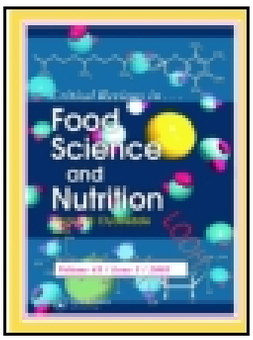

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Critical Reviews in Food Science and Nutrition

ISSN: 1040-8398 (Print) 1549-7852 (Online) Journal homepage: http://www.tandfonline.com/loi/bfsn20

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To cite this article: Joanna Tannous, Nancy P. Keller, Ali Atoui, André El Khoury, Roger Lteif, Isabelle P. Oswald & Olivier Puel (2017): Secondary Metabolism in Penicillium expansum: Emphasis on Recent Advances in Patulin Research, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2017.1305945

To link to this article: http://dx.doi.org/10.1080/10408398.2017.1305945

Accepted author version posted online: 31 Mar 2017.

Penicillium expansum secondary metabolism

Review article

Secondary Metabolism in *Penicillium expansum*: Emphasis on Recent Advances in Patulin Research

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ABSTRACT

The plant pathogenic fungus *Penicillium expansum* is a major concern of the global food industry due to its wide occurrence and ability to produce various mycotoxins, of which the most significant is patulin. Relatively less highlighted in the literature, in comparison with the other food-borne mycotoxins, patulin is one of the main factors in economic losses of vegetables and fruits. Otherwise, patulin is a health hazard which results in both short-term and long-term risks. This review includes knowledge on the biosynthetic mechanisms used for secondary metabolite production in *P. expansum,* with special emphasis on patulin biosynthesis. The abiotic factors triggering the production of patulin and the strategies developed to reduce or prevent the contamination by this mycotoxin are comprehensively discussed. The database presented in this review would be useful for the prioritization and development of future research.

Keywords

Penicillium expansum, secondary metabolites, patulin, biosynthesis, pathogenicity determinants, apples.

Introduction

Penicillium expansum is one of the best-known and most studied molds of the genus *Penicillium* (Andersen et al., 2004). This common fungus is a widespread species that can be found in the natural environment, especially in soil (Çolakoğlu 2002; Demirel et al., 2013) and even indoor air (Gutarowska et al., 2012). Additionally, *P. expansum* can infest a wide range of agricultural products in the field, during harvest, in storage, and during processing (Siddique 2012). Pome fruits (such as apples and pears) and stone fruits (cherries, peaches, plums, etc.) are the primary targets for the toxigenic fungus *P. expansum*. Legumes (beans and peanuts) and cereal grains (such as corn, barley, soybeans, and sorghum) have also been found to be highly favorable for *P. expansum* colonization (Nguyen 2007). In addition, this species is able to grow on non-consumable substrates, such as building materials containing cellulose, especially wallpaper and drywall (Gutarowska et al., 2012) and humid wood blocks (Land and Hult 1987). In food and plant materials, when held at relatively high moisture and moderate temperature for long periods, *P. expansum* is known to produce a large array of secondary metabolites with diverse structures and chemistries. Patulin is the best documented and the most investigated secondary metabolite from *P. expansum* worldwide. Over the last 20 years, several surveys on patulin occurrence and levels in fruit, especially apple-derived products, have been conducted in different countries. Table 1 summarizes the results of the main surveys published in the literature. Patulin was found in samples from all regions probably due to the use of contaminated raw ingredients or the improper storage conditions. In

some regions, such as Turkey, India, Tunisia, and Iran, the average concentrations of patulin in the analyzed samples (N/A, 330, 80, and 48.64 ppb, respectively) and percentage of samples exceeding the tolerated patulin levels of 50 ppb fixed by the European community regulations (43.5%, 16%, 18%, and 29%, respectively) were dramatically high (Gökmen and Acar 1998; Saxena et al., 2008; Zaied et al., 2013; Forouzan and Madadlou 2014) (Table 1). The most plausible explanation of this is that both Mediterranean and tropical climates favor growth of patulin-producing fungi and accumulation of the mycotoxin. However, this suggestion has not yet been either proved or disproved.

Apple infections are most frequently a result of fruit damage by insects, birds, early frost, excessive heat, drought stress, and unfavorable weather conditions just prior to harvest, but may also be due to mechanical damage that occur during transport and handling of fruits (Van Zeebroeck et al., 2007). *Penicillium expansum*, the most serious apple post-harvest pathogen, can penetrate the fruit skin through existing injuries while still in the field, and it continues to grow further and produce patulin during storage (Frisvad and Samson 2004). Several studies provide evidence that rot incidence is closely connected to fruit maturity; apples picked later were found to be more sensitive to rot (Ingle et al., 2000; Valiuškaitė et al., 2006). Patulin is almost absent in intact fruits (Murphy et al., 2006). The destructive rot caused by *P. expansum* is revealed by typical symptoms that are often circular and concentric pale brown, dry, smooth spots (Figure 1). These lesions quickly spread across the surface and also deep inside the fruit tissue (Pitt and

Hocking 2009). The surface of old rots may be covered by white spots that develop to form bluish-green fruiting structures (coremia) (Bencheqroun 2009) (Figure 1).

There are a number of review papers dealing with patulin research worldwide, including patulin biosynthesis, toxicity, and approaches settled for its detection, prevention, and removal at all stages of apple-product processing (Moake et al., 2005; Silva et al., 2007; de Souza Sant'Ana et al., 2008; Puel et al., 2010; Mahunu et al., 2016). None of these reviews provides an insight into the pathogenicity of *P. expansum* in apples and its association with patulin production with the exception of some mention of this topic by Wright (2015). Another review article has lately been published by Barad et al. (2016), with a main focus on the methods of patulin analysis in food and the involvement of this mycotoxin in pathogenicity. However, some aspects of patulin research, particularly the latest preventive measures and remedial procedures developed in order to limit its level in food, as well as its worldwide occurrence, are not documented in these two recent papers. Moreover, only factors influencing patulin production were considered in these reviews. The effects of such parameters on *P. expansum* growth were not discussed, even though reducing fungal growth is fundamental to reduce patulin accumulation. Additionally, most reviews focus on patulin research and very few of them considered other *P. expansum* secondary metabolites (Andersen et al., 2004), which may have, as yet, unknown roles in the virulence of this fungus.

Our present review, therefore, intends to encompass these areas of *P. expansum* research. The overall aim was to summarize and critically analyze the current knowledge

on all the secondary metabolites that have been ascribed to this species, with a special focus on patulin. Particular emphasis is put on patulin biosynthesis, the factors modulating its production and the management practices developed so far to guarantee a safer and more economically viable fruit supply.

Secondary metabolites produced by *Penicillium expansum*

Patulin

Patulin is a naturally occurring mycotoxin biosynthesized through a polyketide pathway by a variety of toxigenic fungal species belonging to the genera *Aspergillus* (Varga et al., 2003; Varga et al., 2007)*, Byssochlamys* (Rice et al., 1977; Dombrink-Kurtzman and Engberg 2006)*, Paecilomyces* (Houbraken et al., 2006; Samson et al., 2009) and *Penicillium* (Frisvad et al., 2004). However, *Penicillium expansum* remains the major contributor to patulin production so far identified (Morales et al., 2008b). This mycotoxin has been described in numerous foodstuffs, yet apples are higher susceptible to infection by *P. expansum* and serve as suitable medium for patulin production (Chen et al., 2004). Some years ago, it had been estimated that the apple blue mold caused by *P. expansum* can result in financial losses of up to $4.5 million annually in the USA alone (Rosenberger 1997). There has been no recent data in this regard. Most types of food processing do not affect the overall stability of this mycotoxin, and patulin was also detectable in apple derivative products ranging from non-fermented apple juices (Özdemir et al., 2009; Reddy et al., 2010) to apple purees (Funes and Resnik 2009). In the food industry, patulin production is frequently, but not exclusively, associated with apple rot. This mycotoxin has occasionally

been isolated from other rotting fruits like pears, apricots, peaches, strawberries, blueberries, cherries, and grapes, but always at lower and less dangerous levels than those observed in apples (Puel et al., 2010). The presence of patulin is not limited to fruits and their byproducts; vegetables and cereals also contribute to a certain patulin intake by humans (Lugauskas 2005).

