without fungi (medium only) were used as negative control.

Figure 3.3. Spore suspension preparation.

Fungal growth was determined by measurement of fungal colonies diameter (in cm) after 3, 5, 8, 10, 15 and 20 days of incubation. The procedure was done in triplicate.

3.4.4. PAT analysis of culture media by HPLC

3.4.4.1. 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. The prepared solutions were stored at 4 ºC.

3.4.4.2. Optimization of PAT analysis

For the optimization and validation of PAT analysis by HPLC, two different methods were tested, one by using ethyl acetate as PAT solvent and the other using methanol. The ethyl acetate method is the method usually described in PAT studies (**Morales et al., 2008; Sargenti et al., 2010; Elhariry et al., 2011; Tannous et al., 2016)**, but is toxic, tedious and expensive. For that reason, extraction with methanol was tested as a rapid and less expensive/toxic alternative.

Analysis of PAT with ethyl acetate: Three PAT solutions at 50 µg/mL, 5 µg/mL and 0.5 µg/mL were diluted in ethyl acetate from the stock solution. Ethyl acetate was then evaporated with gaseous nitrogen and the dried extract was redissolved in the same volume of UP water acidified to pH 4.0 with acetic acid. The solution was filtered with a 0.2 µm syringe filter and analyzed by HPLC. HPLC conditions were the same as described in section 3.3.2, but using acidified water (UP water to pH 4.0 with acetic acid) as mobile phase.

Analysis of PAT with methanol: Three PAT solutions at 50 µg/mL, 5 µg/mL and 0.5 µg/mL were diluted in methanol from the stock solution. The solution was filtered with a 0.2 µm syringe filter and analyzed by HPLC. HPLC conditions were the same as described in section 3.3.2, using methanol and water at 10:90 (v/v) as mobile phase.

3.4.4.3. Method validation

As determined in section 3.4.4.2, HPLC analysis with methanol was selected as the analytical method for PAT detection and quantification. Linearity, limit of detection (LOD) and limit of quantification (LOQ) were determined 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 **(Taverniers et al., 2004)**:

$$LOD = 3 \times (s_a/b) \text{ and } LOQ = 10 \times (s_a/b)$$

where s_a is the standard deviation of the intercept of the regression line obtained from the calibration curve, and b is the slope of the line.

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), which is a measurement of the variation obtained within the replicates tested each day, and is given by the corresponding relative standard deviation.

3.4.4.4. PAT extraction from fungal cultures

After 10 days and 20 days 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 (Figure 3.4).

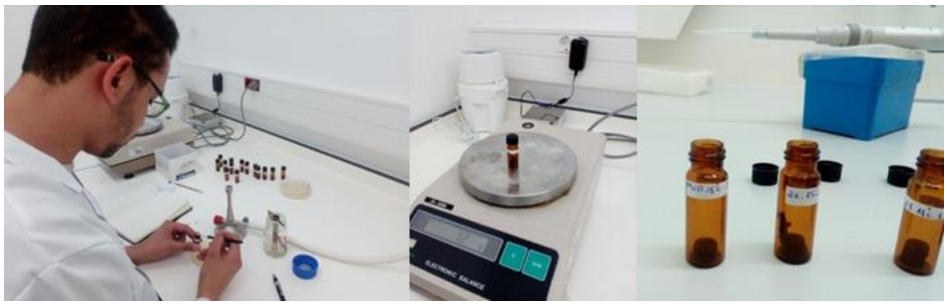


Figure 3.4. PAT extraction from culture media.

3.4.4.5. HPLC analysis

Detection of PAT was done by comparison with the standard as described in section 3.3.2. 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.

3.5. Safety and decontamination procedures

Due to the toxicity of PAT, all the necessary safety considerations were taken into account when handling this substance. Solutions were handled with nitrile gloves under the chemical hood; all disposable materials, including petri dishes with potential PAT contamination, were decontaminated by autoclaving before being disposed; reusable materials were decontaminated by immersion in 10% bleach over-night, immersion in 5% acetone for one hour and washed with distilled water several times.

3.6. 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 analysing Spearman correlation index. In all cases, statistical significance was established at $p \leq 0.05$.

4. RESULTS AND DISCUSSION

4.1. Establishment of the protocol for DNA extraction and PCR amplification

For the purpose of molecular identification of the fungi, two different protocols for DNA extraction and PCR amplification were used. The commercial REDExtract-N-Amp Plant PCR kit with incorporated PCR amplification was tested first for its simplicity, because genomic DNA is extracted in ten minutes and is immediately submitted to PCR amplification. Figure 4.1 shows some of the samples extracted and amplified by the REDExtract-N-Amp Plant PCR kit.

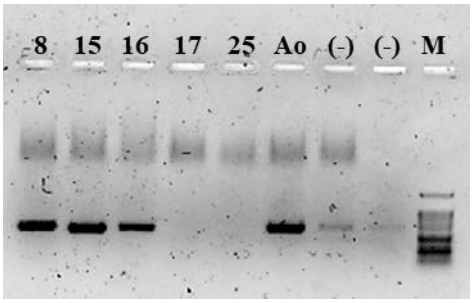


Figure 4.1. PCR products amplified by REDExtract-N-Amp Plant PCR kit with ITSF1-F and ITS4 primers. From 8 to 25: TUN isolates; Ao: *Aspergillus oryzae*; (-): negatives; M: molecular size marker (100 bp ladder).

For this protocol, only 4 out of 13 samples were amplified using the primers ITS1-F and ITS4. For that reason, the classical SDS extraction protocol followed by PCR amplification (as described by **Rodrigues et al., 2009**), already established in the laboratory, was further used for the remaining samples.

Quality and concentration of the genomic DNA obtained by extraction with the SDS protocol is shown in Figure 4.2. Of the twenty-eight samples tested, genomic DNA was detectable for 25 isolates with good quality and enough amount for PCR purposes.