Initially discovered during a screening campaign for new fungal active metabolites, patulin possesses well-documented toxicity (Moake et al., 2005; Clarke 2006). Most of the information on the toxicity of patulin is derived from animal studies; until now, there are no documented cases of acute toxicity reported in humans. The acute toxicity of patulin is generally high; the LD50 values have been demonstrated to range from 10.4 mg/kg bw in dogs (Reddy et al., 1979), 7.60 mg/kg bw in mice, and 5.80 mg/kg bw in rats (Hayes et al., 1979). Effects of acute poisoning such as hematemesis, diarrhea, extreme weakness, lethargy, tachypnea, massive atelectasis, alveolar hemorrhages and others, have been described in different animal species (Tapia et al., 2006).

The chronic exposure to patulin was likewise associated with adverse toxic effects. Patulin has been shown to be genotoxic on hamster V79 cells (Schumacher et al., 2005) and hepatoma cell line HepG2 (Zhou et al., 2009) as well as immunotoxic on various immune system cells (such as alveolar macrophages (Bourdiol et al., 1990) and interdigitating dendritic cells (IDC) of the thymus (Özsoy et al., 2008). Evidence of patulin's teratogenicity was noted in rat and chicken embryos (Ciegler et al., 1976; Smith et al., 1993). Moreover, patulin has been reported to exert cytotoxic effects on several cell types

including human intestinal epithelial cells (Caco-2-14 and HT-29-D4)(Mahfoud et al., 2002), human embryonic kidney cells (HEK 293) (Wu et al., 2008), and liver hepatocellular cells (HepG2) (Ayed-Boussema et al., 2011). The events mediating patulin cytotoxicity are largely dependent on its electrophilic properties, which allow for its irreversible interaction with cellular thiols like glutathione and cysteine (Fliege and Metzler 2000a;b). In light of its acute and long-term toxicity, a patulin level of 0.1 mg/kg b.w. was considered as No Observed Adverse Effect Level (NOAEL), which was adopted by the U.S. Food and Drug Administration (FDA) and the European Commission (EC) to establish the maximum tolerance limits of patulin in apple products (European 2003; 2006; U.S.FDA 2005). The Codex Alimentarius Commission (2003), along with the U.S.FDA (2005), have set regulatory limits of 50 ppb for apple-based products. The European Commission (2006) has gone further and imposed a maximum acceptable level of 10 ppb in infant food commodities.

Other secondary metabolites produced by *P. expansum*

Further to the morphological and physiological criteria, the metabolic profile is one of the most useful and significant taxonomic characters for the identification of *Penicillium expansum* (Frisvad and Filtenborg 1983). Before the advent of molecular biology and the remarkable improvements in analytical methods, a wide range of secondary metabolites has been reported to be produced by *P. expansum*; however, recent studies have narrowed that list (Andersen et al., 2004). Several false attributions occurred due to misidentification of fungal species or chemical compounds. Among these misallocations, we can quote

penicillic acid and cyclopiazonic acid. In pure cultures, citrinin, roquefortine C (Frisvad and Filtenborg 1983), and chaetoglobosins A and C (Larsen et al., 1998), in addition to patulin, have been identified as main *P. expansum* secondary metabolites. Expansolides A and B, communesins A, B, and other minor communesins (Larsen et al., 1998; Andersen et al., 2004; Kerzaon et al., 2009), and cytochalasin(Lugauskas 2005) have likewise been recognized as secondary metabolites produced by *P. expansum.* Moreover, Mattheis and Roberts (1992) identified geosmin as a volatile metabolite of *P. expansum* and showed that it is responsible for the earthy and pungent odor associated with this species. The capability to produce andrastins A, B, and C by *P. expansum* was later confirmed by mass spectrometric analysis (Kim et al., 2012).

It should be noted that these metabolites belong to different chemical classes and they are grouped below based on this classification.

Polyketides

Citrinin

Besides patulin, citrinin is the only polyketide metabolite assigned to *P. expansum*. This mycotoxin first isolated in 1931 from the filamentous fungus *Penicillium citrinum* (Stoev 2008), was originally described as an antibiotic with strong bacteriostatic properties. However, it has not been used as a drug due to its high nephrotoxicity (Xu et al., 2006). In addition to its detection in pure cultures during screening for new bioactive compounds, citrinin has been found to occur naturally in food. The co-occurrence of citrinin and patulin

was described on tomatoes (Harwig et al., 1979) and apples (Martins et al., 2002). Moreover, it should be noted that this mycotoxin is unstable and thermolabile in aqueous solution and there have been several reports on its detoxification (Hirota et al., 2002; Pin-Yen and Cheng-Huang 2002).

Nonribosomal peptides

Communesins and roquefortine C

These two secondary metabolites belong to the family of indole alkaloids. Andersen et al. (2004) have reported the natural co-occurence of communesin B and roquefortine C in apples and cherry juice. Communesins E and F, together with communesins A and B were isolated from the Japanese *Penicillium expansum* Link MK-57. All of them showed insecticidal activity against silkworm larvae (Hayashi et al., 2004). As for roquefortine C, this mycotoxin was first isolated from a strain of *P. roqueforti* and is potentially produced by a range of commonly occurring *Penicillium species* in food and feed. This mycotoxin has previously been characterized as a relatively weak neurotoxin (cited by Scott and Kennedy 1976). In a more recent study by Keblys et al. (2004), roquefortine C was found to be the least toxic mycotoxin, with 1.5 relative inhibition of lymphocyte proliferation.

Hybrid polyketide-nonribosomal peptides

Chaetoglobosins A and C and cytochalasins

Chaetoglobosins and cytochalasins are classified as polyketide/ nonribosomal peptide hybrid compounds that belong to the cytochalasan family of fungal secondary metabolites.

Both chaetoglobosins A and C have been detected in naturally infected apples by *P. expansum* (Andersen et al., 2004). The presence of these two mycotoxins can be lethal to mammalian cells, which act by binding to actin inducing inhibition of cell division. In more recent studies, the cytotoxicity of chaetoglobosins A and C was confirmed against various human cell lines (Thohinung et al., 2010; Zhang et al., 2010; Li et al., 2014). Regarding cytochalasins, they were also widely known for their actin binding characteristics (Cooper 1987). Moreover, several other biological activities of these compounds have been recorded, mainly an anti-angiogenic activity that resulted in inhibition of tumor growth (Huang et al., 2012; Trendowski et al., 2015).

Terpenes

Geosmin and expansolides A and B

Both fungal metabolites geosmin and expansolides belong to the terpene class. Geosmin is a bicyclic terpenoid by-product produced by a number of microorganisms, including several species of cyanobacteria, actinomycetes and fungi (Izaguirre et al., 1982, Schöller et al., 2002, La Guerche et al., 2005). This compound is responsible for the characteristic odor of moist soil, as well as off-flavors in drinking water and foodstuffs (Srinivasan and Sorial 2011). In an earlier study by Young et al. (1996) geosmin has been found to be relatively non-toxic to both invertebrates and mammals. Likewise, the production of this metabolite in biofilms didn't give any indication regarding the existence of apparent toxicity (Bláha et al., 2004). The bioactive compounds expansolides A and B, are tetracyclic sesquiterpene lactones firstly identified in the plant pathogenic fungus *P. expansum*

(Massias et al., 1990). Along with patulin and citrinin, expansolides A and B were detected by HPLC and LC-MS in rotten areas of all apple fruits artificially inoculated with *P. expansum* spores (Watanabe 2008). The toxicity of these metabolites has not been clarified yet.

Andrastins A, B, and C

Andrastins are meroterpenoid compounds biosynthesized from terpene and polyketide (Shiomi et al., 1999). Moreover, these compounds are interesting anticancer drug candidates since they are potent farnesyltransferase inhibitors, primarily of the RAS proteins, which are essential components for controlling cell division and the development of cancer (Shiomi et al., 1999). An antitrypanosomal activity was also reported for Andrastin A (Iwatsuki et al., 2010).

Pathways for the biosynthesis of *P. expansum* secondary metabolites and genes involved

Patulin biosynthesis

Many relevant chemical and molecular aspects of patulin biosynthesis have been studied and extensively described. The patulin biosynthetic pathway, similar to fatty acid synthesis, consists of at least 10 enzymatic conversion reactions initiated by a polyketide synthesis from acetate (Puel et al., 2010). The first stable intermediate in the patulin pathway is 6-methylsalicylic acid. A series of highly organized oxidation-reduction reactions then allows formation of patulin. The currently accepted scheme (Figure 2A) for

patulin biosynthesis is: acetyl coA-precursor → 6-methylsalicylic acid → *m*-cresol → *m*-hydroxybenzyl alcohol → gentisyl alcohol →gentisaldehyde → isoepoxydon → phyllostine → neopatulin → ascladiol → patulin.

Several specific enzyme activities associated with precursor conversions in the patulin pathway have been identified. The 6-methylsalicylic synthase (MSAS) responsible for the first step of the patulin pathway was the first fungal PKS purified from *Penicillium griseofulvum* (syn. *P. patulum* or *P. urticae*) cells and studied *in vitro* by Lynen and Tada (1961). A few years later, Light (1969) purified and characterized 6-methylsalicylic decarboxylase which converts the 6-methylsalicylic acid to *m*-cresol during the second step of the patulin pathway. Two hydroxylase activities that convert *m*-cresol to hydroxylated derivatives: 2, 5-dihydroxytoluene (toluquinol) or *m*-hydroxybenzyl alcohol, with a preference for the latter, were subsequently identified using *P. griseofulvum* cell-free extracts (Murphy et al., 1974). It was later shown that these two hydroxylase activities are catalyzed by two different cytochrome P450 enzymes (Artigot et al., 2009). The latter study showed that one cytochrome P450 allows the conversion of *m*-cresol to *m*-hydroxybenzyl alcohol and that another cytochrome P450 is responsible for the transformation of *m*-hydroxybenzyl alcohol to gentisyl alcohol. This latter P450 modifies *m*-cresol to toluquinol.

Some genes of this cluster have been isolated from patulin-producing species before the advent of whole fungal genome sequencing. The *idh* gene (*patN*) encoding isoepoxydon dehydrogenase was found in both *P. griseofulvum* (Fedeshko 1992), *P. expansum* (White et al., 2006), and *Byssochlamys nivea* (Dombrink-Kurtzman and Engberg 2006; Puel et al.,

2007). Another gene (*patM*) belonging to the patulin cluster and encoding a putative ATP-Binding Cassette (ABC) transporter was found upstream of the *idh* gene in *P. griseofulvum*. An orthologue of this gene has also been isolated from *B. nivea* (Puel 2007). Both the position and orientation of *patM* and *patN* in *B. nivea* are conserved compared to that in *P. expansum* and *A. clavatus* (Puel 2007). A study of Dombrink-Kurtzman (2008) suggested the existence of another gene (*patO*) encoding an isoamyl alcohol oxidase, downstream of the *idh* gene in *P. griseofulvum*.

Molecular analysis of the patulin production in *A. clavatus* (Artigot et al., 2009) and *P. expansum*, by 3 independent research teams (Tannous et al., 2014; Ballester et al., 2015; Li et al., 2015), led to the identification of an approximately 40-kb DNA biosynthetic gene cluster. The latter consist of 15 co-regulated metabolic genes denoted *patA*-*patN*. The positions of these genes differ between both patulin-producing species (Figure 2B).

Orthologs of patulin biosynthetic genes have also been identified in the genome of some non-patulin-producing species like *P. chrysogenum* (van den Berg et al., 2008), *Talaromyces stipitatus* (previously known as *Penicillium stipitatum*). Orthologous genes for 9 of the 15 patulin cluster genes were found in the genome of another post-harvest pathogen, *P. digitatum*, responsible for the green mold of citrus fruits (Marcet-Houben et al., 2012). Recently, the entire genome of the species *Penicillium roqueforti*, commonly used in the cheese industry, has been sequenced (Cheeseman et al., 2014). An overview shows that the structure of the patulin biosynthesis gene homolog cluster in this species is broadly similar to that of *P. expansum*. However, this species has undergone 2 deletion events. The inability

of these species to produce patulin is evidently attributed to the absence of some key genes in these deleted regions of the cluster. In addition, orthologs of 5 patulin pathway genes were identified in the genome of *A. terreus*; among these genes is the one encoding a 6-MSAS. Recent studies predict that this PKS gene and its surrounding genes are responsible for terreic acid biosynthesis in this fungus (Boruta and Bizukojc 2014; Guo et al., 2014)

Some of the genes responsible to express the vital enzymes involved in patulin biosynthesis have been characterized. These include the gene *patK* (6-*msas*) involved in the first step of the patulin biosynthetic pathway (Beck et al., 1990; Wang et al., 1991), the gene *patG* (6-*msad*), which is responsible for the conversion of 6- methylsalicylic acid to *m*-cresol (Snini et al., 2014) and the genes *patH* and *patI* proven to encode the two cytochrome p-450s involved in the third and fourth step reactions of the patulin biosynthetic pathway leading to gentisyl alcohol (Artigot et al., 2009). Most catalytic steps in the conversion of gentisyl alcohol to patulin have not yet been assigned to a specific metabolic gene. The step by which isoepoxydon is converted to phyllostine had long remained the only characterized reaction. The gene *patN* (*idh*) encoding an isoepoxydon dehydrogenase is responsible for this conversion (Fedeshko 1992; Dombrink-Kurtzman and Engberg 2006; Dombrink-Kurtzman 2007; Puel et al., 2007). Recently, the last step in the patulin pathway has been assigned to the *patE* gene encoding a glucose-methanol-choline (GMC) oxidoreductase (Tannous et al., 2016). This same group of researchers has also identified the patulin- specific transcription factor (*patL*). This gene is required for the up-regulation of the patulin pathway genes (Snini et al., 2015).

To date, the function of 8 metabolic genes in the patulin cluster remains unknown. However, searching the conserved domain database (CDD) at NCBI has led to the functional annotation of the proteins encoded by these genes. Therefore, the putative function assigned to each one of the genes are: putative acetate transporter (*patA*), putative carboxyl esterase (*patB*), putative MFS (Major Facilitator Superfamily) transporter (*patC*), putative Zn-dependent alcohol dehydrogenase (*patD*), putative dioxygenase (*patJ*), putative ABC (ATP binding cassette) transporter (*patM*), putative isoamyl alcohol oxidase (*patO*) (Artigot et al., 2009; Tannous et al., 2014). Additional analyses are suggested in order to shed light on the likely involvement of these genes in the uncharacterized patulin biosynthetic steps.