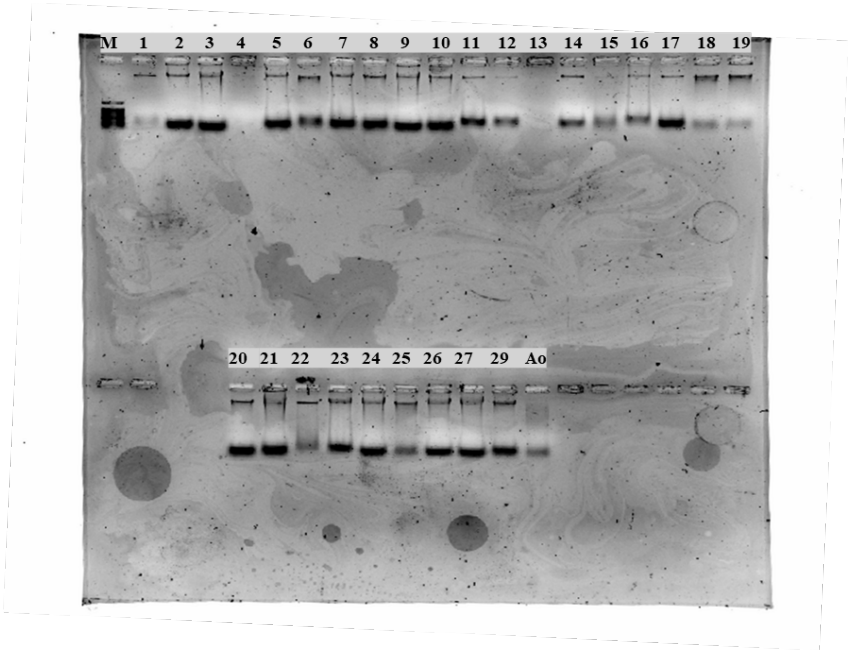


Figure 4.2. Genomic DNA of the 28 samples obtained by the SDS extraction protocol. M: molecular size marker (100 bp ladder); From 1 to 29: TUN isolates; Ao: *Aspergillus oryzae*.

After PCR amplification of the ITS region, DNA bands were found to have the expected size of *ca*. 600 bp (Figure 4.3), but there were 6 failed reactions in 21 samples. While the failed reaction of sample 4 was expected, based on the quality of gDNA, this was not expected for samples 5, 6, 10, 25 and 29, since gDNA was apparently of good quality and amount. These reactions where repeated successfully. On the other hand, sample 13 had a good PCR amplification, even though gDNA was not extracted in high amounts.

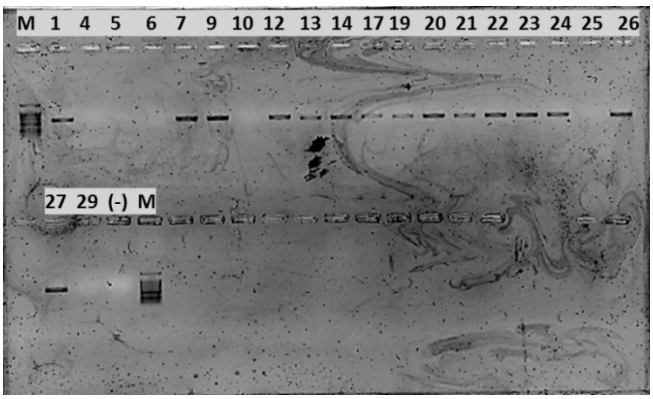


Figure 4.3. PCR product using primers ITS1-F and ITS4 after DNA extraction with the SDS protocol. M: molecular size marker (100 bp ladder); From 1 to 29: TUN isolates; (-): negative.

Given the results obtained by both tested protocols, SDS protocol, although more time and labor demanding, was more efficient than REDExtract-N-Amp PCR kit, and it was able to extract gDNA from almost all samples, being that it was only necessary to repeat extraction of two samples.

4.2. Identification of fungal isolates

The first objective of this study was to identify to the species level a set of 28 fungi previously isolated from Tunisian olive groves and representative of the total mycobiota. These fungi were firstly identified to the genus level based on morphological characteristics and grouped by morphological similarity. Figure 4.4 shows one isolate of each genus identified.

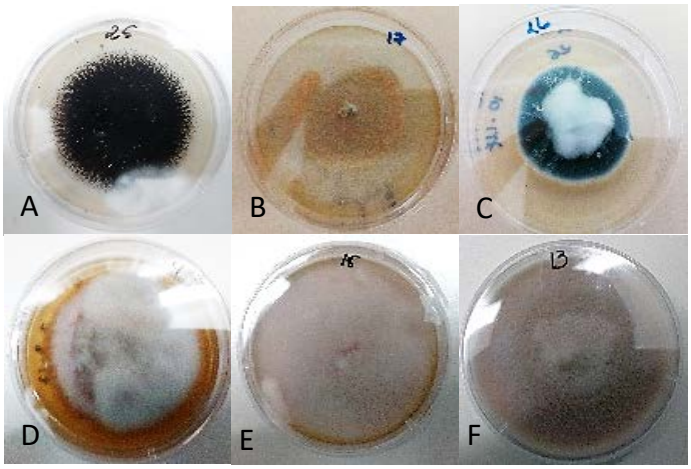


Figure 4.4. Fungal isolates grown on MEA for 7 days at 25 °C, in the dark.
A: *Aspergillus*; **B:** *Tricothecium*; **C:** *Penicillium*; **D:** *Pleospora*; **E:** *Fusarium*; **F:** *Alternaria*.

In the past, identification of fungi was based on phenotypic features, mostly morphological and cultural. However, these characteristics are usually insufficient to identify fungi to the species level. Currently, genotypic identification is used as a complement to phenotypic methods. Typically, genotypic identification of fungi involves the use of conserved sequences within phylogenetically informative genetic targets. Of the various DNA regions tested as markers, the internal transcribed spacer (ITS) is the one mostly used because of its broad utility as a species marker in

taxonomic and ecological studies, when different genera are under study. ITS is thus the currently accepted DNA barcode for fungi **(Begerow et al., 2010).**

After comparison of consensus sequences with the sequences available in the NCBI database, a degree of homology of 99.8 to 100% was obtained between most of the isolates and matching reference strains, as shown in Table 4.1. For some samples, the obtained sequences where not of enough quality to run a BLAST (TUN10 and TUN24), or did not give a match above the established threshold of 97% similarity (TUN3). In these situations, fungi were only tentatively identified at the phenotypic level.

Compared with other agricultural commodities, studies concerning contamination of olives with potentially toxigenic fungi in this matrix are scarce. In this work, the diversity of fungi isolated from olives intended for oil production from Tunisian olive groves was carried. By using an approach of identification by integrating phenotypic and molecular identification methods it was possible to identify the 28 fungi as belonging to six different genera: *Alternaria, Aspergillus, Fusarium, Penicillium, Pleospora and Tricothecium*. Among the isolates, *Penicillium* species were the dominant fungi from Tunisian olives (23 isolates; 82%). The other identified genera showed one isolate each.

Table 4.1. Polyphasic identification of fungi from Tunisian olives.

Code	Phenotypic ID	Molecular ID	% Similarity with reference strains in NCBI
TUN1	*P. expansum*	*P. expansum*	100%
TUN2	*P. crustosum/commune*	*P. crustosum*	100%
TUN3	*P. crustosum/commune*	no match above threshold (possibly *P. camemberti*)	-
TUN4	*Stemphylium* sp.	*Pleospora herbarum* (anamorph *Stemphylium herbarum*)	100%
TUN5	*P. crustosum/commune*	*P. crustosum*	100%
TUN6	*P. polonicum*	*P. polonicum*	100%
TUN7	*P. polonicum*	*P. polonicum*	99.8%
TUN8	*P. crustosum/commune*	*P. crustosum*	99.8%
TUN9	*P. crustosum/commune*	*P. crustosum*	100%
TUN10	*P. crustosum/commune*	No amplification	-
TUN11	*P. crustosum/commune*	*P. crustosum*	100%
TUN12	*P. crustosum/commune*	*P. crustosum*	100%
TUN13	*Alternaria* sp.	*Alternaria alternata*	100%
TUN14	*P. crustosum/commune*	*P. crustosum*	100%
TUN15	*Fusarium* sp.	*Fusarium brachygibbosum* (*F. oxysporum* complex)	100%
TUN16	*P. polonicum*	*P. polonicum*	100%
TUN17	*Trichothecium* sp.	*Trichothecium roseum*	100%
TUN18	*P. polonicum*	*P. polonicum*	99.8%
TUN19	*P. crustosum/commune*	*P. crustosum*	100%
TUN20	*P. expansum*	*P. expansum*	100%
TUN21	*P. polonicum*	*P. polonicum*	100%
TUN22	*P. expansum*	*P. expansum*	100%
TUN23	*P. expansum*	*P. expansum*	100%
TUN24	*P. crustosum/commune*	No sequence	-
TUN25	*Aspergillus* section *Nigri*	*Aspergillus tubingensis* (*A. niger* complex)	100%
TUN26	*P. crustosum/commune*	*P. crustosum*	100%
TUN27	*P. crustosum/commune*	*P. crustosum*	100%
TUN29	*P. polonicum*	*P. polonicum*	99.8%
A.o.	*Aspergillus oryzae*	*A. oryzae*	100%

Few studies have investigated the mycobiota in olives, at the level of potentially toxigenic fungi. A study by **Roussos et al. (2006)** indicates that the dominant mycobiota isolated from olives and olive cake belong to the *Aspergillus* and *Penicillium* genera. *Trichoderma* and *Alternaria* species were also identified, but with lower prevalence. In another study **(Baffi et al., 2012)**, identification of isolates from olives, olive paste and

olive pomace revealed few fungal strains including *Penicillium commune, Penicillium crustosum, Aspergillus fumigatus, Aspergillus niger* and others. Seven species of fungi were identified from olive fruits infested with fruit fly: *Alternaria solani, Aspergillus niger, Cladosporium herbarum, Fusarium solani, P. digitatum, P. italicum and Rhizopus stolonifer* **(Al-Ameiri et al., 2015)**. In the present work, diversity of fungi found in Tunisian olives matches with these previous studies in term of species identification.

Within the 23 *Penicillium* isolates, only three species were identified: *P. crustosum* (13 isolates, 56.5%), *P. polonicum* (6 isolates, 26.1%) and *P. expansum* (4 isolates, 17.4%).

4.3. Mycotoxin production by fungal isolates

All identified fungi were screened for mycotoxin production in Coconut Agar Medium. After incubation for 7 days at 25 °C in the dark, Petri dishes were submitted to long-wave UV light (365 nm). Among all isolates, only 4 of the 28 isolates produced fluorescence on CAM: TUN20, TUN22 and TUN23, corresponding to *P. expansum*, produced a green fluorescence, while TUN6 (*P. polonicum*) showed an orange fluorescence.

As described by **Lin & Dianese (1976)** coconut-based media allow for the screening of mycotoxin production by fungi, because these media generally induce the mycotoxin production, and they usually fluoresce in CAM when observed under long UV light. The method is mostly performed on *Aspergillus* species for aflatoxin detection **(Davis, 1987; Abarca et al., 1988; Rodrigues et al., 2009)** showing a blue fluorescence, but other mycotoxins are also detected. For example, **Mohamed et al. (2013)** detected citrinin on CAM as an intense yellow green fluorescence, later confirmed by HPLC, and ochratoxin A can be detected as blue-green fluorescence (P. Rodrigues, personal communication).

Table 4.2 shows a list of the mycotoxins potentially produced by the fungal species identified in the present study. It is important to refer that not all isolates of a given species produce the same secondary metabolites (including mycotoxins). In fact,

the mycotoxin profile of a fungus and the proportion of mycotoxigenic strains in a given fungal population vary with the matrix and with the ecological niche **(Pitt & Hocking, 2009)**. This is why it is of major importance for mycotoxin risk assessment of a given food matrix or ecological niche to study its fungal diversity, as well as the mycotoxin profile of fungi under specific conditions.