Eventually, the protein encoded by the *patF* gene in *P. expansum* was reported to have 86.4% of similarity with the N-terminal portion of the neopatulin synthase previously purified from *P. griseofulvum* (Fedeshko 1992; Tannous et al., 2015b). The PATF is therefore suggested to be involved in the eighth step of the patulin biosynthesis during which phyllostine is converted into neopatulin (Tannous et al., 2015b).

Biosynthesis of other secondary metabolites by *P. expansum*

Over the past few years, significant advances have also been made in understanding the genetic and biochemical basis of other *P. expansum* secondary metabolite biosynthesis. Besides patulin, complete cluster configurations and sequences are now available for other *P. expansum* secondary metabolites (Figure 3). This was further facilitated by the recent

genome sequencing of three *P. expansum* strains, using the Illumina Hiseq 2000 pair-end approach (Ballester et al., 2015).

Citrinin

A scheme for the biosynthesis of citrinin by *Monascus ruber* was first proposed in the paper of Hajjaj et al. (1999). This biosynthetic pathway was very recently clarified in the same species using gene knockout and heterologous expression strategies (He and Cox 2016). In addition, sequencing efforts did reveal a putative citrinin gene cluster in *P. expansum* which is predicted to be intermediate in size compared to the clusters identified in *Monascus aurantiacus* and *M. purpureus* (Ballester et al., 2015). The putative gene cluster in *P. expansum* is comprised of 9 genes, the 8-kb PKS sequence (*citS*), an oxidoreductase sequence (*citC*), 2 sequences coding for dehydrogenases (*citE*) and (*citD*), a dioxygenase (*citB*), an hydrolase (*citA*), a sequence coding for a Major Facilitator Superfamily (MFS) transporter (*orf5*), and one sequence encoding putative C6 transcription factor (*ctnA*). The genes in the predicted cluster of *M. aurantiacus* that are absent in the *P. expansum* cluster have distant homologs that are located elsewhere in the genome, suggesting that these genes may not be truly involved in citrinin biosynthesis (Ballester et al., 2015). This hypothesis is in accordance with the work of He and Cox (2016) which showed that CitS, CitA-CitE are sufficient for a significant citrinin production.

Communesins

The biosynthetic pathway of the communesins was recently mapped in the paper of Lin et al. (2015) by targeted-gene deletion experiments in *P. expansum*. Communesins synthesis begins by the coupling of tryptamine and aurantioclavine, 2 building blocks derived from L-tryptophan. Several post-modification steps lead to the formation of the different communesins. It is particularly noticeable that communesin B needs the activity of a polyketide synthase. Recently, Lin et al. (2015) reported the identification and characterization of a communesin biosynthetic gene cluster, comprised of 16 genes, from *P. expansum*.

Roquefortine C

The biosynthetic pathway responsible for the production of roquefortine was characterized in *Penicillium chrysogenum*, a closely related species to *P. expansum* (Ali et al., 2013). Lately, a putative short gene cluster leading to the synthesis of roquefortine C was identified in *P. expansum* (Martín and Liras 2015; Banani et al., 2016). This cluster in *P. expansum* is reduced in size compared to other species that produce meleagrin and oxaline, such as *P. chrysogenum* and *P. oxalicum* (Martín and Liras 2015).

Chaetoglobosins

Several isotope labelling experiments have given a clear picture of the biosynthesis of cytochalasans such as chaetoglobosins (Sekita et al., 1973; Vederas et al., 1975; Probst and Tamm 1981). Moreover, a hybrid PKS/NRPS gene cluster comprising 7 genes involved in biosynthesis of chaetoglobosins has also been partially characterized in *P. expansum*

(Schümann and Hertweck 2007). This gene cluster was verified to be involved in chaetoglobosin biosynthesis by RNA-silencing (Schümann and Hertweck 2007); however, it was not found in any of the *P. expansum* strains sequenced in the study of Ballester et al. (2015) and may represent an isolate-specific metabolite.

Geosmin

Behr et al. (2014) provided data on the mechanism of geosmin formation in *P. expansum*. Different pathways (glycolytic, mevalonate, and methylerythritol phosphate-pathways) leading to geosmin production were comprehensively discussed in this study. The geosmin gene cluster has not yet been identified in *P. expansum.*

Andrastin

The biosynthetic pathway of andrastin was reconstituted using a co-expression system in *Aspergillus oryzae*, a fungal expression host (Matsuda et al., 2013). Furthermore, a scheme of the putative andrastin gene cluster of *P. expansum*, which consists of 10 genes, was recently proposed in the paper of Matsuda et al. (2016).

Among the secondary metabolites mentioned above, a special place is reserved for patulin, the most studied *P. expansum* mycotoxin, which has often been associated with contamination of pomaceous fruits. Moreover, patulin belongs to a short list of 6 mycotoxins whose levels in food are subject to regulatory limits in many countries (Puel et al., 2010). Therefore, the rest of the review will be completely devoted to this mycotoxin.

Factors affecting *P. expansum* growth and patulin production in apples

Factors modulating the *in vivo* patulin production by *P. expansum* are identical to those permitting the growth of this species but are usually more restricted. They can be separated into 2 major groups. The first one includes extrinsic factors that refer to the environment surrounding the substrate (storage conditions: temperature, gas composition, and others), while the second group involves intrinsic factors that are characteristic of the substrate itself (water activity (a_W), pH, and other chemical characteristics of the substrate)(McCallum et al., 2002). This variability may also result from the fungal strain itself. Not all *P. expansum* strains have the same ability to produce patulin; genetic background may be the ultimate cause of this variability. On this point, numerous studies have reported differences in patulin accumulation levels between *P. expansum* isolates (McCallum et al., 2002; Neri et al., 2010; Welke et al., 2011; Ballester et al., 2015). Garcia et al. (2011) conducted one of the first studies to assess the impact of intra-specific variability of *P. expansum* on both capability for growth and patulin production using a large number of *P. expansum* isolates (79 isolates). In this study, the authors found that the variability of growth and patulin production among isolates was greater at margin temperatures (~1°C) compared with 20°C. The failure to produce patulin has also been described for some *P. expansum* strains, regardless of the culture conditions (Paster et al., 1995).

Storage temperature and atmospheric composition

Temperature is an exogenous factor of prime importance. Like most other species of the subgenus *Penicillium*, *P. expansum* is a psychrotrophic fungus, able to grow and be the dominant species at low temperatures (Pitt and Hocking 2009; Elhariry et al., 2011). The

effect of temperature on the germination and growth rate of *P. expansum* was modeled by Baert et al. (2007b). On the basis of these analyses, it was reported that cold storage does not prevent deterioration of apples but only delays it. An increase in linear growth rate was observed for all *P. expansum* strains tested over a temperature range of 2°C to 25°C. However, a decrease was noticed while raising the temperature beyond 25°C. This result has been recently reproduced in the modelling study of Tannous et al. (2015a). Otherwise, patulin production by *P. expansum* is also markedly temperature-dependent. Salomao et al. (2009) and McCallum et al. (2002) have reported a higher patulin production by this fungus at temperatures of 20.5°C and 25°C compared to that at 11°C and 4°C, respectively. On the contrary, a stimulation of patulin production was observed by Baert et al. (2007a) when decreasing the temperature from 20 to 10 or 4°C. In the same study, a further decrease in temperature to 1°C resulted in a reduction of patulin production. Likewise, Morales et al. (2008c) reported a weak patulin accumulation at relatively low temperatures (~1°C). Data collected from these studies do not provide a conclusive answer as to the optimum temperature for patulin production by *P. expansum*, but give evidence that cold storage is not a good practice to inhibit this production.