Table 4.2. Some of the most common toxigenic fungi found in olives and other fruits, and their associated mycotoxins.

Fungus	Associated Mycotoxins	Reference
Alternaria alternata	Alternariol (AOH) Alternariol monomethyl ether (AME) Altenuene (ALT) Altertoxins I, II, III (ATX-I, -II, -III) Tenuazonic acid (TeA)	**Bottalico & Logrieco, 1998**
Aspergillus tubingensis	Ochratoxin A	**Lahouar et al. 2017**
Fusarium oxysporum	Fusaric acid Moniliformin Napthoquinone Nectriafurone	**Seifert & Thrane, 2004**
Penicillium crustosum	Penitrem A Roquefortine C	**Bottalico & Logrieco, 1998**
Penicillium expansum	Patulin Citrinin Chaetoglobosin Requefortine C Commenesin A and B	**Frisvad & Samson, 2004**
Penicillium polonicum	Penicillic acid Verrucosidin Nephrotoxic glycopeptides	**Frisvad & Samson, 2004**
Trichothecium roseum	Roseotoxins Trichothecenes Trichothecin	**Batt & Tortorello, 2014** **Domsch et al. 1980** **Richard et al. 2003**

Considering: i) the number of *P. expansum* isolates screened as mycotoxin producers (probably PAT) in the small fungal sample under study; ii) the importance of PAT in foods, mostly fruits; and iii) the fact that, to our knowledge, no studies exist on the risk of *P. expansum* and PAT contamination of olives, the central focus of the study was directed to this mycotoxin. For that reason, all isolates showing fluorescence on

CAM as well as representative isolates of *P. polonicum* and *P. crustosum* were further subjected to HPLC analysis for PAT detection.

Figure 4.5 shows a chromatogram of PAT standard solution at 50 µg/mL, where the toxin can be detected at retention time of approximately 7.5 minutes, in a run of 20 min. Figures 4.6. and 4.7 represent the results of PAT detection from *P. expansum* and *P. polonicum*, respectively. Chromatogram analysis confirmed the production of PAT by three of the four *P. expansum* isolates. No PAT production was detected in the tested *P. polonicum* and *P. crustosum* isolates.

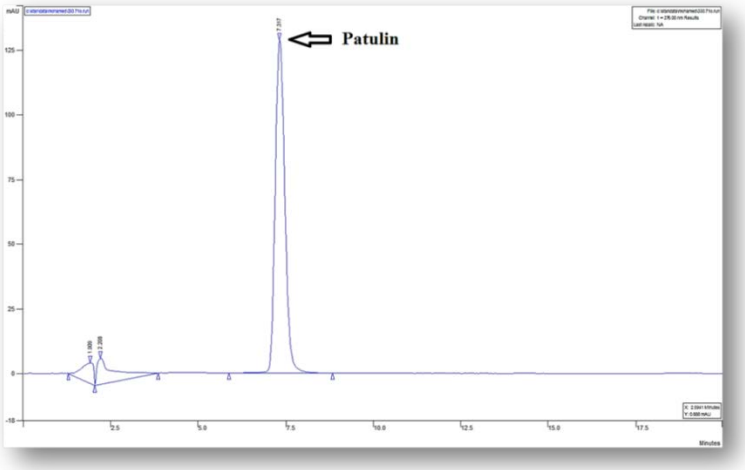


Figure 4.5. HPLC-UV chromatogram of PAT standard solution at 50 µg/mL.

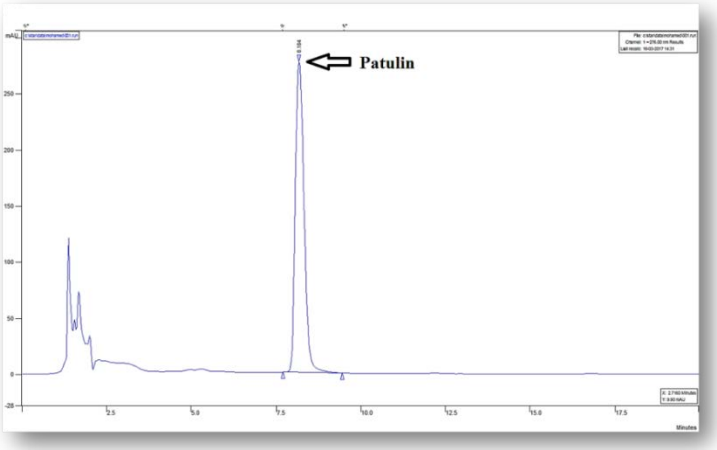


Figure 4.6. Chromatogram of patulin detection from *Penicillium expansum* TUN20 grown on CYA for 7 days, at 25 ºC.

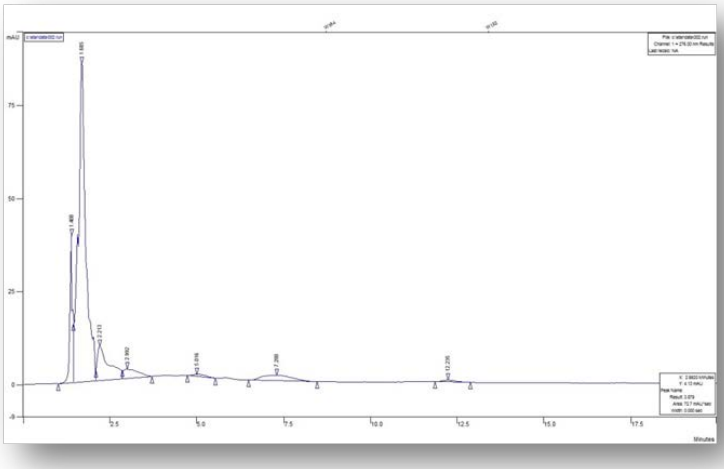


Figure 4.7. Chromatogram of patulin detection from *Penicillium polonicum* grown on CYA for 7 days, at 25 ºC. Patulin is not detected for this fungus.

P. expansum has frequently been defined as an important producer of PAT and citrinin in several fruits, mostly apples and pears **(Gimeno & Martins, 1983; Laido et al., 2001; Martins et al., 2002; Frisvad & Samson, 2004; Morales et al., 2007b, 2008, 2010; Abramson et al., 2009)**. According to our results, *P. expansum* seems to be also an important contaminant of Tunisian olives. Furthermore, 75% of the identified isolates were able to produce PAT on synthetic media (CAM and CYA). Citrinin production by *P. expansum* was not studied.

Based on these results, a second objective was established for this study, which was to assess the risk of PAT contamination of olives related to olive-native *P. expansum*. For this, ecophysiological aspects governing growth and PAT production of olive-native *P. expansum* in olive-based medium under optimal and sub-optimal temperature conditions were studied.

4.4. Ecophysiological studies of growth and PAT production by *P. expansum* in olive-based medium

Few studies of *P. expansum* have been conducted to characterize the growth and the toxigenesis 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 in natural conditions and forecast its potential risks on the fruit and consumer's health **(Tannous et al., 2016)**. This study was carried out on three strains of *P. expansum* initially isolated from Tunisian groves (TUN strains), using a culture collection strain (MUM 10.175) as non-native control (Figure 4.8).

Figure 4.8. Cultures of *Penicillium expansum* used in this study.
A: MUM 10.175; **B**: TUN20; **C**: TUN22; **D**: TUN23.

4.4.1. 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 ($p \leq 0.05$), but significant differences were observed between the MUM strain and the TUN strains. 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 in both media for all temperatures tested are shown in Figure 4.9. Figure 4.10 compares the fungal growth rate, in mm/day, of MUM and TUN strains. Colony diameters were measured throughout the incubation period (at days 0, 3, 5, 8, 10, 15 and 20) and plotted against time (days). For all the tested conditions, the growth curves based on colony diameters were typical of a linear fungal

growth. However, fungal growth was in some cases 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.

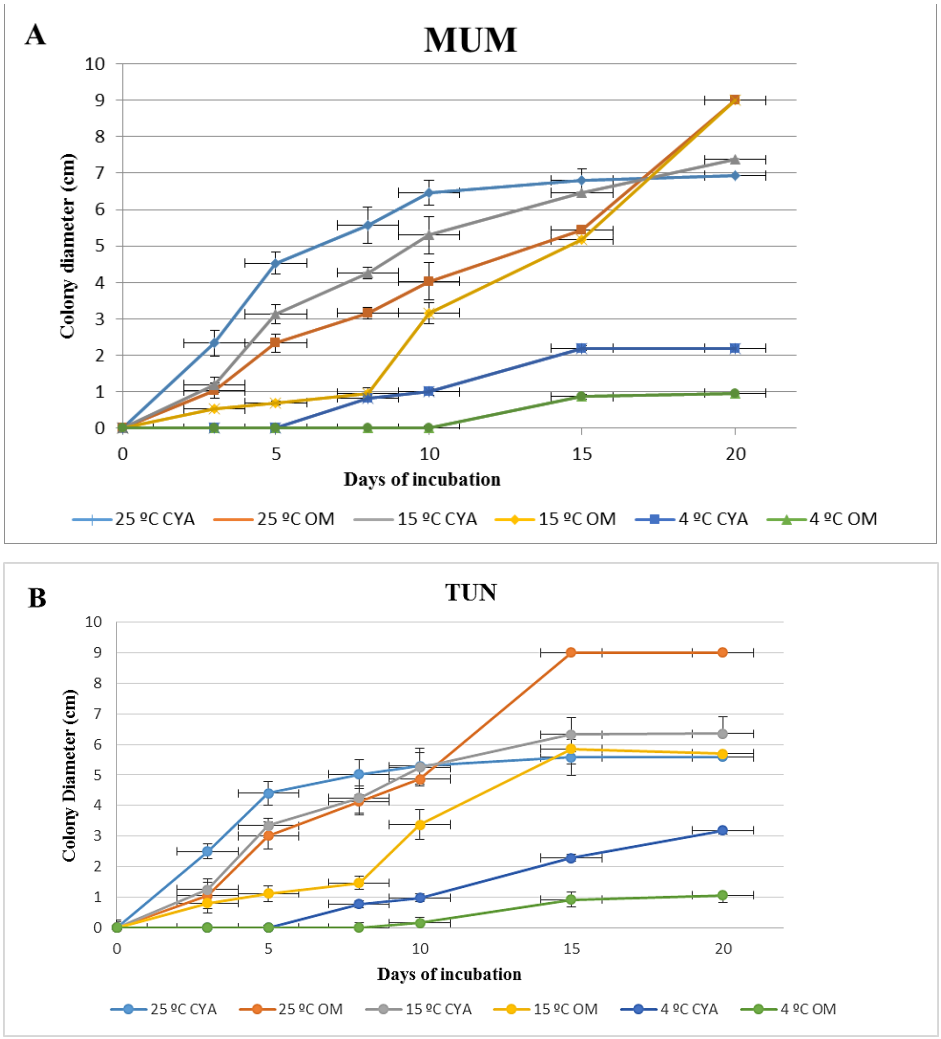


Figure 4.9. Growth curves of *Penicillium expansum* strains. **A**: MUM; **B**: TUN, at 4 °C, 15 °C and 25 °C in CYA and OM.