Besides temperature, other physiological parameters, like atmospheric composition, are also of great influence on the *P. expansum* growth and its patulin production ability. In this regard, Paster et al. (1995) showed that the growth of various *P. expansum* strains, when measuring infected tissue of apples, was not significantly affected by the different oxygen levels tested (2%, 10%, and 20%), indicating that this species has a very low

requirement for oxygen. This finding was in agreement with that of Sitton and Patterson (1992) who demonstrated that the reduced O_2 concentrations were not sufficiently effective in preventing lesion development caused by *P. expansum*; this, nevertheless, seems to be inhibited by an O_2 concentration below 2.3%. In contrast, the growth of the fungus was stimulated by carbon dioxide (CO_2) concentrations up to 15%; but this gas quickly becomes a limiting factor for growth at higher levels, suggesting its use as an effective fungistatic agent during apple storage. Similarly, about 100% of *P. expansum* spores were inactivated after 20 days of treatment in an atmosphere of 13% and 40% of CO_2 during fumigation of fruit cases (Cossentine et al., 2004). The atmospheric composition has also a significant influence on the patulin production by this fungus. A controlled atmosphere packaging with 2% O_2/ 3% CO_2 was found to be an inhibitor of patulin production at a temperature of 25°C. However, this production was restored in a modified atmosphere of 10 to 20% O_2 /2% CO_2 (Paster et al., 1995). Likewise, patulin production has not been reported on apples stored either under a controlled atmosphere of 2.5% O_2 /3.95% CO_2 or 1.5% O_2 / 2.5% CO_2, at a temperature of 1°C. The production was restored after subsequent apple storage at 20˚C (Morales et al., 2007b).

Recently, data from an *in vitro* study by De Clercq et al. (2016) showed that temperature and atmospheric composition affect patulin production by acting at the transcriptional level of the *idh* gene of the patulin biosynthesis cluster.

Host characteristics

Several investigators have examined the effect of initial pH on the growth and patulin production by *P. expansum*. An *in vivo* study of Prusky et al. (2004) revealed that apple cultivars differ in their tolerance to blue rot caused by *P. expansum*. The rot lesions' development was inversely proportional to the initial pH of the fruit. In the same study, the treatment of Golden Delicious apples (initial pH 4.4) with a solution of $NaHC0_3$ triggered an increase of the pH to 7.1, which resulted in a reduction of the fruit decomposition by the fungus (Prusky et al., 2004). In a subsequent study conducted by Li et al. (2010) on PDB (potato dextrose broth), pH 5 was optimal for *P. expansum* spore germination. However, at both pH values (2 and 8), representing the conditions of acid and alkaline stress, the germination was significantly delayed (Li et al., 2010). These findings were recently confirmed by Tannous et al. (2015a).

As for the effect of pH on patulin production by *P. expansum*, the results obtained by Morales et al. (2008a) suggest a strong influence of this factor on the production and the stability of this mycotoxin. In apple juices inoculated with a *P. expansum* strain, the lowest patulin concentration (4.1 mg/mL) was observed at a pH value of 2.5. This accumulation increased significantly (14.2 mg / mL) at pH 3, to reach 44.9 mg/mL at pH 3.5. However, in the pH range 4- 5.5, patulin concentrations were not significantly different. In their study, Marín et al. (2006a) reported that patulin accumulation was significantly higher in Golden Delicious apples than in Fuji; this was attributed to the fact that the latter was less acidic. These results are consistent with those published by Morales et al. (2008b) while incubating both apple varieties at a temperature of 1 ˚C. However, when apples were stored

at 20 ˚C, contradictory results were reported. These findings reveal that the apple cultivar is not always a determining factor in patulin accumulation. It is clear that its production is the result of a synergy between several intrinsic features of fruits and other extrinsic factors related to the environment. It has been published that sugar and organic acid contents of fruits evolved during ripening (Jan et al., 2012). However, the work of Morales et al. (2007a) found no relationship between fruit maturity and patulin accumulation by *P. expansum*.

Although a large influence exists on *P. expansum* germination and patulin accumulation *in vitro* (Mislivec and Tuite 1970; Hocking and Pitt 1979; Judet-Correia et al., 2010), water activity is not a determining factor when it comes to apple contamination as the a_W of all fresh fruits falls in the range 0.97-0.99 (Tannous et al., 2015a). Besides these 2 endogenous factors, other physiological characters of apple cultivars (such as carbohydrate, organic acid, and phenolic compound contents) (Marks et al., 2007; Wu et al., 2007) can exert a strong influence on the ability of the fungus to grow and to produce patulin. In this regard, a recent study of Zong et al. (2015) suggests a link between carbon sources and patulin biosynthesis. However much work is required in order to fully understand the effects of these apple-linked factors on *P. expansum* growth and toxigenesis.

Pathogenicity determinants of *P. expansum*

The understanding of the association between secondary metabolism and *P. expansum* pathogenesis has made remarkable progress over the last few years. In this regard, several groups have investigated the potential role of patulin as a virulence factor in the *P.*

expansum- apple interaction. However, existing studies have sometimes yielded contradictory results. Both Sanzani et al. (2012) and Barad et al. (2014) have consistently demonstrated that patulin is directly associated with *P. expansum* contamination of infested apples. A reduction in the disease incidence and severity was observed on Golden Delicious apples infected, respectively, by the *patK* (*6msas*) and *patN* (*idh*) mutants of *P. expansum*, in comparison with those infected by the wild-type strain. However, both studies were not so conclusive, as the mutants generated by either knock-out or RNAi, respectively, continues to produce residual amounts of patulin (Sanzani et al., 2012; Barad et al., 2014). In contradiction with the previously mentioned reports, the results using knockout mutants in the study of Ballester et al. (2015) clearly demonstrated that neither patulin nor citrinin are required by *P. expansum* to successfully infect Golden Delicious apples. However, in the latter study only the percentage of infected wounds (i.e. disease incidence) was estimated on the fifth day following inoculation, a relatively short period of time to make conclusions. Similar results were noticed thereafter by Li et al. (2015) on Fuji apple cultivars. The authors mentioned that no differences in growth rate were observed between the wild-type and the PeΔ*patL* mutant strains. Likewise, this study is not conclusive as only one apple variety was tested. To remove all doubt in this regard, Snini et al. (2015) have conducted the first exhaustive study to determine whether or not the patulin role as a virulence factor is affected by apple cultivars. In this study, a patulin-nonproducing mutant was generated through the disruption of the patulin-specific transcription factor (*patL*) in *P. expansum*. On 9 out of 13 apple varieties, the growth rate of the PeΔ*patL* strain was significantly lower than that of its parental wild-type. Findings in

this study, conducted over 14 days, suggest that patulin is not essential for the initiation of the disease, but acts as a cultivar-dependent aggressiveness factor for *P. expansum* (Snini et al., 2015).