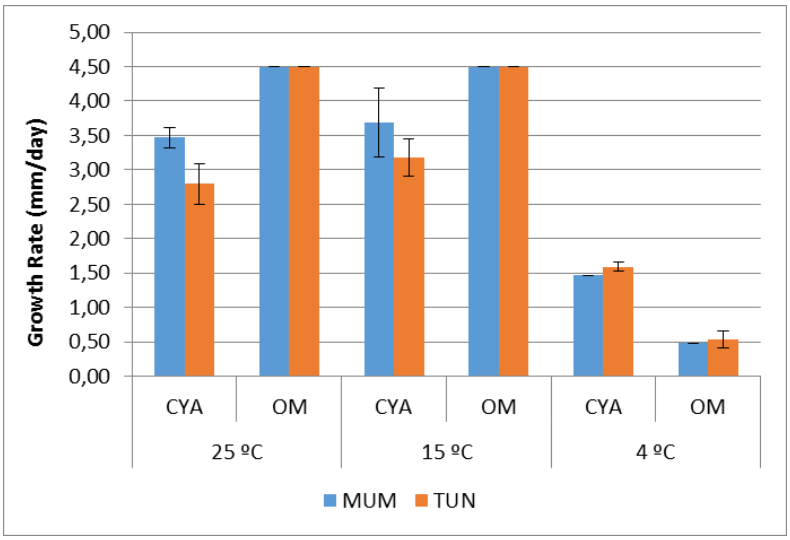


Figure 4.10. Growth rate (mm/day) of MUM and TUN strains at 4 °C, 15 °C and 25 °C, on CYA and OM.

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 (Figure 4.9) and also on fungal growth rate (Figure 4.10) of both fungi. When analyzing growth rate after 20 days of incubation, this parameter was generally significantly higher ($p < 0.05$) on OM for both fungi, for 25 ºC and 15 ºC. If growth throughout time was considered, growth was better on CYA for these temperatures until the 10^{th} day, but between 10 and 20 days of incubation growth trend suffered an inflection, and fungi started to grow faster on OM.

Interestingly, the strains displayed very different colony morphologies on the two media (Figure 4.11). On CYA, growth was abundant (Figure 4.11 A, B and D), whereas on OM growth was poor on mycelium and was mostly based on highly spread (hence the colony diameter) and strongly sporulating synnemata (Figure 4.11 D, E and F), i.e.

large, erect reproductive structures bearing compact conidiophores, which fuse together to form a strand with conidia at the end (Figure 4.12). 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.

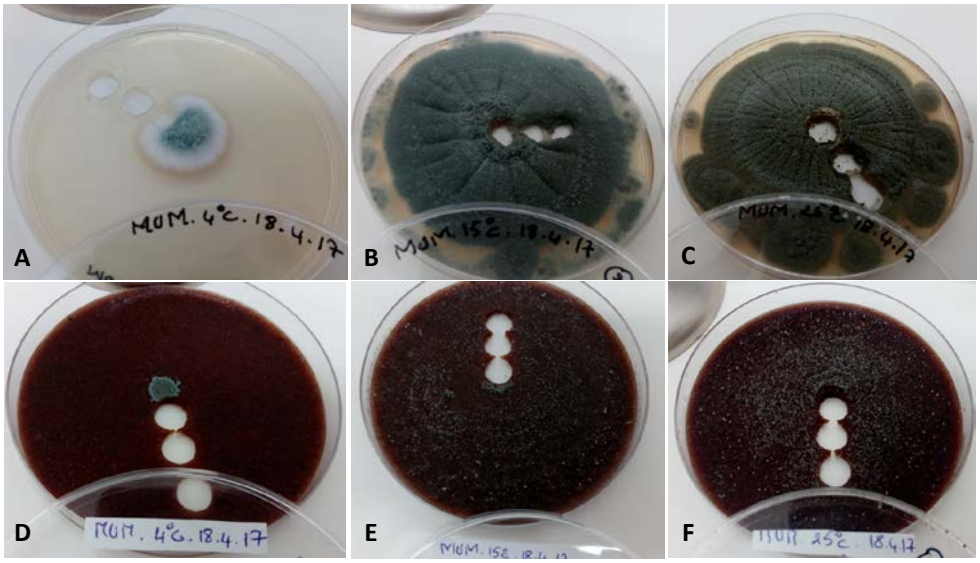


Figure 4.11. Colonies of *Penicillium expansum* MUM 10.175 after 20 days of incubation: **A**, **B** and **C**: growth on CYA at 4 ºC, 15 ºC and 25 ºC, respectively; **D**, **E** and **F**: growth on OM at 4 ºC, 15 ºC and 25 ºC, respectively.

Figure 4.12. Detail of synnemata produced by *Penicillium expansum* MUM 10.175 after 20 days of incubation on OM at 25 ºC.

At 4 ºC, growth followed a similar trend throughout time on both media, but with a growth rate significantly higher on CYA than on OM ($p < 0.05$) for MUM and TUN

strains. Growth rate of TUN was significantly higher than that of MUM on CYA ($p = 0.25$) but not 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 but, to our knowledge, this is the first study considering the growth of this fungus on olives and olive-based matrices. **Tannous et al. (2016)** observed a growth of 6.8 cm of *P. expansum* on CGA (Czapek Glucose Agar) after 7 days of incubation at 25 ºC, but growth of the same fungus on apples was in general slower.

On the other hand, 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 & 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.

Our results lead to the conclusion that OM is not an optimal growth medium for *P. expansum*, and that it can be a limiting factor for fungal spread on olives if 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. Also, olive-native fungi (TUN) don't seem to be more adapted to olive matrix than the MUM culture.