A great number of recent studies focused on the close relationship between the modulation of the host's ambient pH by the fungus and its pathogenicity. During infection, *P. expansum* acidifies apple tissues via the secretion of several organic acids. mainly gluconic acid (GLA) (Prusky et al., 2004; Vilanova et al., 2014). This acidification is induced by the high sucrose concentration found in ripe fruits (Bi et al., 2016). Accumulation of GLA and tissue acidification provide optimal conditions for pectolytic enzyme activity (Prusky and Yakoby 2003). Polygalacturonase (PG) is the major pectolytic enzyme produced in *P. expansum*, which causes pectin depolymerization and tissue maceration (Yao et al., 1996); hence the close association between host acidification and pathogenicity. The oxidation of the β-D-glucose into GLA in this fungus is catalyzed by glucose oxidase (GOX encoded by *gox2*). In their studies, Barad et al. (2012; 2014) generated *gox2*-RNAi mutants, which are weakened in glucose oxidation to GLA. These mutants showed moderate acidification and patulin accumulation and reduced pathogenicity.

Most recently, the global regulator LaeA has been found to be a virulence factor in apple pathogenesis (Kumar et al., 2016). In this study, the deletion of *laeA* in 2 *P. expansum* strains has reduced patulin accumulation and colonization patterns on apples.

Practices and strategies to mitigate patulin contamination

Patulin is a chemical contaminant with a broad spectrum of toxicity. The evidence on its genotoxicity (Schumacher et al., 2005; Glaser and Stopper 2012), cytotoxicity (Burghardt et al., 1992; Mahfoud et al., 2002; Donmez-Altuntas et al., 2013), and its other toxic effects arises from numerous *in vivo* and *in vitro* studies. Unfortunately, the presence of this mycotoxin in food products and beverages cannot be completely eliminated; it can, however, be controlled in order to avoid causing harm to humans. Among the strategies developed in this regard, some are applied upstream of the patulin production as preventive practices against the development of the pathogen prior to harvest, at harvest, or during storage of fruits. However, other strategies are carried out in the post-production phase, as corrective actions, in order to reduce patulin levels in raw materials.

Preventive actions

Physical damage and chilling injuries arising in fruits are considered the main routes of entry for *P. expansum* and other patulin-producing species. Therefore, fruit quality, which depends mainly on the harvesting method, is the first request in order to control patulin levels. In a study of Jackson et al. (2003), patulin was undetectable
in cider produced from 7 varieties of fresh tree-picked apples, while this mycotoxin was detected at concentrations ranging between 40.2 and 374 mg/L in cider pressed from 4 apple varieties collected from the ground. Other physical post-harvest treatments, including high-pressure water-washing washing and sorting of fruits, have proven to be widely effective against fungal contamination and patulin production(Acar et al., 1998). Ultraviolet-C light is another physical application that has recently shown promising

results in reducing postharvest loss of fresh fruits by *P. expansum* (Syamaladevi et al., 2015). However, the efficiency of this treatment is closely dependent on the morphology and hydrophobicity of the fruit skin.

Chemical control against patulin-producing fungi has also been widely applied. The use of fungicides, such as benzimidazole, has contributed to satisfactory results in terms of apple rot reduction during storage and thus to actively decreasing patulin accumulation. However, the excessive non-recommended widespread usage of these chemicals has led to the emergence of *P. expansum*-resistant strains (Rosenberger et al., 1991). A very recent paper by Lai et al. (2017) revealed that exogenous potassium phosphite treatment significantly inhibit the growth and patulin production of *P. expansum* and possess beneficial effects in controlling blue mold on apple fruits. Nowadays, in many countries, public authorities call for massive restraint in the use of fungicides and chemicals due to their harmful impacts on the environment and human health. Therefore, the use of safe chemicals and natural antifungal agents has taken place as an alternative to synthetic fungicides, showing remarkable results in the control of patulin-producing fungi. A combined oxidative treatment made of sodium hypochlorite (NaClO), hydrogen peroxide (H_2O_2), and copper sulfate ($CuSO_4$) resulted in almost complete inhibition of *P. expansum* growth, conidial germination, and fungal infectivity on apples (Cerioni et al., 2013). Recently, propolis, a plant resin made by honeybees, was successfully applied as a natural antifungal agent to inhibit *P. expansum* growth and prevent patulin production (Silici and Karaman 2014; Matny et al., 2015).

Besides the preventive approaches described above with respect to their direct applicability against patulin producers, other indirect tools have been developed. Mathematical models have emerged as powerful tools to predict the behavior of patulin-producing species under different physicochemical conditions. The *P. expansum* growth rate and its patulin production ability have been assessed and modelled as a function of various intrinsic and extrinsic factors (Lahlali et al., 2005; Marín et al., 2006b; Baert et al., 2007b; Judet-Correia et al., 2010; Morales et al., 2008a; Salomao et al., 2009). Recently, a mathematical model was developed by Tannous et al. (2015a) to understand the integrated effect of 3 key factors (temperature, a_W, and pH) on the *P. expansum* growth rate. In the same study, the model applicability was successfully tested on different apple varieties. Therefore, this tool can be used by apple growers and the manufacturers of fruit juices in order to predict the development of *P. expansum* during storage and apple processing (Tannous et al., 2015a).

Molecular techniques used for the early detection of patulin-producing fungi have also been shown to be effective in preventing the occurrence of this mycotoxin in the food chain. In this regard, some PCR and real-time PCR systems have been developed for the detection of patulin-producing species based on the isoepoxydon dehydrogenase *patN* (*idh*) gene involved in patulin biosynthesis. Nevertheless, these tools fail to ensure specificity to patulin producers (Paterson et al., 2000; Dombrink-Kurtzman 2007; Rodríguez et al., 2011).

The recent completion of the patulin cluster sequence in *P. expansum* (Tannous et al., 2014) has put at researchers' disposal a wide range of genes showing greater advantages than the broadly used *idh* gene. Lately, a real-time PCR assay based on the *patF* gene was developed for the quantitative and specific detection of the *P. expansum* species in apples (Tannous et al., 2015b). Blast analysis proved that the occurrence of this gene is limited to patulin producers and 2 other fungi (*Neofusicoccum parvum* and *Macrophomina phaseolina*). Otherwise, this paper describes a simple and efficient protocol for the direct isolation of *P. expansum* DNA from apple tissues. This newly established method brings improvements in terms of time-saving compared to previous practices in which the fungal DNA extraction from apples was preceded by isolation and culturing on agar plates (Alwakeel 2013).

Counteraction and detoxification of patulin

Patulin in food and beverages can be removed, inactivated or detoxified by physical, chemical, and biological means. As a mandatory condition for the application of these treatments, the obtained products must be proven safe for consumers and their essential nutritive value should not be deteriorated.

Physical methods

Treatment by activated charcoal is the first physical treatment known and applied for patulin detoxification(Sands et al., 1976). Although this treatment is well-suited for the treatment of patulin, its general use is limited because of its adverse effects on the

organoleptic and physicochemical properties of juices (color, fumaric acid content, pH, and degree Brix) (Kadakal and Nas 2002). More recently, several studies were conducted using germicidal UV irradiation (Dong et al., 2010; Assatarakul et al., 2012; Tikekar et al., 2013). This treatment has shown promising effectiveness in reducing patulin levels in clarified apple juices. However it was found useless in treating unfiltered cider due to the high levels of suspended particles that absorb UV rays before reaching patulin molecules. Unlike the first treatment with activated carbon, the UV method for decontamination induced no significant changes in chemical and organoleptic properties of the product. Subsequently, pulsed light (PL) was investigated by Funes et al. (2013) as a patulin decontamination method. The exposure of all apple product samples to PL doses between 2.4 and 35.8 J/cm^2 induced a significant decrease in patulin levels. However, further investigations are required for assessing the toxicological safety of the resulting patulin degradation products.

Chemical methods

Chemical decontamination methods have been found more suitable for patulin detoxification in an industrial context. Several previous studies have dealt with the effects of different chemicals on patulin such as ammonia, potassium permanganate, sulfur dioxide, ozone, vitamin B, pyridoxine hydrochloride, and calcium D-pantothenate (Frémy et al., 1995; Ough and Corison 1980; Burroughs 1977; McKenzie et al., 1997; Yazici and Velioglu 2002). Most of these treatments were very beneficial and showed promising results in terms of patulin decontamination.

Biological methods

Despite the satisfactory results reported above, the global trend is now to reduce the use of chemicals through the development of alternative tools. The bio-control based on the use of microorganisms, such as the yeast *Saccharomyces cerevisiae* (Moss and Long 2002) and the Gram-negative bacterium *Gluconobacter oxydans* (Ricelli et al., 2007), has emerged as one of the most important alternatives. This method generates biodegradation products that have recently been found less toxic than the parental mycotoxin (Maidana et al., 2016; Tannous et al., 2017). Nevertheless, usage of these biological methods is limited to products which can be fermented. A very recent study by Chen et al. (2017), described for the first time the use of the antagonistic yeast *Candida guilliermondii* to control patulin contamination. In addition, the latter study reported the application of an iTRAQ-based proteomic analysis that allowed the identification of 30 differential proteins in patulin-treated yeast cells. The findings presented in this study open up new perspectives for research on commercially formulated patulin-degrading enzymes that can be applied in any fruit-derived products. Besides the microorganisms known for their patulin degradation ability, others have been described for their aptitude to adsorb this mycotoxin through their cell wall without being able to degrade it (Hatab et al., 2012). A recent study by Zhu et al. (2015) reported that the use of the yeast *Rhodosporidium paludigenum* inhibits and reduces patulin contamination in apples and pears via two mechanisms: physical adsorption and biological degradation.

Conclusion

Apples are among the world's most consumed and exported fruits, in addition, they are the main component of many foodstuffs intended for infants. For several years, apple growers have been facing a serious pest, *Penicillium expansum,* which causes severe damage to apples and drastic losses in yield. Besides the esthetic damage characterized by brown circular rot, apples contaminated with this fungus become a source of mycotoxins of which patulin is the most serious one. This species is able to produce roquefortine, chaetoglobosins, andrastins, citrinin, communesins, and expansolides. Although biosynthetic clusters of most of these metabolites have been identified, few data exist about their toxinogenesis, toxicity, and involvement in *P. expansum* pathogenicity. In this review, we discuss *P. expansum* as an agricultural and food safety threat and summarize recent advances in secondary metabolite profiling by this fungus. The main focus was to summarize our current knowledge of the biosynthetic pathway leading to patulin production, the established role of this mycotoxin during *P. expansum* infection of apples (fungus--host interactions) and effective control tools used for reducing patulin contamination.

While several studies on *P. expansum* secondary metabolites, particularly patulin, have improved our understanding significantly over the last 3 decades, some knowledge gaps appeared to us in the process of writing this review. As basic research, more work appears desirable (i) to identify additional classes of secondary metabolites produced by *P. expansum*, to characterize their biosynthetic pathways, and describe their role in the pathogenesis of this fungus; (ii) to clarify the role of the remaining patulin biosynthetic

genes whose functions are still unclear; (iii) to further understand the mechanism of genetic regulation of patulin synthesis; and (vi) to find out other physicochemical factors that might regulate *P. expansum* growth and, consequently, patulin production. Otherwise, on an applied research level, further updated surveys on patulin occurrence and levels are required, particularly in countries considered as the largest producers and exporters of apples.

Funding

This review paper is based upon work supported by the National Council for Scientific Research (CNRS), Lebanon under Grant 3443; the Research Council of Saint- Joseph University (Lebanon) under Grant FS38; and CASDAR AAP RT 2015 under Grant 1523, project “Mycotoxines majeures et émergentes dans les filières cidre et vin.”

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Worldwide contamination of apple by-products with patulin

Country	Commodity	Total samples (n)	Average (ppb)	Range (ppb)	**Percentage of samples over the maximum limits**	References
Argentina	Apples and pears by-products (solid and semi-solid consistency)	51	61.7	17--221	5.88%	Funes and Resnik (2009)
Australia	Apple, pear, and mixed fruit products	328	N/A[(a)]	5--1130	22%	Burda (1992)
Belgium	Imported and local apple juices and ciders	50	9 and 3.4, respectively	2.5 --38.8 and 2.6 -- 6.1, respectively	_ [(b)]	**Tangni et al. (2003)**
	organic, conventional and handcrafted apple juices	177	22.2	ND[(c)] --65.7	1.12%	Baert et al. (2006)
Brazil	Processed fruit juices and sound fruits	111 and 38, respectively	17	ND--17	_	Sylos (1999)
	Apple based beverages	134	N/A	3 -- 7	_	Iha and Sabino (2008)
China	Apple products	95	20.4	1.2 -- 94.7	16%	Yuan et al. (2010)

	Apple juices	1987	8.44	N/A	0.2%	Guo et al. (2013)
Greece	Imported and local fruit juices	90	10.54 and 5.57, respectively	2.6 --36.8 and 0.9 -- 11.8, respectively	_	Moukas et al. (2008)
India	Apple juices	50	330	21--1839	16%	Saxena et al. (2008)
Country	Commodity	Total samples (n)	Average (ppb)	Range (ppb)	**Percentage of samples over the maximum limits**	References
Iran	Apple juices Apple juice concentrates	65	48.1 and 61.7, respectively	N/A-285	33% apple juices 56% apple juice concentrates	Cheraghali et al. (2005)
	Apple juices	72	48.64	29.58- 151.2	29%	Forouzan and Madadlou (2014)
	Fruit Juices	161	34.5	5- 190.7	2.5%	Rahimi and Rezapoor Jeiran (2015)
Italy	Apple juices	26	4.5	1-22	_	Versari et al. (2007)
	Pure and mixed apple juices	135	6.42	1.58 -- 55.41	1%	Spadaro et al. (2007)
	Fruit juices	105	28	1-92	13%	Bonerba et al. (2010)
Japan	Apple juices	76	8.89	ND-- 45.6	_	Ito et al.

						(2004)
	Apple juices and mixed juices	188	N/A	6--15	_	Watanabe and Shimizu (2005)
Netherlands	Apple products	63	N/A	N/A	1.58%	Boonzaaijer et al. (2005)
Portugal	Apple-based-foods	144	N/A	1.2 -- 42	_	Barreira et al. (2010)
	Apple fruits and products, Quince fruits	41	70.64 and 56.9, respectively	ND--1500 <LOQ [(d)]--118.3	2.7% apple fruits and products	
11% quince fruits	Cunha et al. (2009)					
Romania	Apple-based juices	50	13.3	0.7--101.9	6%	Oroian et al. (2014)
Saudi Arabia	Imported and local apple juices	51	140	ND -- 152.5	5.88%	Al-Hazmi (2010)
Country	Commodity	Total samples (n)	Average (ppb)	Range (ppb)	**Percentage of samples over the maximum limits**	References
South Africa	Locally produced commercial apple products	60	10	5 -- 45	_	Leggott and Shephard (2001)
South Korea	Fruit Juices	72	11.81	2.8 -- 30.9	_	Cho et al. (2010)
Spain	Apple juices	100	19.4	0.7--118.7	11%	Murillo-Arbizu et al.