The effect of temperature on fungal growth was performed at 4 ºC, 15 °C and 25 °C for both media. These temperature conditions intend to reflect correct storage conditions (4 ºC; refrigerated) and potentially abusive 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 rate on CYA at the end of incubation was higher (but not significantly; $p = 0.822$) at 15 ºC than at 25 ºC for both MUM and TUN strains (Figure 4.10), being that MUM grew significantly faster than TUN at 25 ºC ($p = 0.019$), but not

at 15 ºC ($p = 0.073$). Growth rate on OM was maximum for both temperatures, with 9 cm being achieved at the end of incubation. On the other hand, growth at 4 ºC was significantly reduced when compared with higher temperatures ($p < 0.037$). However, growth rate is not constant throughout this period. In general, the optimal growth temperature for MUM and TUN strains was 25 ºC, at which the fungus exhibited the faster initial growth and the most significant colony growth (Figure 4.9). The exception goes to growth of TUN strains, which was higher at 15 ºC on CYA than at 25 ºC 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 ºC and 25°C **(Lahlali et al., 2005; Baert et al., 2007a; Pitt & Hocking 2009; Tannous et al., 2016).**

At 4 ºC, a lag phase varying from 5 to 10 days was observed in both MUM and TUN fungi, with the longer period corresponding to growth on OM. A lag phase of 6 days was also 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 ºC to 25 ºC, but minimum temperatures for growth have been reported between − 2 and − 6 °C, with growth still quite strong at 0 °C **(Pitt & 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.

4.4.3. Influence of matrix and temperature on PAT production

4.4.3.1. Validation of PAT extraction method and HPLC analysis

Like other forms of chromatography, HPLC have been used for the detection of several types of mycotoxins. In the present study, PAT was extracted with methanol and analyzed by HPLC in a mobile phase composed of methanol and water at 10:90 (v/v).

The validation of the PAT extraction method and analysis by HPLC involves the determination of the recovery rate in CYA and OM, linearity, repeatability of the method, and determination of the limit of detection (LOD) and limit of quantification (LOQ) of PAT. Linearity was studied calculating a PAT calibration curve based on PAT standard solutions ranging from 0.2 µg/mL to 125 µg/mL. The calibration parameters including equations of the linear regression, correlation coefficient (R^2), LOD and LOQ for PAT are shown in Table 4.3.

Table 4.3. Calibration parameters for PAT detection and quantification.

Parameter	**Patulin**
R_t (retention time)	7.2 - 8.2
Calibration curve	y = 234777x - 139622
Correlation coefficient (R^2)	0.9983
Linearity range (µg/mL)	0.2 to 125
LOD (µg/mL)	5.8
LOQ (µg/mL)	19.2

PAT has a low molecular weight and is soluble in water and polar organic solvents like methanol, ethanol, acetone and ethyl acetate. It is quite stable in acid medium, but unstable in alkaline medium **(Li et al., 2017)**.

Sample preparation is critical in the overall analytical process, since it plays a role in analyte extraction, preconcentration and clean-up from coexisting compounds. Consequently, an efficient extraction and clean-up procedure is often required for the

quantitative analysis of PAT at trace levels in highly complex matrices such as food samples. Liquid-liquid extraction with ethyl acetate has been the universal method of sample preparation for PAT analysis and successfully validated through collaborative studies for PAT determination in clear and cloudy apple juices and apple puree **(Beltran et al., 2014; Seo et al., 2015; Zouaoui et al., 2015; Marsol-Vall et al., 2016)**. It was adopted by AOAC International as an official method in 2000. The main steps are generally: extraction with ethyl acetate; clean up by extraction with sodium carbonate solution; extract drying with anhydrous sodium sulfate and evaporation of ethyl acetate. PAT is then resuspended in water pH 4 and determined by reversed-phase liquid chromatography (LC) with UV detection. PAT is detected at UV at 276 nm wave-length **(Morales et al., 2007b)**. Apart from the time-consuming procedure, a big defect of this method is that PAT is not stable in alkaline Na_2CO_3 solution, and the neutralization stage needs to be performed as quickly as possible to avoid losses **(Li et al., 2017)**.

Less commonly but much more practical and faster, methanol has also been used as a PAT extraction solvent **(Christensen et al., 2009)**, with aqueous solution of methanol being used as mobile phase in HPLC analysis. In the present study, PAT extraction with methanol and HPLC analysis with methanol:water mobile phase was compared with the previously described ethyl acetate method. The validation of method using methanol as mobile phase was confirmed by preparing 3 concentrations of PAT (50 µg/mL, 5 µg/mL and 0.5 µg/mL,) with both methods. Unexpectedly, no PAT was detected after ethyl acetate extraction, while detection with methanol resulted in good recoveries and clean chromatograms.

At the level of HPLC analysis, special attention is needed to consider interference from 5-hydroxymethylfurfural (5-HMF) and phenolic compounds in chromatographic methods, due to their similar chromatographic behavior to PAT **(Li et al., 2017)**. Unambiguous identification of PAT could be accomplished by HPLC-UV coupled to DAD, which allowed the confirmation of the PAT peaks by analysis of the UV spectrum.

The recovery rate for each of the two media was confirmed by spiking 1 g of CYA and OM medium with a known concentration of 50 µg/g. The percentual recovery rate and relative standard deviation (RSD) are presented in Table 4.4.

Table 4.4. 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

PAT 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, PAT recovery obtained in our study is within the range of that observed in other studies, for example 92.9% to 100.5% from commercial baby foods in Turkey **(Karakose et al., 2015)**, 74% to 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)**.

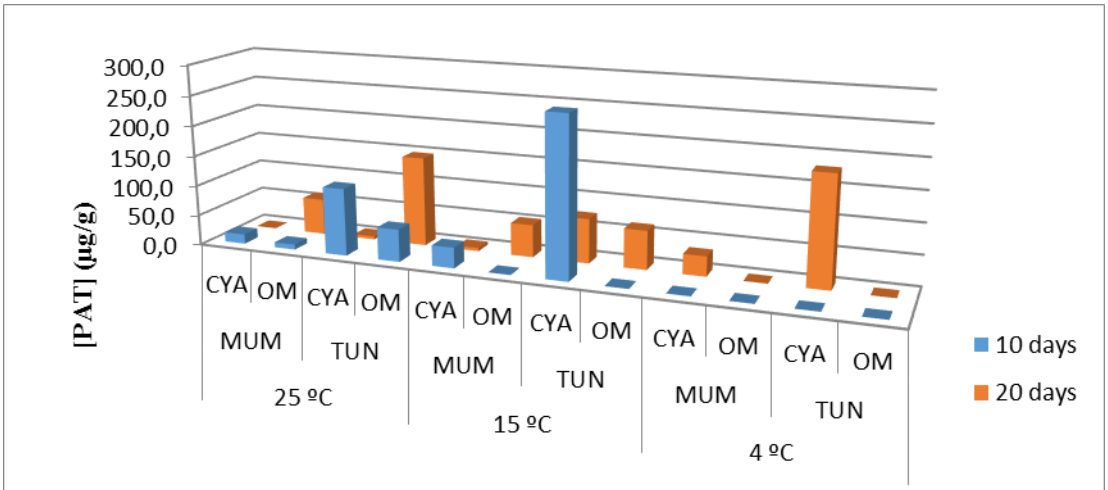
4.4.3.2. PAT detection and quantification on CYA and OM

PAT concentration on each medium (CYA and OM) and each temperature (4 °C, 15 °C, 25 °C) is shown in Figure 4.11. A significant difference in PAT production was generally observed between MUM and TUN fungi ($p < 0.033$) after 10 days of incubation. The amount of PAT after 20 days was significantly different between fungi at 25 ºC ($p = 0.24$), at 15 ºC on OM ($p = 0.33$) and at 4 ºC on CYA ($p = 0.13$), with TUN fungi producing higher amounts of PAT than MUM.

The differential production of PAT 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 *Botrytis 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 produced more PAT when grown on the host from which they originated.

A weak correlation between fungal origin and PAT production on OM and CYA was detected in our study for 10 days (Pearson correlation = 0.309; $p = 0.008$) and for 20 days (Pearson correlation = 0.328; $p = 0.005$), but this correlation was not consistent, since in most cases the amount of PAT produced by TUN fungi was higher on CYA

than on OM. **De Clercq et al. (2016)** found that PAT production was more dependent on the temperature/atmosphere conditions or strain than on strain origin.



	25 ºC				15 ºC				4 ºC			
	MUM		TUN		MUM		TUN		MUM		TUN	
	CYA	OM	CYA	OM	CYA	OM	CYA	OM	CYA	OM	CYA	OM
10 days	15,8	8,5	110,9	53,3	33,9	0,0	261,3	0,4	0,0	0,0	0,0	0,0
20 days	0,0	59,7	5,0	147,3	5,2	53,5	73,1	63,6	32,8	0,0	180,5	0,0

Figure 4.13. Patulin production by MUM and TUN fungi at 25 °C, 15 ºC and 4 ºC after 10 and 20 days of incubation on CYA and OM. (Note: 0,0 corresponds to < LOQ).

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

Our results match those of other studies, including that of **Baert et al. (2007b),** where PAT production was reduced when the temperature decreased from 20 to 10 or

4 °C. Also it was reported by **Paster et al. (1995)** and **Tannous et al. (2016)** that the highest PAT concentrations were attained at around 16 to 17 °C. In a study of PAT production by *P. expansum* in apple puree medium, **De Clercq et al., (2016)** indicated that low temperatures (4 ºC) 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 PAT. After 10 days of incubation, more PAT was detected on CYA than on OM, but an inflection of this trend was observed when the longer incubation period is analyzed. For 20 days of incubation, PAT was generally found in higher amounts on OM, except at 4 ºC. Even though fungi were able to produce PAT on CYA at 4 ºC, no PAT was produced on OM. This temperature seems to be safe for olive storage, at least for a storage period of up to 20 days. On the contrary, storage temperatures of 15 ºC and 25 ºC are abusive conditions for long periods of storage. On this respect, fungi seem to have a long lag phase of PAT production on OM, but once this phase is overcome, high amounts of PAT are produced. Other authors have also reported that the length of time apples are stored at ambient temperature (20 °C) is critical for PAT accumulation **(Morales et al., 2007c; Welke et al., 2011; Baert et al., 2012**).

The other interesting aspect of these results is that, oddly, amounts of PAT on CYA suffered a significant reduction from 10 to 20 days of incubation when considering 25 ºC and 15 ºC ($p < 0.05$). This effect is not observed at 4 ºC 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. The mechanism behind this degradation has not been subject of analysis in the present study. Suggestions have been reported that reduction in PAT production can due to metabolic destruction **(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 amount of aflatoxin B1 detected in black olives (produced by *A. parasiticus*) decreased after 9 days at 30 °C. In a study by **Leontopoulos et al. (2003)**, aflatoxin B1 produced by *A. parasiticus* increased significantly after the 12th day of incubation, while on olives incubated at 20 °C the toxin suffered a massive decrease after the same period of incubation. Citrinin amounts decreased after 40 days 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.

5. CONCLUSIONS

In this work, we were able to reach the following conclusions:

a) Identify the diversity of fungi isolated from olives intended for oil production from Tunisian olives groves: *Alternaria alternata*, *Trichothecium roseum*, *Aspergillus tubingensis*, *Fusarium brachygibbosum*, *Pleospora herbarum*, *Penicillium crustosum*, *Penicillium expansum* and *Penicillium polonicum*.

b) Among the identified *Penicillium* species tested for PAT production, only *P. expansum* was a PAT producer.

c) *P. expansum* MUM and TUN were able to grow on CYA and OM medium at 4 °C, 15 °C and 15 °C with an optimum growth at 25°C.

d) Matrix and temperature had a significant influence on *P. expansum*.

e) *P. expansum* MUM and TUN showed different PAT production abilities was affected by nature of matrix, temperature and time of incubation.

In conclusion, contamination of olives with PAT must be considered a potential risk in the safety and quality plans of the olive producing chain. The olive-based matrix does not seem to be highly adequate for growth and PAT production by *P. expansum* only if adequate temperatures throughout storage (refrigeration) are guaranteed. However, if there is an abuse on storage temperature and longevity, PAT can turn into a real risk.

To our knowledge, this is the first report on the risk of *P. expansum* growth and PAT production on olives and olive-based products. More studies are needed to reinforce the results obtained in the present study.

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