						(2009)
Tunisia	Apple based products (apple juices and baby food)	85	80 and 68, respectively	ND--167	18% juices 28% food intended to infants	Zaied et al. (2013)
Turkey	Concentrated apple juices	215	N/A	7 --376	43.5%	Gökmen and Acar (1998)
	Apple juices	45	N/A	19.1--732.8	44%	Yurdun et al. (2001)
USA (Michigan state)	Apple juice and cider	493	36.9	4.6 -- 467.4	2.2%	Harris et al. (2009)

[a] The authors have not provided the average or the range values (ppb) of their analyses

[b] The obtained results were all below the legislated levels considering foods intended for infants (10 ppb), solid apple based products (25 ppb) and fruit juices (50 ppb)

[c] ND stands for None Detected

[d] Lower than limit of quantification

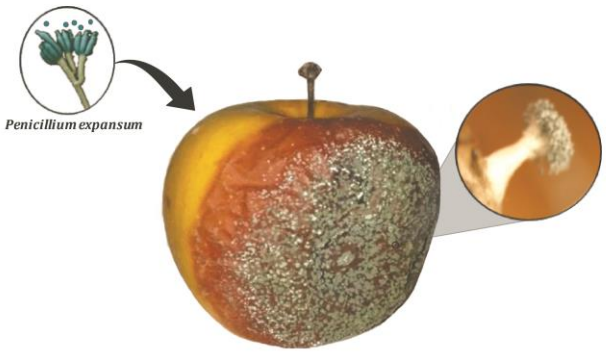

Figure 1: (Left) Blue mold caused by *Penicillium expansum* on Golden Delicious apple. (Top right) Coremia observed on the surface of the rotted area by a stereo microscope, binocular (3X magnification) (Tannous 2015).

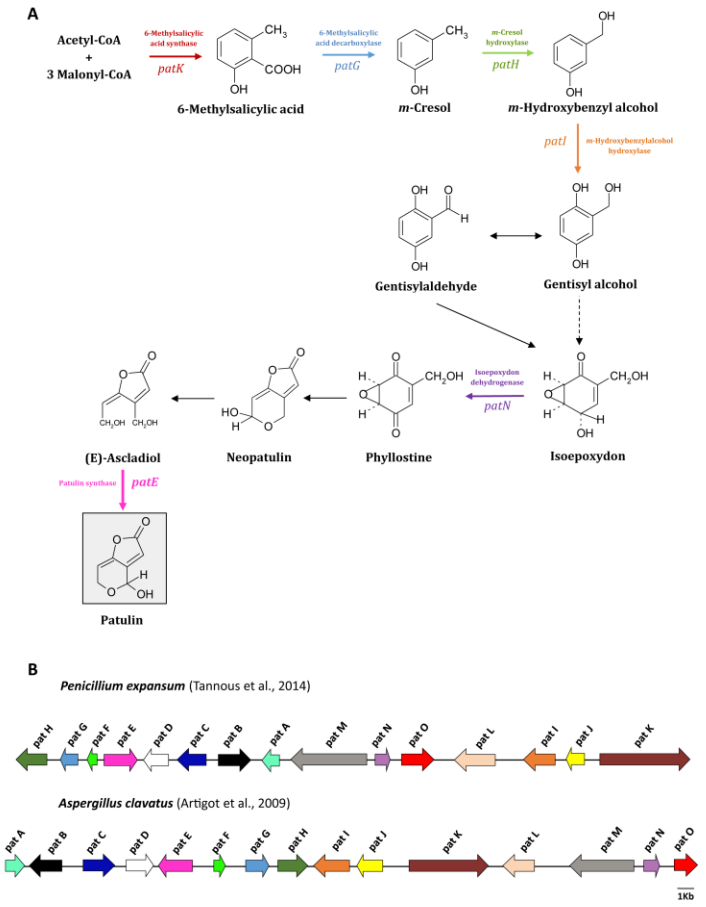

Figure 2: The generally accepted pathway for patulin biosynthesis is shown in panel A (Tannous 2015). The corresponding genes and their enzymes are presented. Arrows indicate the pathway steps from previous precursor to the next intermediate towards the formation of patulin. Arrow colors indicate the relationship between the genes and the bioconversion steps they are involved in. The patulin biosynthetic pathway gene clusters in *Aspergillus clavatus* and *Penicillium expansum* are shown in panel B. The gene names are labeled on top of the clusters. The direction of transcription is indicated by arrowheads and

homologous genes in both species are designated with the same color (Tannous et al., 2014).

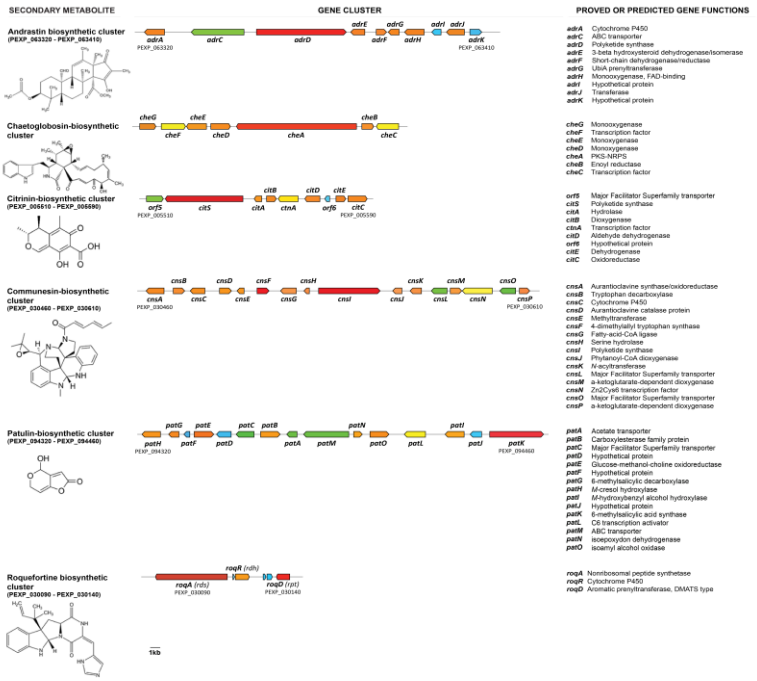

Figure 3: Representation of each biosynthetic cluster identified on the genome of *P. expansum*. Genes are color-coded according to functions of encoded enzymes: Red, structural genes (NRPS, PKS, DMATs); yellow, regulatory genes; green, transporter genes; blue, hypothetical genes and orange, other genes (encoding oxygenases and enzymes involved in precursor synthesis and post-core tailoring modifications). The putative andrastin biosynthetic cluster of *P. expansum* refers to the work of Matsuda et al. (2016), that of chaetoglobosin-biosynthesis (Genbank accession number: AM779763) to the paper of Schümann and Hertweck (2007), the putative citrinin cluster is revised from Ballester et al. (2015) and He and Cox (2016), that of communesin from Lin et al. (2015), whereas the genetic organization of the patulin cluster (Genbank accession number: KF899892) in *P. expansum* refers to the study by Tannous et al. (2014). The putative roquefortine cluster shown in the last panel is adapted from the paper of Banani et al. (2016). Genes are named

according to the original cluster described in *P. chrysogenum* (Ali et al., 2013; Ries et al., 2013). Gene nomenclature adapted by Martín and Liras (2015) is shown in parentheses. Gene accession numbers of the *P. expansum* d1 strain are used for all the gene clusters. No accession numbers are available for the chaetoglobosin gene cluster that is absent in this strain (Ballester et al., 2015)

